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Direct diffusion of anti-Müllerian hormone from both the cranial and caudal regions of the testis during early gonadal development in mice

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Abbreviations

AMH: anti-Müllerian hormone; AMHR2: AMH receptor type 2; AR: androgen receptor; dpc: days post coitum; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; MD: Müllerian duct; MTs: mesonephric tubules; RC: rete cord; rh-AMH: recombinant human AMH; TC: testis cord; WD: Wolffian duct.

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ABSTRACT

INTRODUCTION: The Müllerian duct (MD), the primordium of the female reproductive tract, is also formed in males during the early stage of development, then regresses due to the anti-Müllerian hormone (AMH) secreted from the testes. However, the detailed diffusion pathway of AMH remains unclear. We herein investigated the mechanism by which AMH reaches the middle region of the MD using an organ culture system.

RESULTS: Injection of recombinant human AMH into the testis at the start of MD regression induced diffuse immunoreactivity in the mesonephros near the injection site. When the testis and mesonephros were cultured separately, the diameters of both cranial and middle MDs were significantly increased compared to the control. In the testis–mesonephros complex cultured by inhibiting the diffusion of AMH through the cranial region, the cranial MD diameter was significantly increased compared to the control, and there was no difference in middle MD diameter.

CONCLUSIONS: These results indicate that AMH, which infiltrates from the testis through the cranial region at physiological concentrations, induces regression of the cranial MD at the start of MD regression. They also indicate that AMH infiltrating through the caudal regions induces regression of the middle MD.

INTRODUCTION

In mammals, undifferentiated gonads are sexually dimorphic, and their differentiation into testes or ovaries is controlled genetically.[1,2] The female and male reproductive tracts do not arise from a common primordium as in the gonads, but rather from different primordia. The male and female reproductive tracts are derived respectively from the Wolffian duct (WD) and the Müllerian duct (MD). In early development in both sexes, both of these ducts form in the mesonephros adjacent to the lateral gonad[3,4] (Fig. 1). In the mouse, the WD forms from about 9.0 days post coitum (dpc) to 10.5 dpc[5,6], and the MD from 11.5 dpc to 13.5 dpc, both in a cranial–caudal direction[7-9]. After the sexual differentiation of the gonads, the sexually dimorphic development of the reproductive tract in males is established by testicular secretions such as testosterone and anti-Müllerian hormone (AMH). AMH induces the regression of the MD, while testosterone induces the development of the WD into the epididymides, vasa deferentia, and seminal vesicles[3,10] (Fig. 1). In males, the testis cord (TC), rete cord (RC), and mesonephric tubules (MTs) develop and are connected to the WD. Conversely, due to the absence of these hormones in developing female gonads, the WD regresses and the MD gives rise to the oviduct, uterus, and upper part of the vagina[3,11] (Fig. 1).

Testosterone is a steroid hormone synthesized from Leydig cell–derived androgens by the action of 17β-hydroxysteroid dehydrogenase type 3 (HSD17β3), which mediates the final reaction of testosterone synthesis in Sertoli cells located within the TC.[4,12] Testosterone induces the expression of androgen receptors (AR) in the mesenchyme around the WD and the WD epithelium, then binds to the AR and develops the WD.[13,14] AMH is a peptide hormone belonging to the TGF-β superfamily.[15] AMH is synthesized as a proprotein precursor (proAMH) in Sertoli cells, and in mice *AMH* mRNA expression starts around 12.0 dpc.[16,17] Then, proAMH is cleaved by subtilisin/kexin proprotein convertases, yielding an N-terminal prodomain (AMH_N) and a C-terminal mature domain (AMH_C), the latter of which is capable of binding Anti-Müllerian hormone receptor type 2 (AMHR2).[18-20]

AMHR2, a serine/threonine kinase, is expressed in the coelomic epithelial cells and subjacent mesenchymal cells lateral to the MD.[21-23] In male mice, AMHR2 expression begins around the most cranial part of the MD at 13.0 dpc and reaches the most caudal part by 13.5 dpc.[24] When AMH binds to AMHR2, MD regression is induced,[25] and the MD is eliminated by 18.0 dpc in male mice.[26] In other words, development of the WD requires that testosterone secreted by the testes reaches it, while regression of the MD requires that AMH secreted by the testes reaches the mesenchyme around the MD.

In the case of true hermaphrodites with an ovary on one side and a testis on the opposite side, both WD development and MD regression occur only on the testis side.[27-29] Removal of one gonad of a male rabbit fetus caused WD regression and MD development on the removed side and, on the opposite side, WD development and MD regression.[30] If testosterone and AMH act in an endocrine manner through the circulatory system, the WD should develop and the MD should regress on both sides. Therefore, testosterone and AMH that reach the WD and MD independently of the circulatory system are thought to play important roles in determining the developmental fates of these ducts. It has been shown in an organ culture system that testosterone reaches the WD via RC, which is the primordia of the rete testis and MTs which are the primordia of the efferent ductules, and connect with the RC at the cranial region of gonad–mesonephros complexes.[31] In addition, the high concentrations of testosterone that diffuse down the lumens of the ducts are thought to be important for the development of the WD, since AR expression reflects a higher testosterone gradient.[14]

We previously showed that AMH also diffuses from the testis to the mesonephros without entering the circulatory system during the start of MD regression, and we suggested that AMH infiltrates through the cranial part of the border region between the testis and mesonephros (Fig. 1).[32] This secretion pattern led us to speculate that AMH infiltrating from the cranial region of the testis (cranial testis) into the mesonephros induces the regression of the cranial part of the MD (cranial MD).

However, the detailed mechanism by which AMH reaches the middle part of the MD (middle MD) and beyond remains unclear. In this study we sought to gain further insight into the pathway by which AMH acts on the MD, especially its middle part.

106 RESULTS

107 *AMH localization in mouse embryo*

108 AMH localization in embryonic mouse testis–mesonephros complexes at 12.5, 13.5, and 15.5 dpc
109 was examined by using the anti-AMH antibody. In 12.5, 13.5, and 15.5 dpc mouse sections,
110 immunoreactivity was observed in both the TC and the interstitium of the testis (Fig. 2A-C). In
111 addition, at 13.5 dpc, when RC was observed[33], AMH was weakly detected in the RC (Fig. 2B'), as
112 described previously.[32] The same expression pattern was observed at 15.5 dpc (Fig. 2C'). Weak
113 immunoreactions were also observed in the mesenchyme of the mesonephros at 12.5, 13.5, and 15.5
114 dpc (Fig. 2A-C). These immunoreactions were considered nonspecific because they were detected in
115 the female mesonephros (data not shown).

116

117 *Connection between the testis and mesonephros*

118 We previously analyzed the amounts of AMH in the mesonephros at 13.5 and 14.5 dpc by Western
119 blotting (WB), revealing that the highest amount of AMH in the cranial part occurred at 14.5 dpc.[32]
120 The tunica albuginea, which separates the testis from the mesonephros, is absent in the part of the
121 cranial region where the RC and MTs extend toward the WD.[34] Based on the relationship between
122 the amount of AMH in the mesonephros and the region of tunica albuginea formation, it was
123 speculated that connective tissue may inhibit AMH movement. In the present study, we used aniline
124 blue staining to evaluate the degree of connective tissue formation at 12.5, 13.5, and 15.5 dpc in
125 males. The region with less connective tissue and the connection between the testis and the
126 mesonephros were noted in both the cranial testis and the caudal region of the testis (caudal testis,
127 Fig. 1) at 12.5 and 13.5 dpc (Fig. 3A-D, A'-D'). At 15.5 dpc, whereas the region of connection
128 between the testis and the mesonephros was also observed at the cranial testis (Fig 3E, E'), the
129 connective tissue formation at the boundary region between the testis and the mesonephros

progressed (Fig 3E, F, F') and the boundary region was narrower (Fig. 3E, F) compared with those at 12.5 and 13.5 dpc (Fig. 3A-D).

Dynamics of rh-AMH injected into the testis

Together, our previous result[32] and the above detection of AMH localization indicated that AMH infiltrates from the testis to the mesonephros through the RC. However, it remains unclear whether AMH infiltrates through the mesenchyme at the boundary region between the testis and the mesonephros. Based on the above result of aniline blue staining, we expected AMH to infiltrate from the testis through not only the RC but also the mesenchyme, especially at 12.5 and 13.5 dpc. To investigate the region where AMH can infiltrate from the testis to the mesonephros, we injected recombinant human (rh)-AMH into the testis at 12.5, 13.5, and 15.5 dpc and detected it by using the anti-AMH antibody. Rh-AMH is known to regress the mouse MD and has long been used to investigate MD regression.[35]

When 0.3−0.5 μl of rh-AMH was injected into the cranial or caudal testis at 12.5 dpc, AMH was strongly detected throughout the entire testis, including the interstitium, and especially in the testis tissue near the injection site (Fig. 4A, D). AMH was also detected in the mesonephros near the injection site and seemed to diffuse from the boundary region between the testis and the mesonephros to the MD (Fig. 4A', D'). On the other hand, AMH was not detected in the mesonephros at the pole opposite the injection site even though the section was reacted on the same slide glass on which the section near the injection site was reacted (Fig. 4B, C). The same expression pattern was observed at 13.5 dpc (data not shown).

When 0.8-1 μl of rh-AMH was injected into the cranial testis, the center of the testis, or the caudal testis at 15.5 dpc, AMH was detected in the TC and in the portion of the interstitium near the injection site (Fig. 5A–C). However, in the boundary region between the testis and the mesonephros, AMH was

154 only weakly detected in the RC (Fig. 5A' and B') and was not detected in the mesenchyme (Fig. 5A'–
155 C'). AMH was also not detected in the mesenchyme around the MD. This was the same as at 15.5 dpc
156 (Fig. 2C).
157
158 *Timing of the beginning of culture*
159 On the basis of the above results, we expected AMH to infiltrate the mesonephros from both the
160 cranial and caudal testes at 12.5 and 13.5 dpc and to induce regression of the cranial and middle MD
161 at 13.5 dpc. To examine the AMH diffusion pathway under conditions close to those *in vivo*, we
162 decided to use an organ culture system, and first investigated the timing of the beginning of culture.
163 The degree of MD regression was evaluated by the degree of condensation of the mesenchymal cells
164 around the MD and by the diameter of the cranial and middle MD (Fig. 6A). As in previous reports,[5]
165 the condensation of mesenchymal cells around the MD was not observed at 12.5 dpc, and at 13.5 dpc
166 it was observed in both the cranial and middle MD (data not shown). There was no difference between
167 males and females in the diameters of the cranial and middle MD at 12.5 dpc (Fig. 6B, C). At 13.5
168 dpc, on the other hand, males had significantly smaller cranial ($p<0.001$) and middle ($p=0.005$) MD
169 diameters than females, which do not produce AMH (Fig. 6B, C). Comparing the cranial MD
170 diameters at 12.5 and 13.5 dpc, we found that females had slightly higher diameters at 13.5 than at
171 12.5 dpc ($p=0.107$), whereas males showed the opposite pattern ($p=0.648$, Fig. 6B). The 12.5 dpc male
172 cranial MD diameter was significantly smaller than that of 13.5 dpc females ($p=0.014$, Fig. 6B).
173 Comparing the diameter of the middle MD at 12.5 and 13.5 dpc, we found that in females the diameter
174 at 13.5 dpc was slightly larger than at 12.5 dpc ($p=0.535$), whereas the opposite pattern occurred in
175 males ($p=0.471$, Fig. 6C). In addition, the diameter of middle MD in 13.5 dpc males was significantly
176 smaller than that in 12.5 dpc females ($p=0.015$, Fig. 6C). These results indicated that, in the presence
177 of AMH, the MD had a smaller diameter, or at least did not grow, as development progressed. The

178 results also reconfirmed that AMH already affected the regression of the cranial and middle MD at
179 13.5 dpc. Therefore, the gonad–mesonephros complexes at 12.5 dpc were used for the organ culture.
180
181 *Analysis of AMH in the mesonephros at 12.5 dpc and after incubation*
182 To investigate whether AMH infiltrates from the testis to the mesonephros during incubation, we
183 used WB to analyze the amount of AMH in the mesonephros of the testis–mesonephros complex
184 cultured for 72 hr from 12.5 dpc and at 12.5 dpc (Fig. 7A). The amount of AMH per mesonephros
185 sample cultured for 72 hr from 12.5 dpc was approximately 1.35 times that per mesonephros sample
186 at 12.5 dpc. When the AMH band intensity was quantified by using the band intensity of
187 glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the amount of AMH in the 72-hr cultured
188 mesonephroi from 12.5 dpc was approximately 2.2 times that in the mesonephroi at 12.5 dpc (Fig.
189 7B). These results indicate that physiological concentrations of AMH infiltrate the mesonephros from
190 the testis.
191
192 *AMH diffusion pathway under organ culture conditions*
193 The effect of the incision of a candidate region for AMH infiltration on MD regression was
194 evaluated in the organ culture system. We prepared gonad–mesonephros complexes at 12.5 dpc
195 cultured for 72 hr under the following conditions (Fig. 8A): in Condition 1, the gonad was separated
196 from the mesonephros; in Condition 2, the cranial boundary region between the gonad and the
197 mesonephros was incised; in Condition 3, the caudal boundary region between the gonad and the
198 mesonephros was incised; and in Condition 4, MTs connected with the WD were incised. A control
199 condition was also established by culturing the gonad–mesonephros complexes intact. In all conditions,
200 testes and mesonephroi were larger after culture than before (Fig. 8B). Under all conditions, including
201 the control, in males and females, the MD was elongated to the most caudal portion of the testis at the

beginning of the culture, and after incubation it was elongated to the most caudal portion of the mesonephros (data not shown). Under all these conditions, in males AMH was detected in Sertoli cells in the cultured testis (Fig. 8C). These results confirmed that there were no differences among the conditions in AMH expression in the testes or in MD elongation.

The diameter of the cranial MD after incubation showed a significant increase over the control in Condition 1, in which AMH diffusion from the testis was inhibited, as well as in Condition 2, in which AMH diffusion at the cranial testis was inhibited. On the other hand, there was no significant difference between the control and Condition 3, in which AMH diffusion at the cranial testis was not inhibited (Fig. 8D). These results indicated that AMH infiltrates the mesonephros from the cranial testis and induces the regression of the cranial MD. The middle MD diameter under culture Condition 1 showed a significant increase over the control, while that under Condition 2 was not significantly different from the control (Fig. 8E). These results indicated that AMH infiltrates the mesonephros from the caudal testis and induces the regression of the middle MD. On the other hand, in culture Condition 3, the mean middle MD diameter was slightly larger than that of the control. Nonetheless, the disparity did not yield statistical significance in the Kruskal–Wallis test (p=0.192), which simultaneously assessed all five groups (Fig. 8E). However, considering the significant distinction highlighted by the Mann–Whitney U test (p=0.031), it would be a Type II error to deem the control and culture Condition 3 as equivalent and to assume the absence of AMH infiltration from the caudal testis. Under Condition 4, in which AMH diffusion through the MTs and the WD was inhibited, the cranial MD diameter was not significantly different from the control by the Kruskal–Wallis test (p=0.199, Fig. 8D). However, the two groups may not be equivalent because the Mann–Whitney U test revealed a significant difference (p=0.032). The middle MD diameter was not different from that of the control or from that under Condition 2 (Fig. 8E). From these results, it was speculated that the pathway via the RC, MTs, and WD may be important for cranial MD regression. On the other hand, there is still a possibility that

226 the gel sheet inserted to prevent tissue reattachment also prevented AMH infiltration from the
227 mesenchyme around MTs. In females, neither the cranial nor the middle MD diameter differed
228 significantly among the conditions (Fig. 8F, G).

229 The degree of MD regression was also evaluated by the degree of condensation of mesenchymal
230 cells around the MD. Condensation of mesenchymal cells around the cranial MD was observed in the
231 control and under Condition 3 (Fig. 9A, G). On the other hand, it was not observed under Conditions
232 1, 2, and 4 (Fig. 9C, E, I). Condensation of mesenchymal cells around the middle MD was observed
233 under the control and Conditions 2, 3, and 4 (Fig. 9B, F, H, J), but not under Condition 1 (Fig. 9D).
234 The lumen of the WD was formed and developed in the control and under Conditions 1 to 3 (Fig. 9A–
235 H), but under Condition 4, the WD diameter was smaller and vacuoles were scattered in the epithelial
236 cells of the WD, indicating progressive regression (Fig. 9I, J). In females, there were no morphological
237 differences (Fig. 9K–T). These results were consistent with those obtained by evaluating MD
238 regression in the diameters of the cranial and middle MD (Fig. 8D–G).

DISCUSSION

We previously showed that AMH, although generally classified as a hormone,[20,36] acts in a secretory rather than an endocrine manner.[32] At that time, our immunohistochemistry results suggested that AMH infiltrates through the cranial region between the testis and mesonephros at the start of MD regression (Fig. 1). However, the pathway through which AMH acts on the MD, especially its middle part, remained unclear. This was because nonspecific cross-reactivity is observed by using high concentrations of commonly used anti-AMH antibodies, and it is difficult to specifically detect low concentrations of AMH in the mesonephros. Therefore, in the present study we used an organ culture system to investigate the details of the AMH diffusion pathway.

AMH is synthesized in the testes at 12.0 dpc and not transcribed in the mesonephros.[16,17] AMH was detected in the mesonephros at 12.5 dpc in the present study. Furthermore, our findings indicated that the physiological concentrations of AMH infiltrate the mesonephros from the testis after 12.5 dpc. On the other hand, MD regression was suppressed when the testis and the mesonephros were cultured separately. This means that AMH infiltrating the mesonephros at 12.5 dpc is insufficient to induce MD regression and that such regression requires AMH infiltration after 12.5 dpc.

The results of the organ culture revealed that AMH infiltrates from the cranial testis to the mesonephros at around 12.5 dpc and induces cranial MD regression. Those results also indicated that AMH infiltrates mesonephros from the caudal testis and induces regression of the middle MD (Fig. 10A). No tubular structure extended from the testis to the vicinity of the MD at the caudal testis,[33,37] suggesting that AMH produced in Sertoli cells diffuses into the interstitium in the testes and infiltrates the mesenchyme at the boundary region between the testis and the mesonephros. The pathway of AMH diffusion through the RC was also reconfirmed in this study by immunohistochemistry. It is unclear whether AMH reaches the MTs and WD via the RC after secretion into the luminal side and migration to the RC. However, it is possible that AMH infiltrated the mesenchyme of the mesonephros from the

MTs because the basement membranes of MTs may be discontinuous at the start of the MD.[37] The middle MD diameter measurements in Conditions 2, 3, and 4 indicated that AMH infiltration through the mesenchyme from the caudal testis into the mesonephros has a greater influence on the middle MD regression than the presence of AMH in the vicinity of the MD through the RC, MTs, and WD. Interestingly, when the testis–mesonephros complexes were cultured under the condition in which MTs connected with the WD were incised (Condition 4), the WD regressed. Tong *et al.* (1996) found that testosterone produced in the testis diffused through the RC, MTs, and WD.[31] Therefore, the present result indicated that testosterone passing through the RC, MTs, and WD is more important for the development of the WD than testosterone infiltrating the mesenchyme. The reason for the difference in migration modes between testosterone and AMH is unclear. Further studies are needed to clarify this point.

Our previous findings,[32] the present results of the injection of rh-AMH and the aniline blue staining, suggested that as development progresses, AMH infiltration from the caudal testis to the mesonephros becomes less likely. These collective results also suggested that it may become more important for AMH to reach the vicinity of the MD via the RC, MTs, and WD as development progresses (Fig. 10B). Since the RC, MTs, and WD are not connected to the MD, we speculated that AMH leaks from the RC, MTs, or WD into the mesenchyme of the mesonephros.

AMH is secreted by the granulosa cells of the ovary in adults, and its blood concentration is used as an index of ovarian function.[38] In the embryo, AMH is synthesized in Sertoli cells, regresses the MD as an endocrine hormone,[4,36] and regulates Leydig cell function by paracrine secretion.[36,39] We previously suggested that AMH in the fetal testis infiltrates to the mesonephros.[32] The present study demonstrated that the AMH infiltration from the testis to the mesonephros correlates with the regression of the MD. The present results indicated that, especially at the start of MD regression, AMH

286 migrates from the entire boundary region between the testis and the mesonephros. The extent of the
287 effect of AMH in the blood on early MD regression is unclear.[20,32,36] Freemartinism has been observed
288 in cattle. Shared circulation is a common occurrence in most cattle twins. For opposite-sex twins,
289 AMH passes from the male to the female through the blood vessels and influences the female MD.[40]
290 Therefore, it is assumed that AMH also passes through the blood vessels in mice. However,
291 freemartinism is not observed in mice; the structure of the placenta differs between mice and cattle. In
292 mice, true hermaphrodites, i.e., those with a testis on one side and an ovary on the other, experience
293 MD regression on the testis side, while no regression occurs on the ovary side.[27-29] In addition, Jost
294 (1953) showed that in male rabbit fetuses, MD develops only on the castrated side.[30] These reports
295 suggest that infiltrating AMH is more important than blood AMH, at least at the start of MD regression.
296 To achieve complete MD regression, AMH has to be present during the period before and after the
297 start of AMHR2 expression.[41] We speculated that AMH needs to reach around the MD early after MD
298 formation in order to induce MD regression, and thus a direct pathway around the MD may be
299 important. The mechanism underlying AMH infiltration is unclear, but we assume that AMH secreted
300 into the extracellular space diffuses according to a concentration gradient. Furthermore we suspect
301 that, rather than simple free diffusion, there might be a requirement for AMH-binding activity or a
302 scaffolding effect to facilitate rapid dispersion.

303 Organ culture systems have long been used to investigate the development of gonads and accessory
304 reproductive organs.[42-44] Various methods have been adopted in these organ culture studies, such as
305 culturing on agarose blocks, filters, or droplets; for research into accessory reproductive organs,
306 culturing on filters has been widely used.[7,45,46] In general, organ culture allows for a wide variety of
307 experimental interventions, including the addition of agonists and antagonists, partial excision of
308 organs, and combined culture with other organs.[7,45-48] Injection experiments have shown that
309 testosterone acts in an infiltrative manner during WD development.[31] In the case of the MD, the

addition of AMH to the culture medium induces regression of the duct.[35,49,50] In the present study, partial excision of the border region between the gonad and the mesonephros revealed the details of the migration pathway; this, too, is possible only in organ culture systems. On the other hand, the morphology of the mesonephros caudal to the testis, especially near the urogenital sinus, was not well conserved, and thus the AMH diffusion mechanism could not be investigated. It is assumed that the establishment of a culture system that can conserve this region will enable us to elucidate in detail the mechanism underlying MD regression.

In conclusion, this study revealed that physiological concentrations of AMH infiltrate the mesonephros from both the cranial and caudal testes, at least at the start of MD regression. The results also suggested that AMH infiltration through the mesenchyme from the caudal testis into the mesonephros may play a more important role in middle MD regression than AMH reaching the vicinity of the MD via the RC, MTs, and WD at the start of MD regression.

EXPERIMENTAL PROCEDURES

Animals

Both male and female ICR mice were purchased from SLC Japan (Hamamatsu, Japan) and maintained as described elsewhere.[32,51] Fetuses at 12.5, 13.5 and 15.5 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 was designated as 0.5 dpc. 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.

Injecting rh-AMH into the testis

Approximately 0.3–1 μl of recombinant human (rh)-AMH (100 μg/ml, 1737-MS; R&D Systems, Minneapolis, MN) was injected into the cranial testis, the center of the testis and the caudal testis at 12.5, 13.5 or 15.5 dpc using a glass capillary (approximately 40 μm in diameter). Then, the testis–mesonephros complex was placed on an agar block in 150 μl of Dulbecco's Modified Eagle Medium (DMEM 4.5 g/l glucose with L-Gln; Nacalai Tesque, Kyoto, Japan) containing HEPES at 4°C for 1 hr.

Organ culture

A small gel cassette with a gap of about 90 μm was prepared by stacking two pieces of scotch tape (BK-12N; 3M Japan, Tokyo). Approximately 300 μl of 4% agarose gel was spread into a sheet using the gel cassette and dried. Rectangular pieces (0.8×5 mm) were cut from the agar sheet with a scalpel blade (no.11; Feather Safety Razor, Osaka, Japan).

To clarify the pathway by which AMH diffused to each region, the gonad–mesonephros complexes at 12.5 dpc were prepared in five conditions (Fig. 8A). Then, as described elsewhere,[32,51] each sample

346 was placed in a groove of an agarose gel block created using the needle sections of 21-gauge needles
347 (Terumo, Tokyo) and cultured for 72 hr. A gel sheet (one of the small pieces from the agar sheet) of
348 approximately the same length as the incision was inserted to prevent the tissue from reattaching (Fig.
349 5A). Pieces of aluminum foil or vinyl, although impermeable, were not suitable for culture because
350 they adhered to the tissue (data not shown). Then, a gel sheet was placed on each gonad–mesonephros
351 complex to maintain the natural shape of the gonads by adding weight.
352
353 *Histological analysis*
354 The gonad–mesonephros complexes before and after culture were fixed in Zamboni fixative
355 containing 4% paraformaldehyde at 4°C for 24 hr. The specimens were dehydrated with an ethanol
356 series followed by xylene and then embedded in paraffin. Then, 5 μm-thick sections at 25 μm intervals
357 were cut by a sliding microtome and placed on slide glasses that had been precoated with 2% 3-
358 aminopropyltriethoxysilan (Shin-Etsu Chemical, Tokyo) and stored at -18°C until use.
359 After deparaffinization and hydration, the sections were stained with hematoxylin and eosin (HE)
360 or aniline blue. The latter staining involved overnight mordanting with an equal volume mixture of
361 2.5% phosphotungstic acid and 2.5% phosphomolybdic acid, followed by a 10-second wash with 1%
362 acetic acid. The sections were immersed in aniline blue solution for 2 hr, washed with 1% acetic acid
363 for 10 seconds, and then fractionated with isopropyl alcohol.
364 Immunohistochemical staining was carried out as described previously.[32] For the primary antibody,
365 anti-Amh goat polyclonal antibody (1:6,000; #sc-6886, Santa Cruz Biotechnology, Dallas, TX, USA)
366 was used. For the secondary antibody, peroxidase-conjugated donkey anti-goat IgG (1:200, ab97112;
367 Abcam, Cambridge, U.K.) was used.
368
369 *Western blotting*

We used male mesonephroi at 12.5 dpc (n=22) and cultured them for 72 hr under control conditions (n=30). Normal ovaries at 12.5 dpc (n=5) were used as a negative control for AMH, and testes cultured for 72 hr under the control condition (n=5) were used as a positive control for AMH. The collected samples were snap-frozen in liquid nitrogen and stored at −80°C until use. WB was carried out as described previously.[32] WIDE-VIEW Prestained Protein Size Marker III (#230-02461, Wako, Tokyo, Japan) was used as a guide for cutting. For the primary antibody, anti-AMH goat polyclonal antibody (1:800) or anti-GAPDH mouse monoclonal antibody (1:4,000; #016-25523, Wako) was used. For the secondary antibody, an HRP-labeled anti-goat IgG donkey antibody (1:20,000; #705-035-147, Jackson Immuno Research Laboratories, West Grove, PA, USA) or HRP-labeled anti-mouse IgG rat antibody (1:20,000; #415-035-166, Jackson Immuno Research Laboratories) was used.

Measuring MD diameter

The short diameters of the cranial and middle MD were measured in four HE-stained sections for each sample, and the mean value for each region was calculated (Fig. 6A). We evaluated the diameter of the MD using the short diameter rather than the average of the long and short diameters in order to minimize measurement error in case the MD was cut diagonally. The degree of MD regression was compared using a Kruskal–Wallis test and a Steel–Dwass test or Mann–Whitney *U* test (BellCurve for Excel, version 3.23; SSRI, Tokyo). The results were considered significant when the *P*-value was less than 0.05.

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Fig. 1 Schematic diagram of Müllerian duct (MD) and Wolffian duct (WD) in the developing mouse. The MD and WD form in both sexes during early development. During the developmental stage when anti-Müllerian hormone (AMH) expression is initiated within the testis cord, the elongation of the WD is finalized, and the elongation of the MD extends to approximately the most caudal region of the testis. At the start of MD regression, the elongation of the MD is almost completed (part of the pink dotted line). As development progresses, the MD regresses and the WD develops in males. In females the WD regresses and the MD develops inversely. In this study, approximately the cranial half of the gonad is defined as the cranial gonad and the caudal half as the caudal gonad. The MD located at the same level as the cranial gonad was defined as cranial MD, the MD located at the same level as the caudal gonad as middle MD, and the MD located posterior to the caudal end of the gonad as caudal MD.

Fig. 2 Immunoreactivity of anti-Müllerian hormone (AMH) at 12.5 (A), 13.5 (B), and 15.5 dpc (C). AMH was strongly detected in Sertoli cells in the testis cord (TC; A, B, and C). A weak immunoreaction was observed in the rete cord (RC) where it connects with the TC (dashed line in B' and C') and in the interstitium of the testis (A–C). A weak immunoreaction was also observed in the mesenchyme of the mesonephros at 12.5, 13.5, and 15.5 dpc (A–C). Tes, testis; Mes, mesonephros.

Fig. 3 Aniline blue staining for transverse sections of testis–mesonephros complexes at 12.5 (A and B), 13.5 (C and D), and 15.5 dpc (E and F) in males. The regions with less connective tissue (between arrows of the same color in A'–E') were observed at the connection between the testis and mesonephros at each dpc. At 15.5 dpc (E and F), the tunica albuginea (red bars in B', D', and F') was thick and the boundary region between the testis and the mesonephros was narrow. Tes, testis; Mes, mesonephros.

Fig. 4 Immunoreactivity of anti-Müllerian hormone (AMH) on sections of recombinant human (rh)-AMH–injected testis at 12.5 dpc. Rh-AMH was injected into the cranial testis (A and B) and into the caudal testis (C and D). A (A') A strong positive reaction was observed throughout the entire testis, including the interstitium. Positive reactions were detected from the boundary region between the cranial testis and the mesonephros to the vicinity of the Müllerian duct (MD). B (B') The caudal section was negative in the mesonephros. C (C') The section of the cranial region was negative in the mesonephros. D (D') A strong positive reaction was observed throughout the entire testis, including the interstitium. Positive reactions were detected from the boundary region between the caudal testis and the mesonephros to the vicinity of the MD. WD, Wolffian duct; Tes, testis; Mes, mesonephros.

Fig. 5 Immunoreactivity of anti-Müllerian hormone (AMH) on sections of a recombinant human (rh)-AMH–injected testis at 15.5 dpc. Rh-AMH was injected into the cranial testis (A), the center of the testis (B), or the caudal testis (C). A positive reaction was observed in the testis cords and the interstitium near the injection site (A–C). Weak positive reactions were detected in the rete cord (arrowheads: A', B'). AMH was not detected in the mesenchyme at the boundary region between the testis and the mesonephros (A'–C'). Arrows indicate the rh-AMH injection site inside the testis (A–C). Tes, testis; Mes, mesonephros.

Fig. 6 Diameters of the cranial and middle Müllerian duct (MD) at 12.5 and 13.5 dpc. (A) The small diameters of the cranial and middle MD were measured at intervals of 25 μm for four HE-stained sections to evaluate the degree of MD regression. (B) At 13.5 dpc, males had a significantly smaller cranial MD diameter than females ($p<0.001$). The diameter of the cranial MD in males at 12.5 dpc was significantly smaller than that in females at 13.5 dpc ($p=0.014$). (C) At 13.5 dpc, the middle MD

591 diameter in males was significantly smaller than that in females (p=0.005). The diameter of the middle
592 MD in males at 13.5 dpc was significantly smaller than that in females at 12.5 dpc (p=0.015). The
593 rhombus in the scatter plot indicates the mean ± SD. Numbers in parentheses are the sample numbers.
594 Data are presented as means ±SD. WD, Wolffian duct; MTs; Mesonephric tubules.
595
596 Fig. 7 Quantitative analysis of anti-Müllerian hormone (AMH) in the male mesonephros at 12.5 dpc
597 or in the cultured mesonephros. (A) AMH expressed in the mesonephros at 12.5 dpc or of the testis–
598 mesonephros complex cultured for 72 hr from 12.5 dpc by Western blotting. Normal ovaries at 12.5
599 dpc were used as a negative control for AMH, and cultured testes under the control were used as a
600 positive control for AMH. AMH bands (12.5 kDa) were detected in male mesonephroi at 12.5 dpc, the
601 cultured mesonephroi and the cultured testes. The glyceraldehyde-3-phosphate dehydrogenase
602 (GAPDH) bands (37 kDa) were detected in all samples. (B) The amounts of AMH in the mesonephroi
603 at 12.5 dpc and cultured mesonephroi. The band intensity of AMH was quantified by using the values
604 of the GAPDH bands. When the amount of AMH in the mesonephroi at 12.5 dpc was set at 1, the
605 amount in the cultured mesonephroi was 2.2. Mes, mesonephroi; Tes, testis; Ov, ovary.
606
607 Fig. 8 Culture conditions and diameters of cultured Müllerian duct (MD). (A) Culture conditions.
608 Control: gonad–mesonephros complexes were cultured. Condition 1: gonad and mesonephros were
609 separated. Condition 2: the cranial boundary region between gonad and mesonephros was incised.
610 Condition 3: the caudal boundary region between gonad and mesonephros was incised. Condition 4:
611 Mesonephric tubules (MTs) connected to the Wolffian duct (WD) were incised. In Conditions 1–4, a
612 gel sheet was inserted in the incision to prevent tissue from reattaching. At the beginning of culture,
613 the MD was elongated to the position of the most caudal part of the testis. (B) Under all conditions,
614 testes and mesonephroi exhibited enlargement after 72 hr of culture. (C) AMH immunoreaction in

615 cultured testes under all five culture conditions (Conditions 1 to 4 and control). AMH was detected in
616 Sertoli cells in the testis cords under all conditions. (D–G) Diameters of cultured MD. Diameters of
617 the cranial (D, F) and middle (E, G) MD of male and female mice under each condition after incubation.
618 In males, the cranial MD diameter was significantly increased under Conditions 1 (p=0.030) and 2
619 (p=0.027) compared to the control (D). The middle MD diameter significantly increased under
620 Condition 1 (p=0.024, E). Females showed no significant differences among conditions in the cranial
621 or middle MD (F and G). The rhombus in the scatter plot indicates means ± SD. Circles are outliers.
622 Numbers in parentheses are the sample numbers. Data are means ±SD. Tes, testis; Mes, mesonephros.
623
624 Fig. 9 HE staining for the cultured gonad–mesonephros complexes of male (A–J) and female (K–T)
625 mice. (A and B) Under the control condition, condensation of the mesenchymal cells was observed
626 around the cranial and middle Müllerian duct (MD). (C and D) Under Condition 1, condensation of
627 the mesenchymal cells was not observed around the cranial or middle MD. (E and F) Under Condition
628 2, condensation of the mesenchymal cells was observed around the middle MD and not around the
629 cranial MD. (G and H) Under Condition 3, condensation of the mesenchymal cells was observed
630 around the cranial and middle MD. (I and J) Under Condition 4, condensation of the mesenchymal
631 cells was observed around the middle MD and not around the cranial MD. In the epithelial cells of the
632 Wolffian duct (WD), vacuoles were scattered (I' and I'', arrows). (K–T) Condensation of the
633 mesenchymal cells was not observed around the cranial and middle MD under any conditions.
634
635 Fig. 10 Schematic diagram of the pathway of anti-Müllerian hormone (AMH) diffusion from the testis
636 to the mesonephros in the developing mouse. (A) In the periods before and after the start of the
637 regression of the Müllerian duct (MD), AMH infiltrates the mesonephros through both the cranial and
638 caudal testes. (B) As development progresses and the formation of connective tissue between the testis

639 and mesonephros advances, AMH infiltration from the caudal testis to the mesonephros becomes less
640 likely, and AMH diffuses to the rete cord (RC) and may also diffuse to mesonephric tubules (MTs)
641 and the Wolffian duct (WD). The gray dotted line indicates that the MD is regressing.
642

Fig. 1

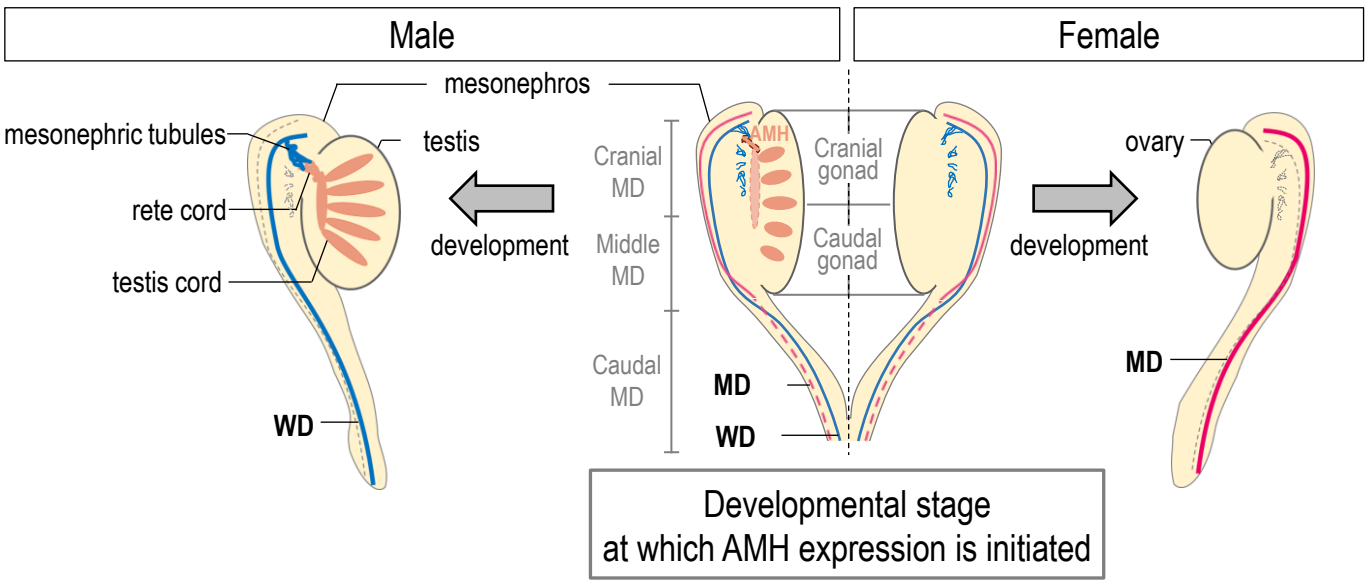

Male
Female
mesonephros
mesonephric tubules
testis
rete cord
testis cord
development
WD
Cranial MD
Middle MD
Caudal MD
AMH
Cranial gonad
Caudal gonad
MD
WD
development
ovary
MD
Developmental stage
at which AMH expression is initiated

Fig. 2

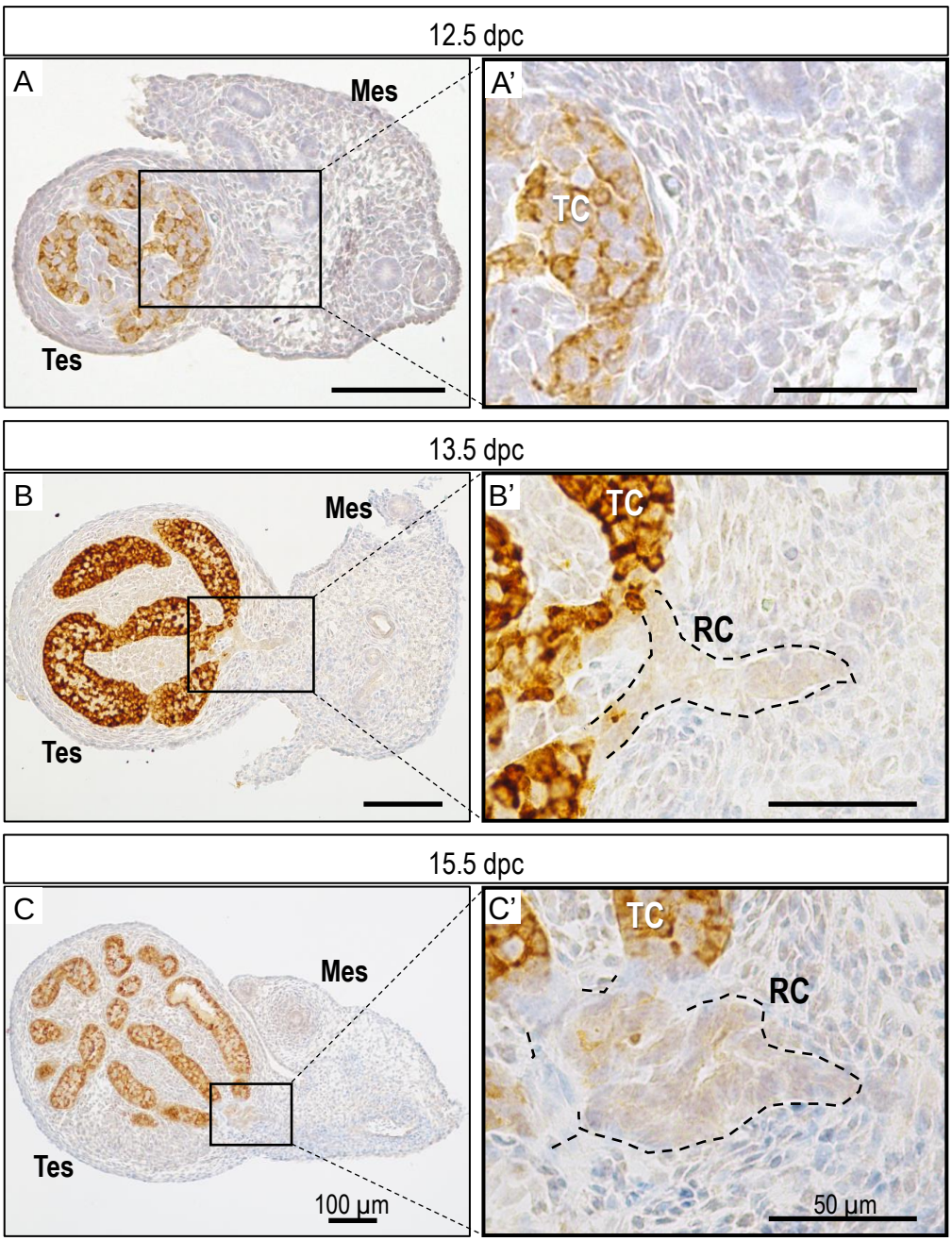

12.5 dpc
A
Mes
Tes
A'
TC
13.5 dpc
B
Mes
Tes
B'
TC
RC
15.5 dpc
C
Mes
Tes
100 μm
C'
TC
RC
50 μm

Fig. 3

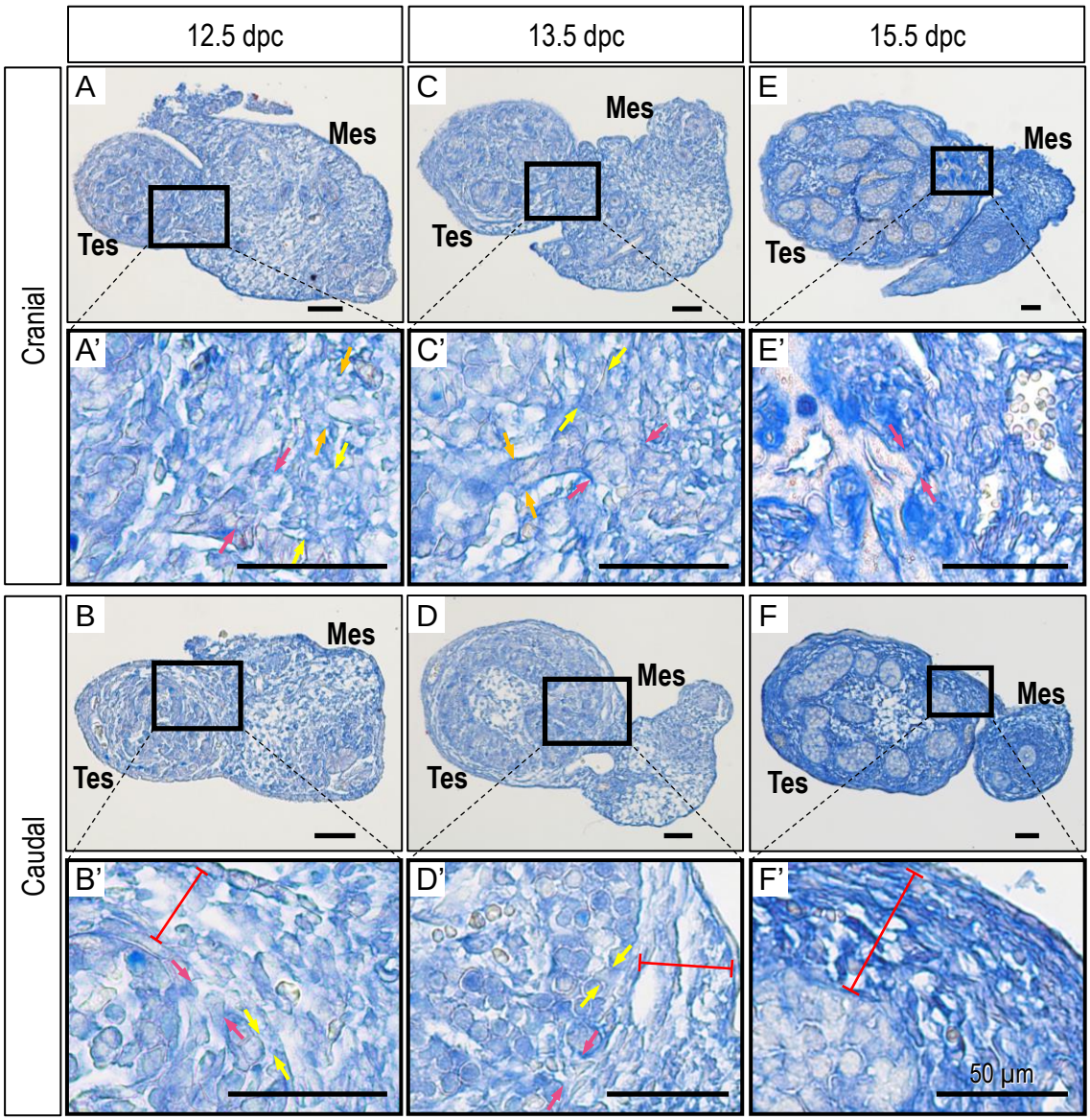

12.5 dpc
13.5 dpc
15.5 dpc
Cranial
Caudal
A
A'
B
B'
C
C'
D
D'
E
E'
F
F'
Mes
Tes
50 μm

Fig. 4

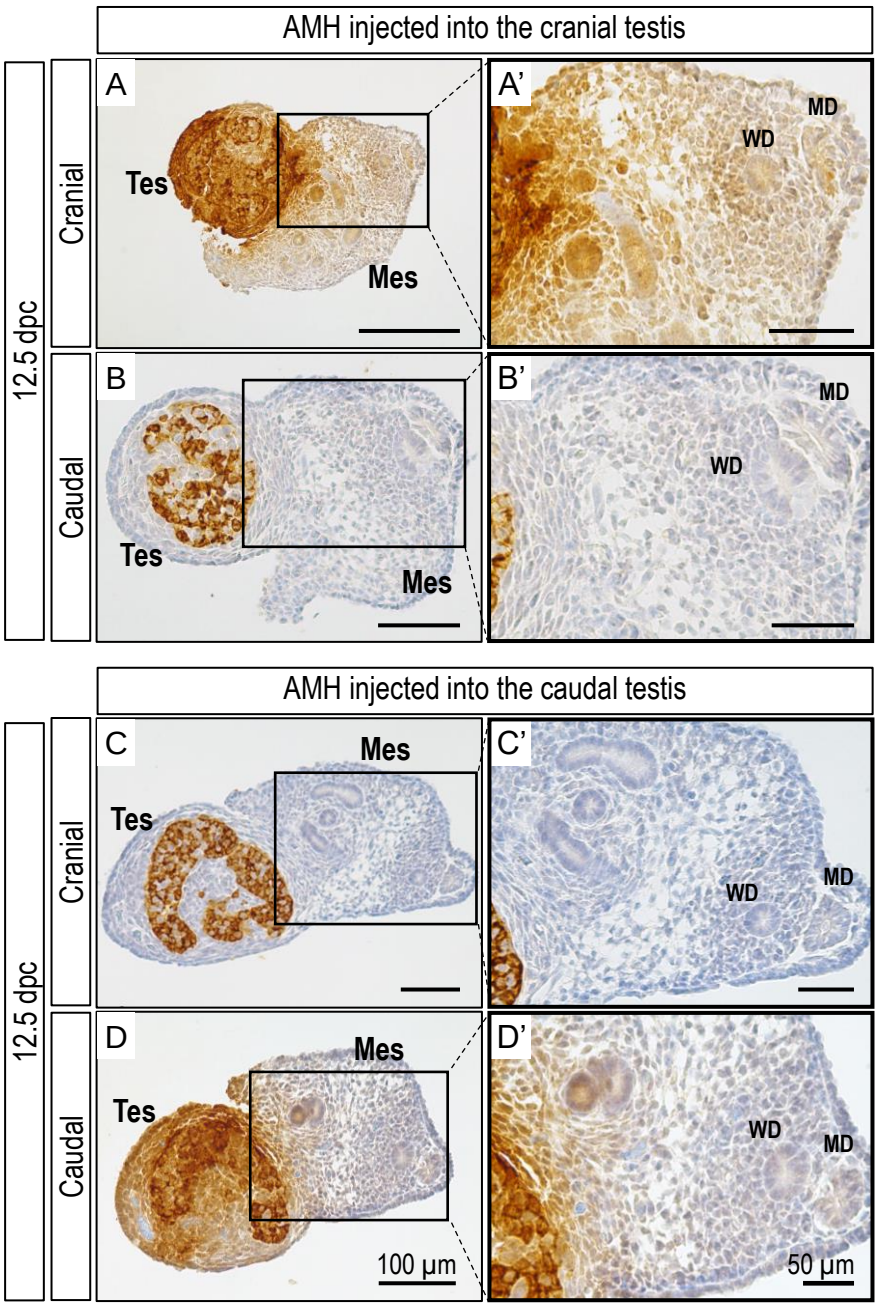

AMH injected into the cranial testis
12.5 dpc
Cranial
Caudal
A
A'
B
B'
Tes
Mes
MD
WD
AMH injected into the caudal testis
C
C'
D
D'
100 μm
50 μm

Fig. 5

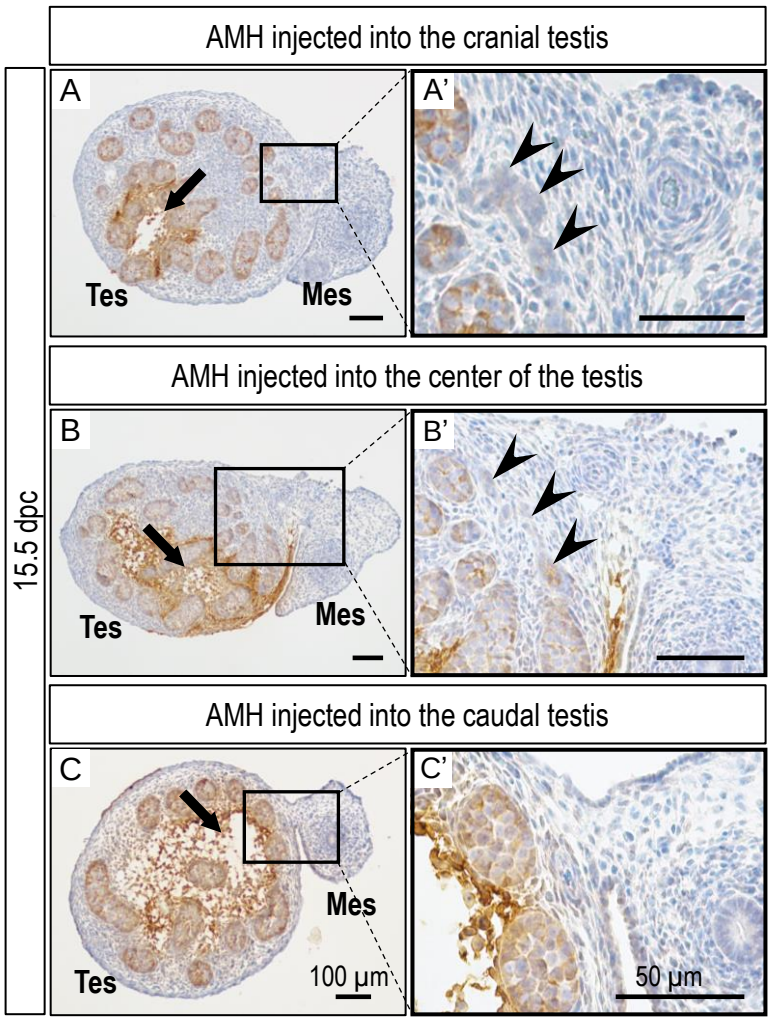

AMH injected into the cranial testis
A
A'
Tes
Mes
AMH injected into the center of the testis
B
B'
Tes
Mes
AMH injected into the caudal testis
C
C'
Mes
Tes
100 μm
50 μm
15.5 dpc

Fig. 6

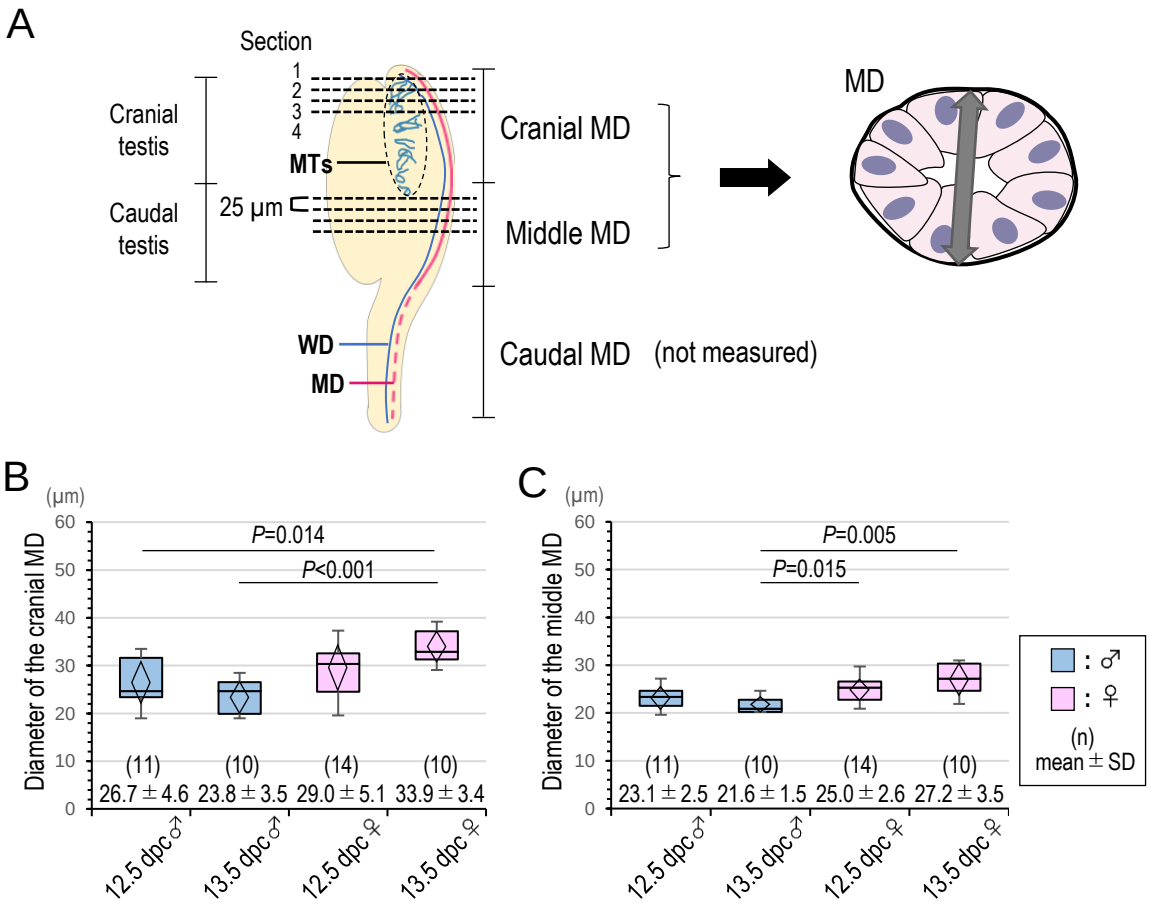

A
Section
1
2
3
4
Cranial testis
Caudal testis
MTs
25 μm
WD
MD
Cranial MD
Middle MD
Caudal MD (not measured)
MD
B
(μm)
Diameter of the cranial MD
P=0.014
P<0.001
(11) 26.7 ± 4.6
(10) 23.8 ± 3.5
(14) 29.0 ± 5.1
(10) 33.9 ± 3.4
12.5 dpc♂
13.5 dpc♂
12.5 dpc♀
13.5 dpc♀
C
(μm)
Diameter of the middle MD
P=0.005
P=0.015
(11) 23.1 ± 2.5
(10) 21.6 ± 1.5
(14) 25.0 ± 2.6
(10) 27.2 ± 3.5
12.5 dpc♂
13.5 dpc♂
12.5 dpc♀
13.5 dpc♀
: ♂
: ♀
(n)
mean ± SD

Fig. 7

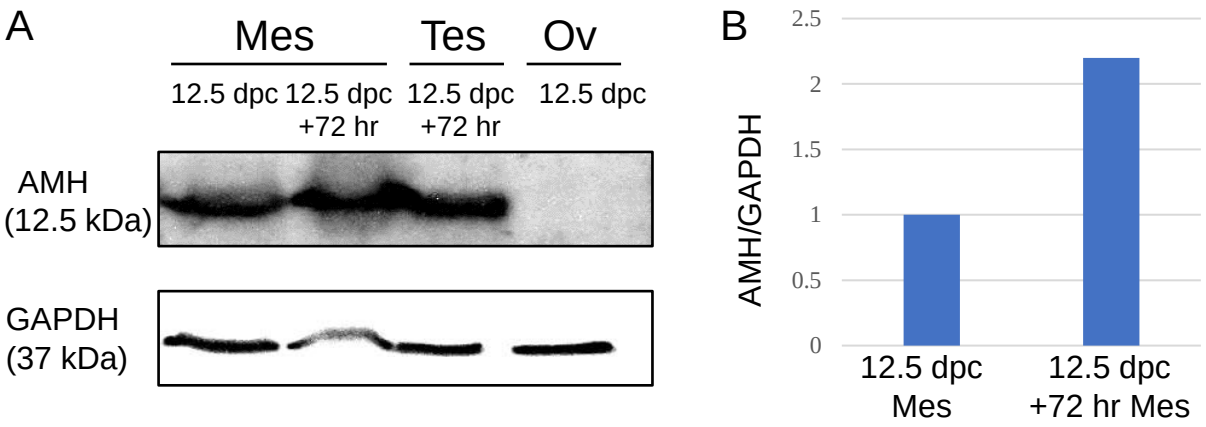

A
Mes
Tes
Ov
12.5 dpc
12.5 dpc +72 hr
12.5 dpc
+72 hr
12.5 dpc
AMH
(12.5 kDa)
GAPDH
(37 kDa)
B
AMH/GAPDH
2.5
2
1.5
1
0.5
0
12.5 dpc Mes
12.5 dpc +72 hr Mes

Fig. 8

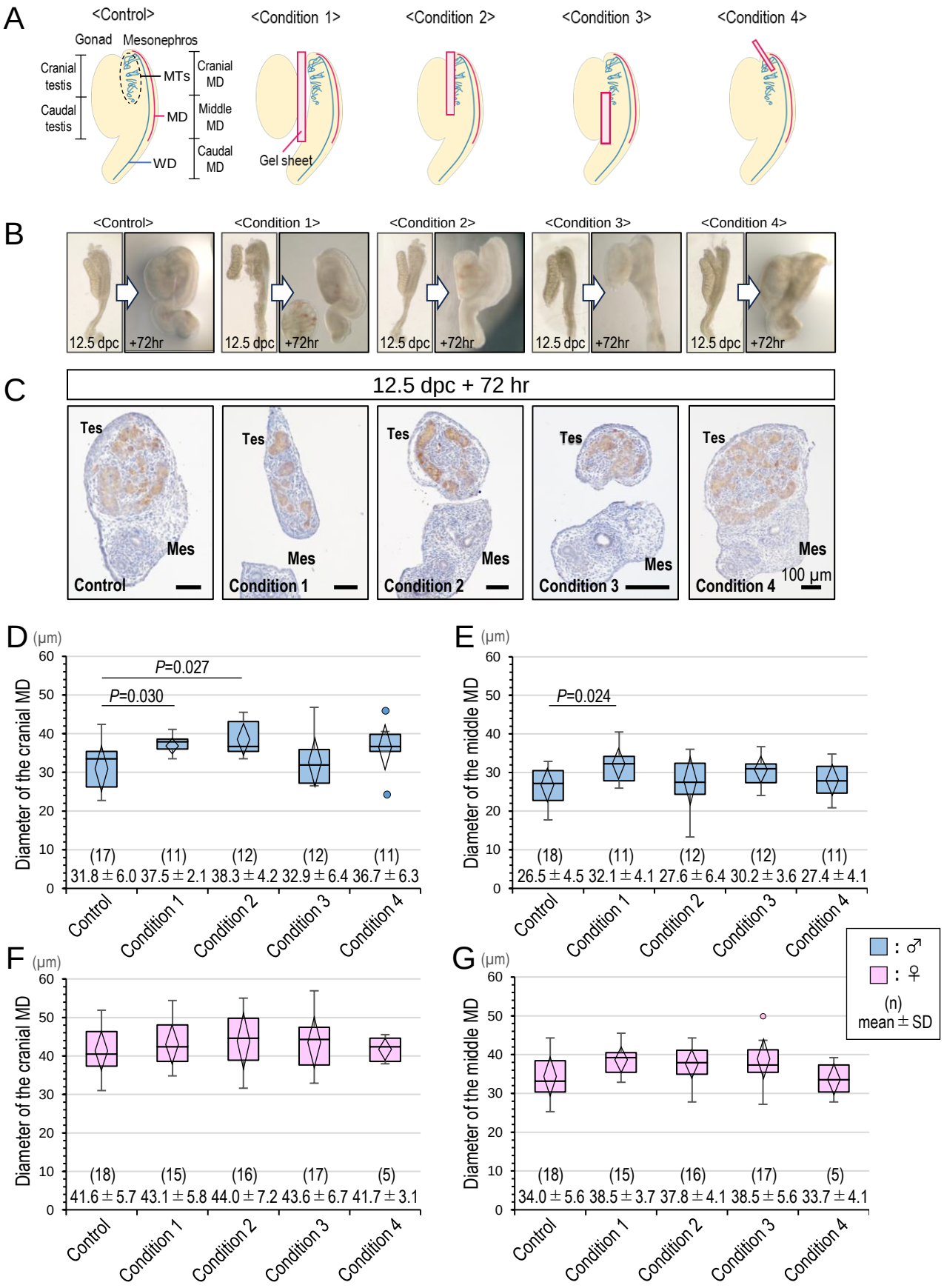

A
<Control>
<Condition 1>
<Condition 2>
<Condition 3>
<Condition 4>
Gonad
Mesonephros
Cranial testis
Caudal testis
MTs
MD
WD
Cranial MD
Middle MD
Caudal MD
Gel sheet
B
<Control>
<Condition 1>
<Condition 2>
<Condition 3>
<Condition 4>
12.5 dpc
+72hr
C
12.5 dpc + 72 hr
Tes
Mes
Control
Condition 1
Condition 2
Condition 3
Condition 4
100 μm
D
(μm)
Diameter of the cranial MD
P=0.027
P=0.030
(17) 31.8 ± 6.0
(11) 37.5 ± 2.1
(12) 38.3 ± 4.2
(12) 32.9 ± 6.4
(11) 36.7 ± 6.3
E
(μm)
Diameter of the middle MD
P=0.024
(18) 26.5 ± 4.5
(11) 32.1 ± 4.1
(12) 27.6 ± 6.4
(12) 30.2 ± 3.6
(11) 27.4 ± 4.1
F
(μm)
Diameter of the cranial MD
(18) 41.6 ± 5.7
(15) 43.1 ± 5.8
(16) 44.0 ± 7.2
(17) 43.6 ± 6.7
(5) 41.7 ± 3.1
G
(μm)
Diameter of the middle MD
(18) 34.0 ± 5.6
(15) 38.5 ± 3.7
(16) 37.8 ± 4.1
(17) 38.5 ± 5.6
(5) 33.7 ± 4.1
: ♂
: ♀
(n)
mean ± SD

Fig. 9

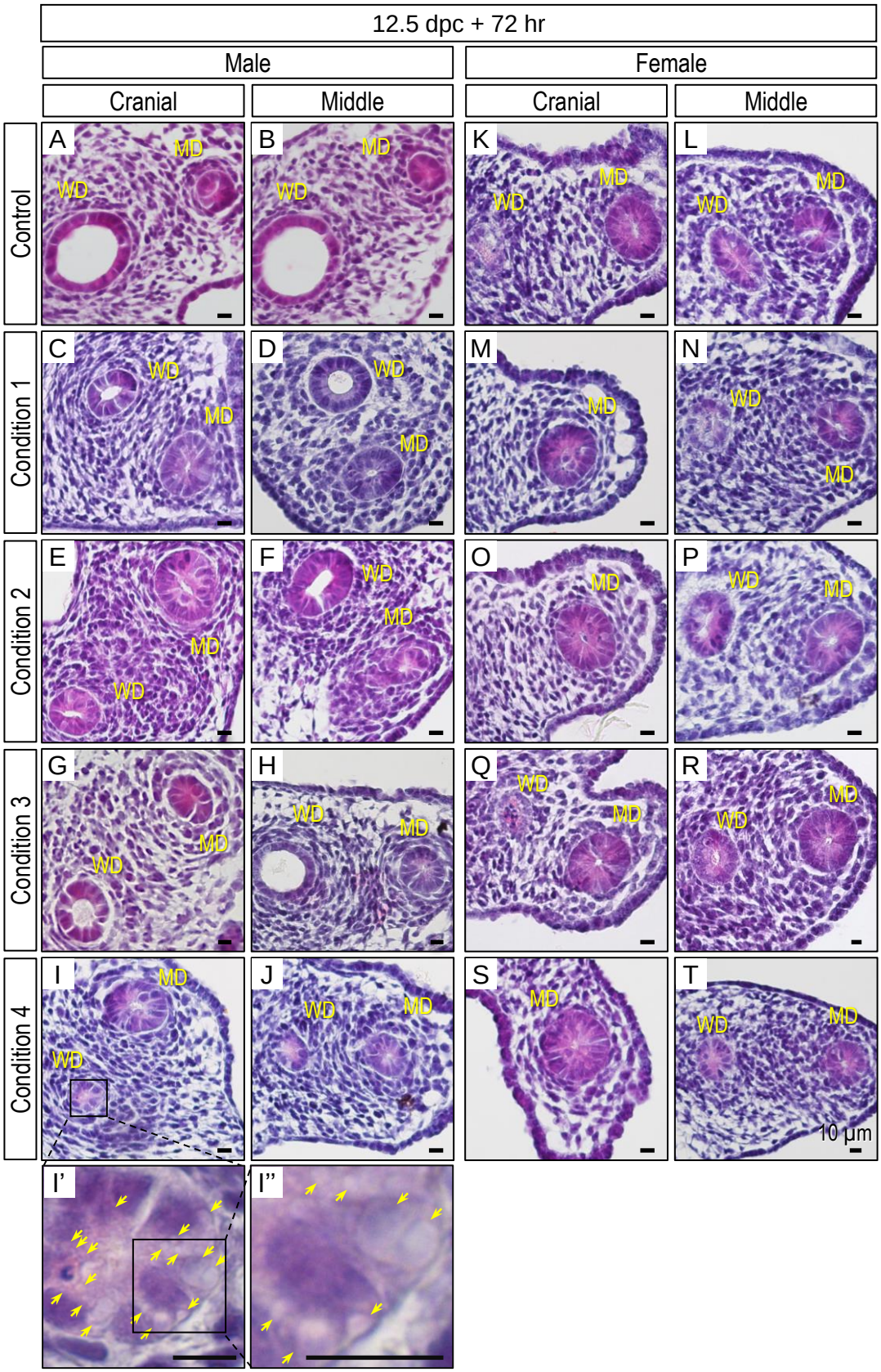
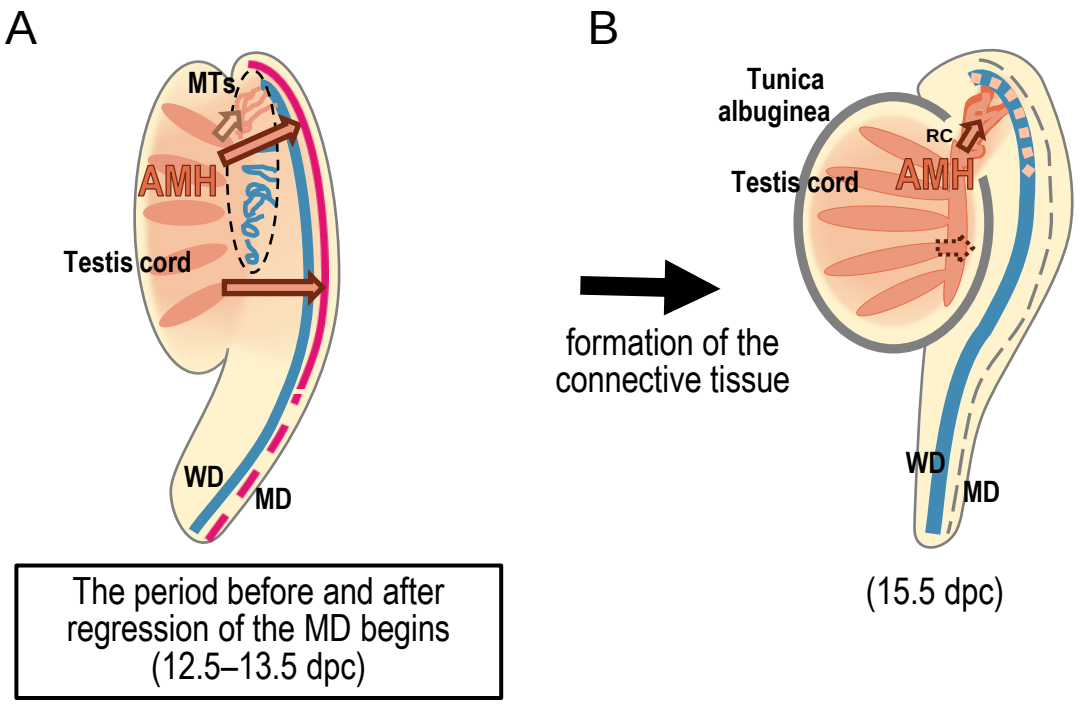

12.5 dpc + 72 hr
Male
Female
Cranial
Middle
Cranial
Middle
Control
Condition 1
Condition 2
Condition 3
Condition 4
A
B
K
L
C
D
M
N
E
F
O
P
G
H
Q
R
I
J
S
T
I'
I''
WD
MD
10 μm

Fig. 10


A
MTs
AMH
Testis cord
WD
MD
The period before and after regression of the MD begins (12.5–13.5 dpc)
B
Tunica albuginea
RC
Testis cord
AMH
formation of the connective tissue
WD
MD
(15.5 dpc)