学位論文

Photoperiod-regulated expression of sex differentiation-related genes and its association with AMH signaling system in the testis of the adult quail

（成体ウズラの精巣における光周期による性分化関連遺伝子の発現制御とその AMH 情報伝達系への関与）

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Abstract

Gonadal sex differentiation proceeds by the interplay of various genes encoding transcription factors and secretory factors in a complex network. The sex differentiation-related genes have been reported to be expressed not only during early sex differentiation but also in the adult gonads. In addition, the studies using conditional knockout mice suggest that they are involved in the adult gonadal functions. On the other hand, few studies have been conducted from the viewpoint of gene network.

In the present thesis, I analyzed the expression profile of various sex differentiation-related genes in the testis of the adult Japanese quail (*Coturnix japonica*), whose testicular functions are dramatically changed by altering photoperiod, as a first step to understand their network in the adult gonad. Anti-Müllerian hormone (*AMH*) was significantly upregulated in the regressed testis induced by the short-day condition. The expression of the transcription factors that promote *AMH* expression in mammals (*SF1*, *SOX9*, *WT1*, and *GATA4*) was also increased in the regressed testis. The putative binding sites for these transcription factors were present in the quail *AMH* promoter region. Moreover, AMH-specific type II receptor (*AMHR2*) showed an expression pattern similar to its ligand during the testicular changes induced by photoperiod. These results suggest that the sex differentiation-related genes function via AMH in the adult testis.

AMH is a member of the transforming growth factor beta (TGF-β) superfamily. Most of this superfamily members are known to contribute to testicular functions such as spermatogenesis. There are complex signaling cross-talks in this superfamily because their receptors and intra-cellular signaling molecules called SMADs are shared among different kinds of ligands. Therefore, I also analyzed the expression of other transforming growth factor beta (TGF-β) superfamily members and their receptors to examine their association with the AMH signaling. The expression of the ligands and receptors of TGF-β family, and follistatin and betaglycan, which inhibit activin signaling, was increased in the regressed testis. In addition, their expression was

decreased at Stage III, when spermatocytes appeared in the seminiferous tubules, during the testicular changes induced by the long-day condition. These results suggest that AMH is involved in the regulation of spermatogonial proliferation and differentiation together with other TGF-β superfamily members.

In conclusion, the results obtained in this study strongly suggest that the sex differentiation-related genes work in a network that leads to the *AMH* expression and participate in the regulation of spermatogenesis in the adult quail testis.

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Introduction

The genes involved in gonadal sex differentiation

The gonads are the organ that plays an essential role in vertebrate reproduction and functionally different between in males and females. The male gonad, the testis, and the female gonad, the ovary, produce sex-specific gametes and hormones. These differences are formed through gonadal sex differentiation during early embryonic development. The genes implicated in gonadal sex differentiation have been characterized in humans and mice (reviews: Wilhelm *et al*., 2007, Eggers *et al*., 2014) since the discovery of the testis-determining gene *SRY* (Sex-determining Region on the Y chromosome) (Sinclair *et al*., 1990).

Most of the sex differentiation-related genes encode transcription factors. Wilms tumor 1 (WT1) and steroidogenic factor 1 (SF1 also known as NR5A1) are important for initial formation of the bipotential gonad (Kreidberg *et al*., 1993, Luo *et al*., 1994). SRY-box 9 (SOX9) (Vidal *et al*., 2001, Barrionuevo *et al*., 2006) and GATA binding protein 4 (GATA4) (Manuylov *et al*., 2011) are involved in testis differentiation. DSS-AHC critical region on the X chromosome protein 1 (DAX1 also known as NR0B1) was initially thought to have anti-testis functions (Bardoni *et al*., 1994, Swain *et al*., 1998), but it was also found to be essential for testis differentiation (Meeks *et al*., 2003a, b). It is suggested that DAX1 acts in a narrow window, and its activity and concentration are critical for normal testis differentiation (Ludbrook *et al*., 2004, Eggers *et al*., 2014). In humans, doublesex and mab-3 related transcription factor 1 (*DMRT1*) maps to a region of chromosome 9 that is associated with XY sex-reversal (Veitia *et al*., 1997). It is required for postnatal testis differentiation in the mouse (Raymond *et al*., 2000). Forkhead box L2 (FOXL2) is implicated in a human BPES syndrome that is characterized by eyelid abnormality and premature ovarian failure (Crisponi *et al*., 2001), and it is essential for granulosa cell differentiation in the mouse (Schmidt *et al*., 2004).

In addition to these transcription factors, secretory factors are also involved in gonadal sex

differentiation. Fibroblast growth factor 9 (FGF9) is a signaling molecule essential for testis differentiation (Colvin *et al*., 2001). Prostaglandin D_2, a product converted from prostaglandin H_2 by prostaglandin D_2 synthase (PTGDS), activates *Sox9* transcription and nuclear translocation (Moniot *et al*., 2009). Wingless-type MMTV integration site family member 4 (WNT4) and R-spondin1 (RSPO1) are signaling molecules involved in ovarian differentiation (Vainio *et al*., 1999, Jeays-Ward *et al*., 2003, Tomizuka *et al*., 2008). These genes found in mammals have been reported to be expressed during gonadal sex differentiation in other vertebrate classes (reviews: Morrish and Sinclair 2002, Cutting *et al*., 2013).

Gonadal sex differentiation proceeds by the interplay of these genes in a complex network (reviews: Wilhelm *et al*., 2007, Cutting *et al*., 2013, Eggers *et al*., 2014). The gene network is illustrated in Fig. I-1. For example, *Sox9* expression upregulated by *Sry* in males is maintained by *Fgf9* and *Ptgds* by establishing feed-forward loops (Wilhelm *et al*., 2007, Eggers *et al*., 2014). *Sox9* acts synergistically with *Sf1*, *Wt1*, and *Gata4* to upregulate the expression of anti-Müllerian hormone (*Amh*), a hormone required for the regression of Müllerian ducts in males (Lasala *et al*., 2004). In ovarian differentiation, *Rspo1* is required for *Wnt4* expression, and they function via the activation of beta-catenin (Eggers *et al*., 2014). *Wnt4* is known to repress the male pathway by upregulating the expression of *Dax1* (Jordan *et al*., 2001, Mizusaki *et al*., 2003), which represses the expression of *Amh* (Tremblay *et al*., 2001) and steroidogenic genes (Lalli *et al*., 1998). In addition, the mutually antagonistic interactions between the testicular and ovarian pathways ensure the maintenance of sex. *Rspo1* and *Wnt4* suppress the male pathway through inhibition of *Sox9* and *Fgf9* expression. In contrast, *Sox9* and *Fgf9* can inhibit the female pathway by suppressing *Rspo1* and *Wnt4* expression (Kim *et al*., 2006, Eggers *et al*., 2014).

Expression of the sex differentiation-related genes during postnatal gonadal development and in the adult gonads

The sex differentiation-related genes have been reported to be expressed during the postnatal gonadal development and even in the adult gonads. Their expression has thus far been examined mainly in mammals. For example, SOX9 was expressed in rat Sertoli cells throughout testicular development, and its expression showed spermatogenic cycle-dependence in the adult testis (Fröjdman *et al*., 2000). It was expressed in the mature ovary depending on the follicular cycle in the mouse (Notarnicola *et al*., 2006). WT1 was expressed in the somatic and germ cells of the developing testis and ovary in the tammar wallaby. In the adult gonads, its expression was dynamically regulated during spermatogenesis and oogenesis (Pask *et al*., 2007). GATA4 was expressed in Sertoli and Leydig cells from fetal to adult testis in the mouse (Ketola *et al*., 2002). In the adult female mouse, *Gata4* mRNA was abundantly expressed in granulosa cells of primary and antral follicles (Heikinheimo *et al*., 1997). SF1 and DAX1 were expressed throughout testicular development in the rat and human, and DAX1 expression in the adult rat Sertoli cells showed spermatogenic cycle-specific pattern (Kojima *et al*., 2006). In the human ovary, DAX1 and SF1 expression in granulosa cells increased after the preantral follicular stage. In theca cells, SF1 expression increased according to the development of the follicle (Sato *et al*., 2003). SF1 was expressed in the adult testis and ovary in the tammar wallaby (Whitworth *et al*., 2001). DMRT1 was expressed in Sertoli and germ cells during postnatal testicular development and in the adult testis in the mouse (Lei *et al*., 2007). In female adult mouse, it was expressed in the granulosa cells of all developing follicles in the ovary (Pask *et al*., 2003). DMRT1 was expressed from fetal to adult testis and ovary in the tammar wallaby (Pask *et al*., 2003).

Moreover, seasonal changes of the expression of sex differentiation-related genes in the adult gonads have been reported from several species. For example, *SOX9* expression was increased in

the inactive testis in the non-breeding season, while that of *WT1*, *SF1*, and *DMRT1* was increased in the active testis in the Iberian mole (Dadhich *et al*., 2011). *sox9* and *dmrt1* were highly expressed when spermatogenesis was active in the catfish (Raghuveer and Senthilkumaran 2009, 2010) and lambari fish (Adolfi *et al*., 2015). High expression of *DMRT1* during active spermatogenesis was also reported in the lacertid lizard (Capriglione *et al*., 2010) and rainbow trout (Marchand *et al*., 2000).

Functional analysis of the sex differentiation-related genes using conditional knockout mice

The studies using knockout (KO) mice after sex differentiation suggest that the sex differentiation-related genes are involved in the gonadal functions after early sex differentiation period. For example, conditional *Sf1* KO mouse testis showed a delay in Sertoli cell maturation, fewer proliferative germ cells, and larger number of apoptotic cells (Kato *et al*., 2012). Conditional KO mice of *Gata4* showed age-dependent testicular atrophy and loss of fertility coincident with the decreases in the quantity and motility of sperm (Kyrönlahti *et al*., 2011). Spermatogenic failure was observed in the adult testis of conditional *Sox9* null mutant mice (Barrionuevo *et al*., 2009). In female mice, conditional *Wnt4* KO resulted in premature ovarian failure (Prunskaite-Hyyryläinen *et al*., 2014).

Moreover, gonadal dysfunction has been observed in the mice even when sex differentiation-related genes were deleted in the adult gonads. For example, germ cell death and blood–testis barrier disruption were observed in *Wt1* deficient adult mouse testis (Wang *et al*., 2013). In this mouse, the expression of the genes related to steroidogenesis and testosterone production was also decreased (Chen *et al*., 2014). Loss of *Dmrt1* in mouse Sertoli cells activated *Foxl2* expression and reprogramed Sertoli cells into granulosa cells even in adult (Matson *et al*., 2011). Similar to *Dmrt1* in the testis, inducible deletion of *Foxl2* in the adult mouse ovarian follicles led

to the upregulation of testis-specific genes including *Sox9* and reprogramed the granulosa and theca cell lineages into Sertoli-like and Leydig-like cell lineages occurred with testosterone levels comparable to those in normal males (Uhlenhaut *et al*., 2009).

These studies suggest that the sex differentiation-related genes are involved in the adult gonadal functions. On the other hand, few studies have not been conducted from the viewpoint of gene network. From this situation, I felt it necessary to analyze the expression profile of various sex differentiation-related genes to understand their network in the adult gonad.

Japanese quail as the experimental animal for study in the adult gonad

As the experimental animal in this study, I chose the Japanese quail (*Coturnix japonica*). They are a long-day breeder, and their testicular morphology and activity are rapidly and dramatically changed by photoperiod. Their testicular weight can change about hundred-fold within 30 days (Follett and Farner 1966) (Fig. I-2). This change largely reflects the degree of spermatogenic progression (the amount of seminal fluid and germ cell numbers) and is much greater and faster than those in other seasonal breeders. For example, even in the Djungarian hamster, which is known to show great photoperiodic response among mammals, the change of testicular weight is about 20-fold in 11 weeks (Meachem *et al*., 2005). This dynamic testicular change in the quail is reversible and accompanies tissue regression and reconstruction (Eroschenko and Wilson 1974). These features should be a great advantage to elucidate the expression profile of the sex differentiation-related genes associated with the gonadal changes.

Aim of the present thesis

From the background described above, the present thesis aimed to clarifying the expression profile of various sex differentiation-related genes associated with photoperiod-regulated changes

of the quail testis as a first step to understand their network in the adult gonad. I analyzed the expression of various sex differentiation-related genes encoding transcription factors and secretory factors in the adult testis of the quail at long-day or short-day condition. From the results of the expression analysis, I focused on the AMH signaling system. AMH is a member of the transforming growth factor beta (TGF-β) superfamily including TGF-βs, activins, inhibins, and bone morphogenetic proteins (BMPs). Most of this superfamily members are known to contribute to testicular functions such as spermatogenesis (review: Itman *et al*., 2006).

The ligands of TGF-β superfamily bind to two different serine/threonine kinase receptors referred to as type I and type II, and phosphorylate the intracellular signaling molecules called SMADs (contraction of the *Caenorhabditis elegans* protein, SMA, and the *Drosophila* protein, MAD). The phosphorylated SMADs translocate into the nucleus and regulate the expression of target genes by interacting with various co-factors (reviews: Miyazawa *et al*., 2002, Shi and Massagué 2003) (Fig. I-3A).

There are complex signaling cross-talks in this superfamily because their receptors and SMADs are shared among different kinds of ligands (reviews: Miyazawa *et al*., 2002, Shi and Massagué 2003) (Fig. I-3B). Moreover, mutually antagonistic relationship between the two groups of SMADs (SMAD1/5/8 and SMAD2/3) has been reported (Zeisberg *et al*., 2003, Matsumoto *et al*., 2012). Therefore, I also analyzed the expression of the TGF-β superfamily members that are involved in spermatogenesis and their receptors during the testicular changes to examine their association with the AMH signaling.

The results obtained in this study strongly suggest that the sex differentiation-related genes are involved in the regulation of spermatogenesis via AMH in the adult quail testis.

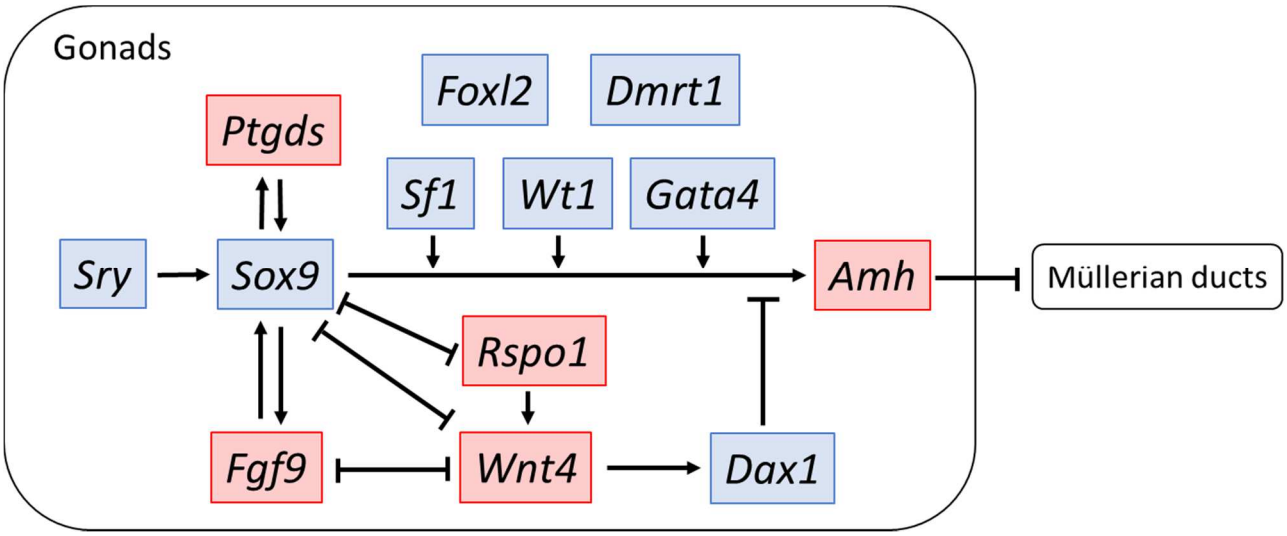


Figure I-1. Network of the genes involved in gonadal sex differentiation in mice

The genes encoding transcription factors are indicated as blue boxes, and those encoding secretory factors are indicated as red boxes. In males, the testis-determining gene *Sry* upregulates the expression of *Sox9*. *Sox9* expression is maintained by *Fgf9* and *Ptgds* by establishing feed-forward loops. *Sox9* and *Fgf9* inhibit the female pathway by suppressing *Rspo1* and *Wnt4* expression. In contrast, *Rspo1* and *Wnt4* suppress the male pathway through inhibition of *Sox9* and *Fgf9* expression. *Sox9* acts synergistically with *Sf1*, *Wt1*, and *Gata4* to upregulate the expression of *Amh*, a hormone required for the regression of Müllerian ducts. *Dax1*, which is upregulated by *Wnt4*, represses *Amh* expression by disrupting the synergistic action of *Sf1*, *Wt1*, and *Gata4*. This schematic diagram is modified from Cutting *et al*., 2013 and Eggers *et al*., 2014.

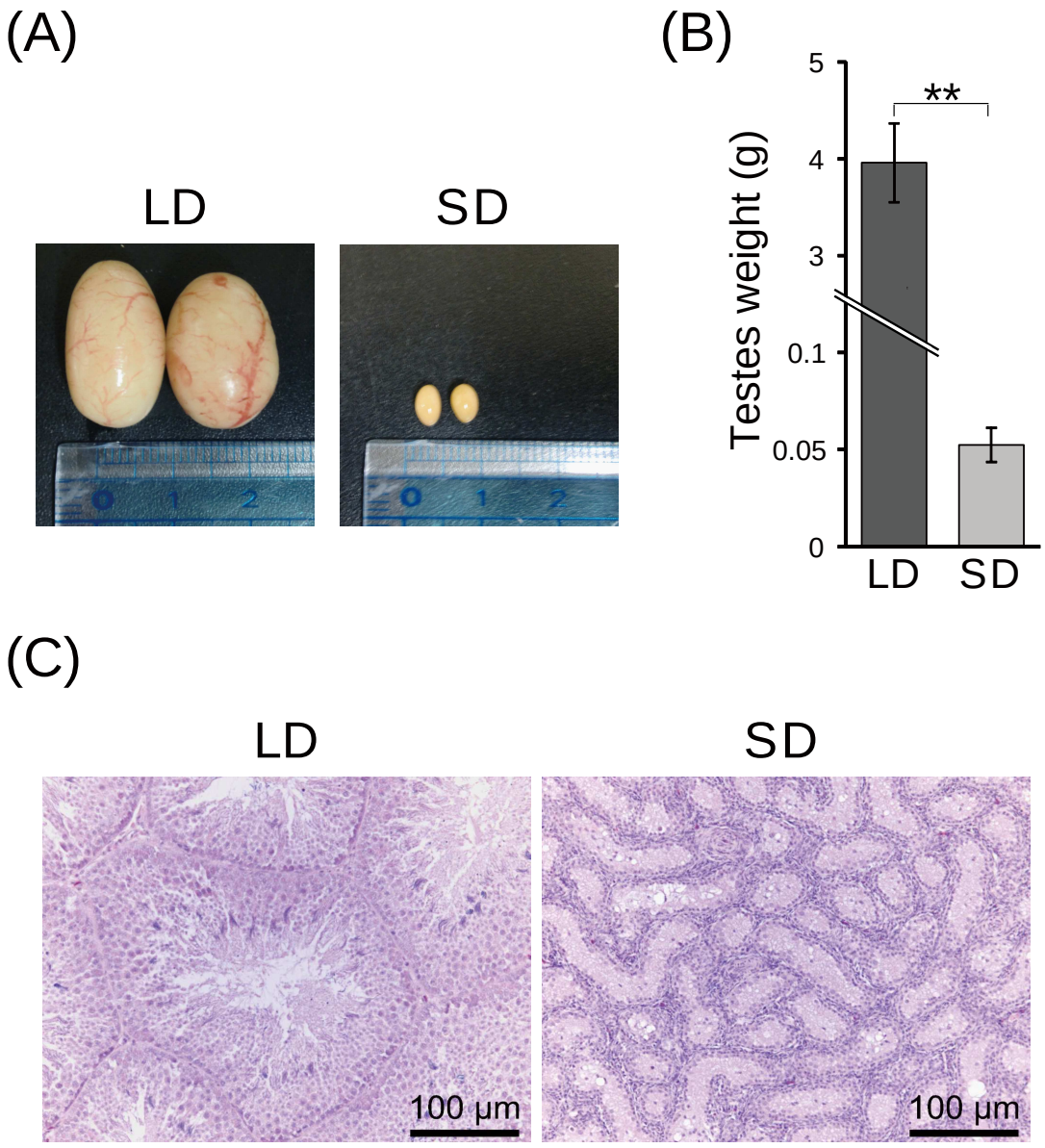
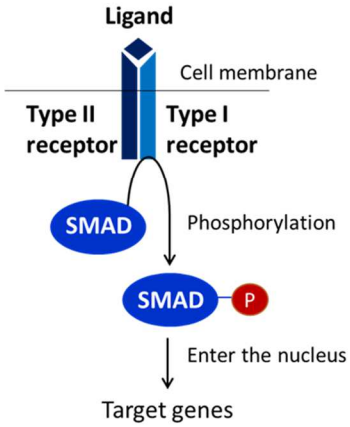


Figure I-2. Testicular changes in the Japanese quail induced by photoperiod

The adult birds of 7 weeks of age were reared under the long-day condition (LD group) or short-day condition (SD group) for 4 weeks. (A) The change of testicular size. (B) The change of testicular weight. Results are shown as mean ± SEM. (n=6 / group). ** $p<0.01$ (Mann Whitney's *U* test). (C) Histological change of the testis. The testes were stained with hematoxylin and eosin.

(A)



(B)

Ligands	AMH	BMPs	TGFBs		Activins (β+β)	Inhibins (α+β)
Type II receptors	AMHR2	BMPR2	TGFBR2		ACVR2A, ACVR2B	
Type I & Co receptors	ALK2, ALK3, ALK6		ALK1 Endoglin	ALK5	ALK4, ALK7	Betaglycan
SMADs	SMAD1/5/8			SMAD2/3		Not activate SMAD

Figure I-3. Signaling system of the TGF-β superfamily

(A) Schematic diagram of the signaling pathway. The ligands of TGF-β superfamily bind to two different serine/threonine kinase receptors referred to as type I and type II, and phosphorylate the intracellular signaling molecules called SMADs. The phosphorylated SMADs translocate into the nucleus and regulate the expression of target genes. (B) The relationship among the ligands, receptors, and SMADs (modified from Miyazawa *et al*., 2002 and Shi and Massagué 2003).

Materials and Methods

Animals

Mature Japanese quail, *Coturnix japonica*, were used in this study. Birds were provided food and water *ad libitum*. All animal procedures were approved by the Committee on Animal Care and Use (School of Science, The University of Tokyo) (Approval no. P12-04) and were carried out in accordance with the guideline of the Life Science Committee at the University of Tokyo. Six-week-old birds were obtained from a local supplier (Motoki, Saitama, Japan). They were reared in individual cages under a long-day condition of 16-h light and 8-h dark (lights on at 6:00, off at 22:00) for a week. For tissue distribution analysis, the adult birds of 7 weeks of age were used. For the expression analysis between the active and regressed testis, the adult birds of 7 weeks of age were reared under the long-day condition (LD group) or a short-day condition (SD group) of 8-h light and 16-h dark (lights on at 10:00, off at 18:00) for 4 weeks and then sacrificed.

For the analysis during the process of testicular changes induced by photoperiod, fertilized eggs were obtained from the supplier. Newly hatched Japanese quail were reared in mixed sex groups under a continuous lighting (24-h light) for 3 weeks. After 3 weeks of age, male birds were reared under the short-day condition for 4 weeks until they became adult. At 7 weeks of age, they were transferred to the long-day condition to develop their testes. They were sacrificed at 0, 2, 4, 7, 10, and 15 days after transferring to the long-day condition.

Animals were killed by rapid decapitation, followed by complete bleeding. Tissues and organs were immediately dissected, frozen in liquid nitrogen, and stored at -80 °C until use. Testes were fixed by immersion in 4% paraformaldehyde in phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4; pH 7.4) at 4 °C overnight. The fixed testes were washed with PBS, dehydrated in a graded ethanol series (70%, 80%, 90%, 99.5%, and 100%), cleared in xylene, embedded in paraffin, and cut at 5 μm-thick. The paraffin sections were used for hematoxylin and eosin staining, *in situ* hybridization, and immunohistochemistry.

Classification of testicular stages

The morphological changes of the quail testis induced by photoperiod were examined by microscopic observation of the paraffin sections stained with hematoxylin and eosin. The different types of germ cells in the seminiferous tubules were identified by their location and morphology as follows. Spermatogonia: located on the basal position in the seminiferous tubules. Spermatocytes: apart from the basal position and located on the adluminal position, and their nuclei and cytoplasm are larger than those of spermatogonia. Spermatids (from round to elongated shapes): located on more adluminal position and much smaller than spermatocytes. Spermatozoa: metamorphosed from spermatids and released into the lumen of the seminiferous tubules. The testes were classified into the stages based on the germ cell types in the seminiferous tubules as below (referring to Mather & Wilson 1964). Stage II: spermatogonia, Stage III: spermatogonia and spermatocytes, Stage V: spermatogonia, spermatocytes, spermatids, and spermatozoa. The classification of the stages was confirmed by the expression analysis of spermatocyte marker *DMC1* (Yoshida *et al*., 1998) and spermatid marker *ZFAND3* (also known as *TEX27*, Otake *et al*., 2011).

RNA extraction and cDNA synthesis

Total RNA was extracted using ISOGEN (Nippon Gene, Tokyo, Japan). The cDNAs used as templates for RT-PCR were synthesized from 3 µg of denatured total RNA using 5 µM oligo (dT) primer and 200 U of M-MLV Reverse transcriptase (Promega, Madison, WI) in a 20-µl reaction volume with incubation at 42 °C for 1.5 h and then 70 °C for 15 min. cDNA templates for 3’ RACE were synthesized in the same way using oligo (dT)-adaptor primer. cDNA templates for 5’ RACE were synthesized from 3 µg of denatured total RNA using 5 µM oligo (dT) primer and 200

U of PrimeScript II Reverse transcriptase (TaKaRa, Shiga, Japan), adding 1 M betaine (Sigma-Aldrich, St. Louis, MO) for improving GC-rich amplification, in a 20-µl reaction volume with incubation at 42 °C for 30 min followed by 50 °C for 1 h and finally 70 °C for 15 min. The cDNA templates were incubated with 60 U of Ribonuclease H (TaKaRa) at 37 °C for 20 min and then 70 °C for 10 min. To add poly (C) at the 5'-terminus, cDNA templates for 5' RACE were incubated with 7 U of Terminal Deoxynucleotidyl Transferase (TaKaRa) and 200 µM dCTP at 37 °C for 20 min and then 70 °C for 10 min. PCR reaction was performed using an adaptor primer with poly (G) region at its tail designed to bind to the poly (C) region of the cDNA. PCR amplification was performed in a 20-µl reaction mixture containing an adaptor primer at 1 µM, 0.25 U of TaKaRa Ex Taq (TaKaRa), each dNTP at 250 µM, Ex Taq Buffer, and 1 M betaine. The PCR condition was as follows: 94 °C for 5 min, 10 cycles of incubation at 94 °C for 30 s, 70 °C for 30 s, 72 °C for 10 min, and finally 72 °C for 5 min.

Expression analysis by RT-PCR

One microliter of each six-fold-diluted cDNA from testis samples was amplified using the primers for various genes and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as an internal control for the cDNA (Table 1). The primers were designed to span at least one intron based on the homologies of cDNA sequences from the chicken and other vertebrates. PCR amplifications were performed in a 20-µl reaction mixture containing each primer at 1 µM, 0.25 U of TaKaRa Ex Taq, each dNTP at 250 µM and Ex Taq Buffer. The PCR condition was as follows: 94 °C for 5 min, 25 or 30 or 35 cycles of incubation at 94 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min, and finally 72 °C for 5 min. The amplified products were electrophoresed on 1.5% agarose gel and stained with ethidium bromide. The specificity of the PCR was confirmed by sequence analysis.

Real-time PCR

Five microliter of each 120-fold-diluted cDNA from testis samples was amplified in a 20-μl reaction mixture containing each primer at 0.3 μM and LightCycler 480 SYBR Green I Master (Roche Diagnostic, Basel, Switzerland) using LightCycler 480 Instrument II (Roche Diagnostic). The primers were designed to span at least one intron based on the sequencing results of the conventional RT-PCR products and sequences of the Japanese quail in the NCBI database (Table 1). For negative control, PCR was also conducted using RNA sample without the RT reaction. The PCR condition was as follows: 95℃ for 5 min, 45 cycles of incubation at 95 ℃ for 10 s, 60 ℃ for 10 s, 72 ℃ for 10 s. The PCR product was confirmed using melting curve analysis. For each analysis, a standard curve was generated from the serial dilutions of the standard cDNA from the active and regressed testis samples. For normalization, the expression of three housekeeping genes, *GAPDH*, beta-actin (*ACTB*), and peptidylprolyl isomerase A (*PPIA*), was examined. However, they showed expressional changes between the active and regressed testis. Therefore, the data were normalized to the geometric mean of *GAPDH* and *PPIA* (the most stable combination) following the report that suggests the geometric mean of multiple housekeeping genes as an accurate normalization factor (Vandesompele *et al*., 2002).

Prediction of the putative transcription factor binding sites in the *AMH* promoter region

The DNA sequences from stop codon of *SF3A2* (an upstream gene next to *AMH*) to start codon of *AMH* were obtained from the quail and chicken genome data using NCBI database. GenBank accession number of the genome sequences, the region used for analysis, and the length are as follows: quail, LSZS01001175.1 (region: from 91138 to 92072, 935bp); chicken, AADN04000558.1 (region: from 141475 to 142893, 1419bp). The putative transcription factor

binding sites were predicted by MatInspector software in Genomatix Software Suite v3.6 (Genomatix Software GmbH, Munich, Germany) (Cartharius *et al*., 2005). The setting is as follows. Library selection: Transcription factor binding sites (Weight matrices), Library version: Matrix Library 9.4, Matrix group: General Core Promoter Elements and Vertebrates, Matrix families: matches to individual matrices, Core similarities: 0.75, Matrix similarities: optimized.

_In_ situ hybridization and immunohistochemistry

To examine the expression sites of *AMH* and germ cell markers (*DMC1* and *ZFAND3*) in the testis of the Japanese quail, *in* situ hybridization was performed using digoxigenin (DIG)-labeled RNA probes (Roche Diagnostics). For the probe synthesis, the ORF sequence of *DMC1* was identified from the quail by RT-PCR using *DMC1*-SE01 and AS01 (Table 1) which were designed based on the homologies of *DMC1* cDNA sequences from various vertebrates. For the templates of RNA probes, the testicular cDNA was amplified using each primer set (Table 1). The amplified product was cloned into a pTAC-2 vector (BioDynamics, Tokyo). Sense and antisense RNA probes were synthesized by T7 RNA polymerase (TaKaRa) with the templates and DIG labeling mix (Roche Diagnostics). The paraffin sections (5 μm thick) of the testis on slide glasses were deparaffinized by xylene, rehydrated in a graded ethanol series (100%, 95%, and 70%), and immersed in PBS. The sections were treated with protenase K (1 μg/ml, 30 min, 37°C) and hybridized with 1 μg/ml sense or antisense RNA probe in hybridization buffer (50% formamide, 3X SSC, 200 μg/ml yeast tRNA, 10% sodium dextran sulfate, 1X Denhardt solution, 10 mM Tris-HCl; pH7.5, 1 mM EDTA; pH8.0) overnight at 60°C. The sections were washed twice with 2X SCC containing 50% formamide for 15 min at 60°C and then treated with 20 μg/ml RNase A for 30 min at 37 °C. The sections were washed twice with 2X SSC and 0.5X SSC for 15 min at 60°C, respectively, followed by washing three times with maleic buffer (0.1 M maleic acid, 0.15 M

NaCl, 0.1% Tween 20; pH 7.5) for 15 min at room temperature, and they were then blocked with 1.5% blocking reagent (Boehringer Mannheim, Mannheim, Germany) in maleic buffer for 1 h at room temperature. They were incubated with 1/1000 diluted anti-DIG antibody conjugated to alkaline phosphatase (Roche Diagnostics) overnight at 4°C, followed by washing three times with maleic buffer for 15 min at room temperature, and visualized with NBT/BCIP reaction. After desired signals had been detected, the sections were immersed in PBS to stop the reaction.

Immunohistochemistry of DEAD-box helicase 4 (DDX4 also known as VASA) was performed in the same section after *in* situ hybridization of *AMH* to identify germ cells in the seminiferous tubules. Rabbit anti-CVH (chicken *VASA* homolog) antibody (kindly provided by Dr. Toshiaki Noce) was used. The specificity and cross-reactivity of the antibody on the Japanese quail was characterized by a previous study (Tsunekawa *et al*., 2000). For antigen retrieval, the sections after NBT/BCIP reaction were incubated with sodium citrate buffer (10 mM sodium citrate; pH 6.0) for 20 min at 100 °C, and then cooled to room temperature. To inactivate endogenous peroxidase activity, the sections were treated with 0.3% H_2O_2 for 30 min at room temperature, and then blocked with 2% normal horse serum in PBS for 1 h at room temperature. The sections were incubated with rabbit anti-CVH antiserum (1:1000) overnight at 4 °C. After washing with 0.05% Tween 20 in PBS (PBST), the sections were incubated with biotinylated goat anti-rabbit IgG antibody (1:200; Vector, Burlingame, CA) for 1 h at room temperature. They were then incubated with VECTASTAIN ABC (Vector) for 40 min at room temperature, washed in PBST, and incubated with 3,3’-diaminobenzidine. After desired signals had been detected, the sections were immersed in PBS to stop the reaction. They were then dehydrated in a graded ethanol series (70%, 95%, and 100%), cleared in xylene, and coverslipped.

Molecular cloning of *AMHR2* cDNA from the Japanese quail by RACE and RT-PCR

Sense and antisense degenerate primers were designed based on the homologies of *AMHR2* cDNA sequences from various vertebrates. Partial sequence was obtained by degenerate PCR from the testicular cDNA using *AMHR2*-dSE01 and dAS01 (Table 1). Then, sense and antisense primers for 3' and 5' RACE were designed from the partial sequence. 3' and 5' RACE was performed from the testicular cDNA using *AMHR2*-SE01 and the adaptor primer for 3' RACE, and the adaptor primer for 5' RACE and *AMHR2*-AS01 (Table 1), respectively. Sense and antisense primers for confirming the sequence were designed based on the results of 3' and 5' RACE. For the confirmation, RT-PCR using *AMHR2*-SE03 and AS03, 3' RACE using *AMHR2*-SE02, and 5' RACE using *AMHR2*-AS02 were performed (Table 1). All of the PCR amplifications were performed in a 20-µl reaction mixture containing each primer at 1 µM, 0.25 U of TaKaRa Ex Taq, each dNTP at 250 µM, Ex Taq Buffer, and 1 M betaine for GC-rich amplification. Each PCR condition was as follows: 94 °C for 5 min, 35 cycles of incubation at 94 °C for 30 s, 60 °C for 30 s, 72 °C for 1.5 min, and finally 72 °C for 5 min. The amplified products were separated by electrophoresis in 1.5% agarose gel and visualized using ethidium bromide staining. The DNA fragments were extracted using phenol and chloroform, cloned into a pTAC-2 vector, and sequenced. The sequencing of the partial ORF was conducted independently three times to avoid potential PCR amplification errors.

Comparison of the amino acid sequences of AMHR2 from various amniotes

The CLUSTAL W program (version 2.1) (Chenna *et al.*, 2003) (http://clustalw.ddbj.nig.ac.jp/index.php?lang=ja) was used with default settings to align the amino acid sequences of AMHR2 from various amniotes. Based on the quail *AMHR2* cDNA sequence, those of the chicken (*Gallus gallus*) and Tibetan ground tit (*Pseudopodoces humilis*) were predicted from the genomic DNA sequences in the WGS (whole-genome shotgun) database

using NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (GenBank accession number: chicken, BK009411; Tibetan ground tit, BK009412). Their cDNA sequences and deduced amino acid sequences were used for the phylogenetic analysis and amino acid alignment, respectively. The transmembrane domain and protein kinase domain of AMHR2 were predicted using TMHMM Server (version 2.0) (Krogh *et al*., 2001) (http://www.cbs.dtu.dk/services/TMHMM/) and Pfam database (version 29.0) (Finn *et al*., 2016) (http://pfam.xfam.org/), respectively. The N-glycosylation sites were also predicted by NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/). The amino acid identity and similarity, based on the number of identical residues or conservative substitutions, were calculated with GeneDoc software (version 2.7). The GenBank accession numbers of AMHR2 amino acid sequences used in the comparison are as follows: Japanese quail, AMY51360.1; American alligator, XP_006273407.1; Green anole, XP_008101873.1; Human, NP_065434.1; Cattle, NP_001192257.1; Armadillo, XP_004467499.2; Rabbit, NP_001076263.1; Mouse, NP_653130.2.

Molecular phylogenetic analysis

For the generation of phylogenetic tree, codon alignment of the nucleotide sequences of *AMHR2* and other TGF-β superfamily type II receptors from various amniotes was constructed using CLUSTAL W program embedded in MEGA software (version 7.0.14) (Kumar *et al*., 2016) with default settings. Molecular phylogenetic tree was constructed using the neighbor-joining method with MEGA. Human *IGF1R* was used as an out group. Bootstrap values were calculated with 1000 replications to estimate robustness of the internal branches. The GenBank accession numbers of cDNA sequences used for the phylogenetic analysis are listed in Table 2.

Tissue distribution analysis of *AMH* and *AMHR2*

One microliter of each six-fold-diluted cDNA from the testis, brain, thyroid gland, adrenal gland, kidney, small intestine, liver, heart, skeletal muscle (from the male), ovary and oviduct (from the female) of the adult Japanese quail was amplified using specific primers for *AMH*, *AMHR2*, and *GAPDH* as an internal control for the cDNA (Table 1). The primers were designed to span at least one intron. PCR amplifications were performed as described above. For negative control, PCR was also conducted using RNA sample without the RT reaction. The PCR condition was as follows: 94 °C for 5 min, 25 or 30 or 35 cycles of incubation at 94 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min, and finally 72 °C for 5 min. The amplified products were electrophoresed on 1.5% agarose gel and stained with ethidium bromide. The specificity of the PCR was confirmed by sequence analysis. The typical data from three independent analyses was shown as a result.

Statistical analysis

All data are expressed as mean ± SEM. The expression levels of the genes between the active and regressed testis were analyzed by Mann-Whitney's *U* test. The expression levels of the genes among the testicular stages were analyzed by Kruskal–Wallis test, followed by *post hoc* Steel–Dwass test. All the analyses were conducted using KyPlot 5.0 software (KyensLab, Tokyo).

Table 1. Oligonucleotide primers used for RT-PCR, RACE, and real-time PCR

Gene	Accession number	Name	Nucleotide sequence	Usage
SF1	AB254390.1	*SF1*-SE01	5'-AGATGGCCGACCAGACACTA-3'	RT-PCR
		SF1-AS01	5'-AGTCCATGTGAGAACTGCACTC-3'	RT-PCR
WT1	NM_205216.1	*WT1*-SE01	5'-CTCCGGTGTACGGCTGTC-3'	RT-PCR
		WT1-AS01	5'-CTAAAGACTCCATGGGTGTGTATTC-3'	RT-PCR
SOX9	NM_204281.1	*SOX9*-SE01	5'-CCCCAACGCCATCTTCAA-3'	RT-PCR
		SOX9-AS01	5'-CTGCTGATGCCGTAGGTA-3'	RT-PCR
GATA4	NM_001293106.1	*GATA4*-SE01	5'-ATGCCTGTGGACTCTACATGAAGC-3'	RT-PCR
		GATA4-AS01	5'-CAGGAGTCCTGTTTGGAGCTG-3'	RT-PCR
DAX1	NM_204593.1	*DAX1*-SE01	5'-AGAACGTGCCCTGCTTCCAG-3'	RT-PCR
		DAX1-AS01	5'-AGCATTTCCAAAAGCATGTCATCCATG-3'	RT-PCR
DMRT1	AF123456.2	*DMRT1*-SE01	5'-CGCGTCTGCCCAAGTG-3'	RT-PCR
		DMRT1-AS01	5'-GATGGAAGGGATGTCCGAATGA-3'	RT-PCR
FOXL2	NM_001012612.1	*FOXL2*-SE01	5'-ACGTGGCCCTGATCGCCATG-3'	RT-PCR
		FOXL2-AS01	5'-GGTAGTTGCCCTTCTCGAACATGTC-3'	RT-PCR
AMH	AY904049.1	*AMH*-SE01	5'-GTCCTGCACCTGGAGGAAGTG-3'	RT-PCR and probe synthesis
		AMH-AS01	5'-GCACCGGAGTCATCCTGGTGAA-3'	RT-PCR and probe synthesis
		AMH-SE02	5'-CACAGTCTGTTCTCCGACCA-3'	Real-time PCR
		AMH-AS02	5'-CCTTTGCTTCCCACTTCACT-3'	Real-time PCR
PTGDS	NM_204259.1	*PTGDS*-SE01	5'-ATCGGCCTGGCCTCCAACTC-3'	RT-PCR
		PTGDS-AS01	5'-CTCCTTGGTGCGGCTGTAGAG-3'	RT-PCR
		PTGDS-SE02	5'-GTCACTGCAGATGGCAACAT-3'	Real-time PCR
		PTGDS-AS02	5'-TCTTCTCACACTGATCACCCTTG-3'	Real-time PCR
FGF9	NM_204399.1	*FGF9*-SE01	5'-ATCAGTATAGCAGTGGGCCTGGTC-3'	RT-PCR
		FGF9-AS01	5'-GCGACAAAGTTTGGCAACAGTGGAG-3'	RT-PCR
WNT4	NM_204783.1	*WNT4*-SE01	5'-AGCAACTGGCTGTACCTGGC-3'	RT-PCR
		WNT4-AS01	5'-CCAGACCACTGGAAGCCCTG-3'	RT-PCR
		WNT4-SE02	5'-GGCAAGGTGGTAACACAAGG-3'	Real-time PCR
		WNT4-AS02	5'-TGCTGAAGAGATGGCGTAGA-3'	Real-time PCR
RSPO1	NM_001318444.1	*RSPO1*-SE01	5'-GCTCTTCATCCTTCTGGAGAGGAAC-3'	RT-PCR
		RSPO1-AS01	5'-GCCCCCACTCACTCATTTCACATTG-3'	RT-PCR
HSD3B	NM_205118.1	*HSD3B*-SE01	5'-GTCTCGCTCGTCATCCACAC-3'	RT-PCR
		HSD3B-AS01	5'-CTCATGTGAGGAGTGTCATCTGAG-3'	RT-PCR
CYP17A1	NM_001001901.2	*CYP17A1*-SE01	5'-ATCTTTGGGGCTGGCGTGGAGAC-3'	RT-PCR
		CYP17A1-AS01	5'-CAGGCCAGGAAGAGGAAGAGCTC-3'	RT-PCR
CYP19A1	NM_001001761.2	*CYP19A1*-SE01	5'-GAATTCTTCCCAAAACCGAATGAG-3'	RT-PCR

Table 1. (Continued)

Gene	Accession number	Name	Nucleotide sequence	Usage
		CYP19A1-AS01	5'-GCACCGTCTCAGAAGAGTCACCAG-3'	RT-PCR
LHCGR	NM_204936.1	*LHCGR*-SE01	5'-ACCTCCCTGTGAAAGTGATCCCATCACA-3'	RT-PCR
		LHCGR-AS01	5'-ACTGCAGCTGGCACCATAGAG-3'	RT-PCR
STAR	NM_204686.2	*STAR*-SE01	5'-ATGGAGCAGATGGGCGACTGGAAC-3'	RT-PCR
		STAR-AS01	5'-GTCTGGGACAGGACCTGGTTGATG-3'	RT-PCR
CYP11A1	NM_001001756.1	*CYP11A1*-SE01	5'-ATCCTCTTCAGCCTCCTTGTGCAGG-3'	RT-PCR
		CYP11A1-AS01	5'-GCGACGACCCAGACACTGCCGTGGC-3'	RT-PCR
AMHR2	KU715092	*AMHR2*-dSE01	5'-AARCCWGGWRTBGCHCAYCGAGAYCTGAGCAG-3'	degenerate PCR
		AMHR2-dAS01	5'-ATYTCCCAYARCACYAGGGCCARRGAGTARAYRTC-3'	degenerate PCR
		AMHR2-SE01	5'-AGAACGTGCTGGTTCGGCAGGA-3'	3' RACE
		AMHR2-SE02	5'-CTCTGCAGGAGCTGCTGGAGGATTGTTG-3'	3' RACE
		AMHR2-SE03	5'-AGATGGACACGGGCTCAGATGGGTCGGAC-3'	RT-PCR to confirm ORF
		AMHR2-SE04	5'-ACCAGGAGCTGTGGCGAGACGGTCTGTAC-3'	RT-PCR
		AMHR2-AS01	5'-AGCAGCGAAGGTCCAGGCTCT-3'	5' RACE and RT-PCR
		AMHR2-AS02	5'-GGGCTCTGAGCTCCCGGCGTCTCTGTG-3'	5' RACE
		AMHR2-AS03	5'-GCATTAACATCAGTCAGAGTCGCTGCTG-3'	RT-PCR to confirm ORF
		AMHR2-SE05	5'-CTGTGGCGAGACGGTCTG-3'	Real-time PCR
		AMHR2-AS04	5'-ACGTTCTGGCTGCTGAGATC-3'	Real-time PCR
DMC1	KU975604	*DMC1*-SE01	5'-GATCAATACTTTATTGCCTGCTCTTTCAC-3'	RT-PCR to confirm ORF
		DMC1-AS01	5'-ACAGCGACCTTCACAAACCTTCATC-3'	RT-PCR to confirm ORF
		DMC1-SE02	5'-ATCTGGATACCAGGATGATGAGGAATC-3'	probe synthesis
		DMC1-AS02	5'-AGCTGTCACACAAAGAGTATGAGATAGCT-3'	probe synthesis
		DMC1-SE03	5'-CGACAACAGAAACTGGCTCA-3'	Real-time PCR
		DMC1-AS03	5'-TCACAAACACAGCCACGTTAT-3'	Real-time PCR
ZFAND3	JF740026.1	*ZFAND3*-SE01	5'-GACTCCAGCTGACCGGCCTGGAAT-3'	probe synthesis
		ZFAND3-AS01	5'-GGCTGACTTTGTCCTAAATAAGGCT-3'	probe synthesis
		ZFAND3-SE02	5'-CATGCCTCATTAACCACACCT-3'	Real-time PCR
		ZFAND3-AS02	5'-TGTCATTACTGTCATCCAGTAGCC-3'	Real-time PCR
GAPDH	NM_204305.1	*GAPDH*-SE01	5'-TGTGACTTCAATGGTGACAG-3'	RT-PCR
		GAPDH-AS01	5'-CAGATCAGTTTCTATCAGCC-3'	RT-PCR

Table 1. (Continued)

Gene	Accession number	Name	Nucleotide sequence	Usage
		GAPDH-SE02	5'-TCCACTGGGGCTGCTAAG-3'	Real-time PCR
		GAPDH-AS02	5'-CGGAAAGCCATTCCAGTAAG-3'	Real-time PCR
PPIA	KP129283.1	*PPIA*-SE01	5'-GCTCCCAGTTCTTCATCTGC-3'	Real-time PCR
		PPIA-AS01	5'-GCCTCCACCACGTTCATC-3'	Real-time PCR
BMP2	NM_204358.1	*BMP2*-SE01	5'-GCAGCTTCCACCACGAAGAAGT-3'	RT-PCR
		BMP2-AS01	5'-ATATTAATACGGTGATGGTAGCTGCTGTTG-3'	RT-PCR
		BMP2-SE02	5'-GCAGCTTCCACCACGAAG-3'	Real-time PCR
		BMP2-AS02	5'-GAGGTGATAGACTCCTCATTAG-3'	Real-time PCR
BMP4	NM_205237.3	*BMP4*-SE01	5'-CTTCCACCATGAAGAGCACCTGGAGA-3'	RT-PCR
		BMP4-AS01	5'-CTCCCGGTACAGCCGCAGCTC-3'	RT-PCR
BMP7	XM_417496.5	*BMP7*-SE01	5'-CTTCGTCAACTTGGTGGAGCATGACAG-3'	RT-PCR
		BMP7-AS01	5'-CGCTGGTCGCTGCTGCTGTTCTC-3'	RT-PCR
BMP8B	XM_003642583.3	*BMP8B*-SE01	5'-CGACACCGTGGTCAGCCTCGT-3'	RT-PCR
		BMP8B-AS01	5'-GTCACCATGAAGGGCTGCTTGGAG-3'	RT-PCR
BMPR2	NM_001001465.1	*BMPR2*-SE01	5'-CCAGGAAATGCTTCAGAACCAGTTCAGTG-3'	RT-PCR
		BMPR2-AS01	5'-AGTGCAGCGGCTCAGTGGAGATGAC-3'	RT-PCR
		BMPR2-SE02	5'-GCCACAAATAGTTTGGATAGCA-3'	Real-time PCR
		BMPR2-AS02	5'-GGAGTTTTCACACGTTTCTTGA-3'	Real-time PCR
ALK2	NM_204560.1	*ALK2*-SE01	5'-CTGCCATCCCGCAGTGTGCAAGA-3'	RT-PCR
		ALK2-AS01	5'-CTCCAACATTTGAAGCAATGAGTCCCTC-3'	RT-PCR
		ALK2-SE02	5'-TGGAGTGCTGCCAAGGATAC-3'	Real-time PCR
		ALK2-AS02	5'-TGATTAGTGTTTCCATGCTGTAAC-3'	Real-time PCR
ALK3	NM_205357.1	*ALK3*-SE01	5'-ATGAAGGTTCAGACTTCCAGTGCAAG-3'	RT-PCR
		ALK3-AS01	5'-AATGCTTCATCTTGTTCCAGGTCACG-3'	RT-PCR
		ALK3-SE02	5'-ACATTACCACCGCTTGATAGTAC-3'	Real-time PCR
		ALK3-AS02	5'-GACTGCCATCCAACGAATGCT-3'	Real-time PCR
ALK6	NM_205132.1	*ALK6*-SE01	5'-CCACACCAAAGAAGATCTATTGAATG-3'	RT-PCR
		ALK6-AS01	5'-GAAGTAGCAGAATATGATGATAAGCACCAG-3'	RT-PCR
		ALK6-SE02	5'-GCTGCCACCACTGAAAAATC-3'	Real-time PCR
		ALK6-AS02	5'-AGCAGGGCCTTATGGTGAAT-3'	Real-time PCR
TGFB1	NM_001318456.1	*TGFB1*-SE01	5'-AGCCACAGCATCTTCTTCGTGTTCAACG-3'	RT-PCR
		TGFB1-AS01	5'-AGCGGCCATGCAGGTACCGCCA-3'	RT-PCR
TGFB2	NM_001031045.3	*TGFB2*-SE01	5'-CCATCCCACCAAGCTATTACAGCCT-3'	RT-PCR
		TGFB2-AS01	5'-CAACCATTCTCCTTCAGCTCTTGTTTTAAC-3'	RT-PCR
		TGFB2-SE02	5'-TCGGAGCAGCGGATAGAG-3'	Real-time PCR
		TGFB2-AS02	5'-TGCTGTCAATGTAGCGCTGT-3'	Real-time PCR

Table 1. (Continued)

Gene	Accession number	Name	Nucleotide sequence	Usage
TGFB3	NM_205454.1	*TGFB3*-SE01	5'-GCATTTGCCCAAAAGGTGTCACCTCCAA-3'	RT-PCR
		TGFB3-AS01	5'-GTCTGCACATTCCTGCCACTGAGGTA-3'	RT-PCR
		TGFB3-SE02	5'-AGCAGAGTTCCGGGTGCT-3'	Real-time PCR
		TGFB3-AS02	5'-CGCTGCTTTGCTATGTGCT-3'	Real-time PCR
TGFBR2	NM_205428.1	*TGFBR2*-SE01	5'-AGTGCAAGAGCAACTGCAACATCACTTC-3'	RT-PCR
		TGFBR2-AS01	5'-CTGTGTTGTGGTTGATGTTGTTGGCACA-3'	RT-PCR
ALK1	XM_015300268.1	*ALK1*-SE01	5'-GCAAGGTGTGCTTCGTCAGCAAGAG-3'	RT-PCR
		ALK1-AS01	5'-GCGGTGCTGGGCCAGCTTCCA-3'	RT-PCR
		ALK1-SE02	5'-TGCAACGCCAGCATGTGCAA-3'	Real-time PCR
		ALK1-AS02	5'-ATCAGCAGGAGGAGGTTGGA-3'	Real-time PCR
ALK5	NM_204246.1	*ALK5*-SE01	5'-CAAAGGACAACTTTACCTGTGTGACAGATG-3'	RT-PCR
		ALK5-AS01	5'-GACTAGAAGCTGGTCTTCCTGGAGTAG-3'	RT-PCR
		ALK5-SE02	5'-GAACTTCCAATTCCAACACCA-3'	Real-time PCR
		ALK5-AS02	5'-AAGCAGACAGGTCCAGCAAT-3'	Real-time PCR
ENG	NM_001080887.1	*ENG*-SE01	5'-CCCATCAAGGACGTGGTGAACATCAC-3'	RT-PCR
		ENG-AS01	5'-GTAGGTGAAGCGGAAGCGCCAGA-3'	RT-PCR
		ENG-SE02	5'-ACACCCTGCTCAGCCACT-3'	Real-time PCR
		ENG-AS02	5'-ACAGGCTGAGGATGAGCTC-3'	Real-time PCR
INHA	NM_001031257.1	*INHA*-SE01	5'-CAGGTGATTCTCTTCCCTTCCACAGA-3'	RT-PCR
		INHA-AS01	5'-CTCCGATGGGCGCTGCAGCAG-3'	RT-PCR
		INHA-SE02	5'-AGGACACATCCCAGGTGATTC-3'	Real-time PCR
		INHA-AS02	5'-TCTTCCTCCAGCAGCTTGTC-3'	Real-time PCR
INHBA	NM_205396.1	*INHBA*-SE01	5'-GTTGTGGAGCAAACCTCAGAAATCATCAC-3'	RT-PCR
		INHBA-AS01	5'-CAGATGTTAACTTTGCCATCACACTCCAG-3'	RT-PCR
		INHBA-SE02	5'-GGAAGAAGAGCAGAAATGAATGAAG-3'	Real-time PCR
		INHBA-AS02	5'-CCTTGGAAATCTCAAAGTGCAAC-3'	Real-time PCR
INHBB	NM_205206.1	*INHBB*-SE01	5'-GTCTCCGAGATCATCAGCTTCGCCGA-3'	RT-PCR
		INHBB-AS01	5'-AGATTGGTCCTGCCATCGCACTCCAG-3'	RT-PCR
		INHBB-SE02	5'-GTCTCCGAGATCATCAGCTTC-3'	Real-time PCR
		INHBB-AS02	5'-TTCTGGTTCCCTTCATTCGAG-3'	Real-time PCR
FST	NM_205200.1	*FST*-SE01	5'-ACCACTTCATGGAAGATCACACAG-3'	RT-PCR
		FST-AS01	5'-ACACACCGAGGTTTGTTCTTCTTGTTC-3'	RT-PCR
		FST-SE02	5'-ACGTCAACGACAACACGCTC-3'	Real-time PCR
		FST-AS02	5'-AGTCCACGTTCTCACATGTTTC-3'	Real-time PCR
ACVR2A	NM_205367.1	*ACVR2A*-SE01	5'-CAGAAATGGAGGTCACACAGCCAACTTC-3'	RT-PCR
		ACVR2A-AS01	5'-AGCAACTGCAGTGGCTTCAAACCCATCA-3'	RT-PCR
ACVR2B	NM_204317.1	*ACVR2B*-SE01	5'-ACTTGCCTGAAGTAACTGGCCCAGAAG-3'	RT-PCR

Table 1. (Continued)

Gene	Accession number	Name	Nucleotide sequence	Usage
		ACVR2B-AS01	5'-GAGCTGTAGAGGTTTTAGGCCAACCAG-3'	RT-PCR
ALK4	XM_015300266.1	*ALK4*-SE01	5'-ATCGATTTAATGGTTCCCAGCGGACAC-3'	RT-PCR
		ALK4-AS01	5'-GTCCTTCGACAGGCACATTTCACAAGA-3'	RT-PCR
ALK7	XM_422170.5	*ALK7*-SE01	5'-ACCGACTTCTGCAACAACATCACCCTC-3'	RT-PCR
		ALK7-AS01	5'-GCTGACCAGGTTGCACTCGGAGA-3'	RT-PCR
BETA GLYCAN	NM_204339.1	*BETAGLYCAN*-SE01	5'-CAGGTGGATATAATCGTTGACATTAAACCA-3'	RT-PCR
		BETAGLYCAN-AS01	5'-CTGGAGGAAGAGAATGGTCTTCTTCATC-3'	RT-PCR
		BETAGLYCAN-SE02	5'-GGTCAACTGGGTTATCAAGTC-3'	Real-time PCR
		BETAGLYCAN-AS02	5'-ATAGTCATGGATCGTTCTGTTTC-3'	Real-time PCR

Abbreviations: R=A or G; W=A or T; Y=C or T; B=C or G or T; H=A or C or T

Table 2. GenBank accession numbers of cDNA sequences used for phylogenetic analysis

Gene	Species	Accession number	Gene	Species	Accession number
AMHR2	Human	NM_020547.2	*ACVR2A*	Human	NM_001278579.1
	Cattle	NM_001205328.1		Cattle	NM_174227.3
	Armadillo	XM_004467442.2		Armadillo	XM_004480425.2
	Rabbit	NM_001082794.1		Rabbit	XM_017342835.1
	Mouse	NM_144547.2		Mouse	NM_007396.4
	Japanese quail	KU715092		Japanese quail	XM_015869263.1
	Chicken	BK009411		Chicken	NM_205367.1
	Tibetan ground tit	BK009412		Tibetan ground tit	XM_005519591.2
	American alligator	XM_006273345.2		American alligator	XM_006277126.2
	Green anole	XM_008103666.1		Green anole	XM_003224978.2
BMPR2	Human	NM_001204.6	*ACVR2B*	Human	NM_001106.3
	Cattle	NM_001304285.1		Cattle	NM_174495.2
	Armadillo	XM_012525295.1		Armadillo	XM_004478392.2
	Rabbit	XM_008258989.2		Rabbit	XM_008253728.2
	Mouse	NM_007561.4		Mouse	NM_007397.3
	Japanese quail	XM_015868188.1		Japanese quail	XM_015853039.1
	Chicken	NM_001001465.1		Chicken	NM_204317.1
	Tibetan ground tit	XM_014249468.1		Tibetan ground tit	XM_005527760.2
	American alligator	XM_014594187.1		American alligator	XM_014608936.1
	Green anole	XM_008114966.1		Green anole	XM_003227810.2
TGFBR2	Human	NM_001024847.2	*IGF1R*	Human	NM_000875.4
	Cattle	NM_001159566.1			
	Armadillo	XM_004470876.2			
	Rabbit	NM_001177748.1			
	Mouse	NM_009371.3			
	Japanese quail	XM_015853978.1			
	Chicken	NM_205428.1			
	Tibetan ground tit	XM_005519145.2			
	American alligator	XM_006263606.2			
	Green anole	XM_008121651.1			

Results

Expression analysis of the sex differentiation-related genes and steroidogenic genes in the testis of the quail at long-day or short-day condition

The adult quail were reared under the long-day or short-day condition for 4 weeks. The average weight of the regressed testis induced by the short-day condition was decreased by about 80-fold compared to that of the long-day control (Fig. I-2). Expression of various sex differentiation-related genes (the transcription factors and secretory factors) was analyzed in the testis of the quail at long-day or short-day condition. Among the transcription factors, the expression of *SF1*, *WT1*, *SOX9*, *GATA4*, and *DAX1* was increased in the regressed testis induced by the short-day condition, while that of *FOXL2* was decreased and *DMRT1* expression was not changed (Fig. R-1A). Among the secretory factors, the expression of *AMH*, *PTGDS*, and *WNT4* was increased in the regressed testis, while that of *FGF9* and *RSPO1* was not changed (Fig. R-1B). The expressional change of *AMH* was the largest among the secretory factors when their expression was quantified by real-time PCR analysis (Fig. R-1C).

The genes related to steroidogenesis were also examined as indicators of adult testicular functions. The expression of *CYP19A1* (*P450AROM*) was decreased in the regressed testis, but that of *HSD3B* and *CYP17A1* (*P450C17*) was not changed (Fig. R-1D). The expression of *LHCGR*, *STAR*, and *CYP11A1* (*P450SCC*) in the regressed testis showed individual differences (Fig. R-1E. analyzed by increasing the number of samples).

Prediction of the putative transcription factor binding sites in the *AMH* promoter region

To examine whether the binding sites of the transcription factors involved in sex differentiation are present in the quail *AMH* promoter region, I analyzed the putative promoter sequence using MatInspector software. For the analysis, the DNA sequences from stop codon of *SF3A2* (an upstream gene next to *AMH*) to start codon of *AMH* were obtained from the quail

genome data using NCBI database. In the quail *AMH* promoter region, there were several putative transcription factor binding sites, such as one SF1 site, three SOX9 sites, two WT1 sites, and one GATA4 site (Fig. R-2, upper panel). It also contained a common binding site for androgen receptor (AR), glucocorticoid receptor (GR), and progesterone receptor (PR) and a binding site for CLOCK/BMAL1 heterodimer. In the same way, I also analyzed the putative promoter sequence of the chicken, which is a model animal for study on sex differentiation in avian species, for the comparison with that of the quail. The chicken sequence contained one SF1 site, four SOX9 sites, two WT1 sites, one GATA4 site, one PR site, one common site for AR, GR, and PR, and four CLOCK/BMAL1 sites (Fig. R-2, lower panel).

Synteny analysis of *AMH* gene

In some teleost species like medaka, *amh* gene lies in a chromosomal region that contains *clock* gene and reproductive and cell cycling genes, suggesting the presence of a functional cluster (Paibomesai *et al*., 2010). To examine their distribution in the quail and chicken chromosomes, I performed synteny analysis using NCBI database. There was a conserved synteny of *LINGO3*, *OAZ1*, *AMH*, and *DOT1L* on chromosome 28 (Fig. R-3). However, *CLOCK* and *KIT* were located on the different chromosome (Chr 4) like in the mouse and zebrafish. *DDX59*, *KIF14*, *NR5A2*, and *LHX9* were also located on the different chromosome (Chr 8) like in the mouse. These results indicate that there is no functional cluster including *CLOCK*, *AMH*, and other genes involved in reproduction in the quail and chicken.

Identification of *AMH* expressing cells in the testis

In situ hybridization analysis was performed to examine the *AMH* expressing cells in the adult testis. Immunohistochemistry of DDX4 (also known as VASA) was performed in the same

section to identify the germ cells in the seminiferous tubules. The expression of the spermatocyte marker *DMC1* (Yoshida *et al*., 1998) and spermatid marker *ZFAND3* (also known as *TEX27*, Otake *et al*., 2011) was also analyzed to examine the germ cell types in the regressed testis. Since *DMC1* cDNA was not cloned, its ORF sequence was identified from the quail by RT-PCR for probe synthesis (GenBank accession number: KU975604). *AMH* was strongly expressed in Sertoli cells (DDX4 negative) in the regressed testis (Fig. R-4H, J), while it was weakly expressed in the active testis (Fig. R-4D, I). *DMC1* and *ZFAND3* were expressed in the active testis (Fig. R-4B, C), whereas they were not in the regressed testis (Fig. R-4F, G), suggesting that the germ cell types in the regressed testis are only spermatogonia. No signal was detected when each sense probe was used as a negative control.

Identification of *AMHR2* cDNA from the Japanese quail

To examine the expression of the AMH-specific type II receptor (*AMHR2*), its partial cDNA sequence was identified from the Japanese quail by RT-PCR and RACE (GenBank accession number: KU715092). However, I could not identify the full ORF sequence in this study probably because of the high GC content (about 70%), which might have affected the reverse transcription and PCR reaction. The deduced amino acid sequence of the identified cDNA contained a transmembrane domain and protein kinase domain predicted by using TMHMM Server and Pfam database, respectively (Fig. R-5). The amino acid sequence of the quail AMHR2 was compared with those of other amniotes (Fig. R-6). The protein kinase domain was well conserved among amniotes. The amino acid identity and similarity, based on the number of identical residues or conservative substitutions, were calculated with GeneDoc software. The protein kinase domain of the quail AMHR2 showed 84-94% identity (89-97% similarity) with those of other birds, 56-67% identity (70-74% similarity) with those of reptiles, and 54-55% identity (70-71% similarity)

with those of mammals. Molecular phylogenetic tree was constructed based on the ORF nucleotide sequences of *AMHR2* and other TGF-β superfamily type II receptors from various amniotes using the neighbor-joining method. Bootstrap values were calculated with 1000 replications to estimate robustness of the internal branches. The identified sequence from the quail clustered with *AMHR2* sequences from other amniotes (Fig. R-7), showing that it is the quail ortholog of *AMHR2*.

Expression analysis of *AMHR2*

I performed tissue distribution analysis of *AMHR2* together with *AMH* by RT-PCR to examine where they are expressed in the adult quail. *AMH* and *AMHR2* were expressed primarily in the testis and ovary. Their expression was detected in the brain and slightly in the thyroid gland, adrenal gland, kidney, and heart by increasing the number of the PCR cycle (Fig. R-8A). The expression of *AMHR2* was examined in the testis of the quail at long-day or short-day condition. *AMHR2* expression was increased in the regressed testis (Fig. R-8B).

Expression analysis of *AMH* and *AMHR2* during the process of testicular changes induced by the long-day condition

The expression of *AMH*, *AMHR2*, and germ cell markers was analyzed during the process of testicular changes induced by the long-day condition. The expression of *AMH* and *AMHR2* was decreased gradually after transferring to the long-day condition (Fig. R-9A). The expression of *DMC1* and *ZFAND3* was increased from 7 days and 15 days after the long-day treatment, respectively (Fig. R-9A). The data were analyzed by the testicular stages classified based on the germ cell types in seminiferous tubules (Fig. R-9B) to examine their association with spermatogenesis. The expression of *AMH* and *AMHR2* was decreased at Stage III, and that of

AMHR2 was further decreased at Stage V. *DMC1* expression was increased at Stage III and further increased at Stage V. *ZFAND3* expression was slightly increased at Stage III and further increased at Stage V (Fig. R-9C).

Expression analysis of the ligands and receptors of TGF-β superfamily in the testis of the quail at long-day or short-day condition

The expression of the TGF-β superfamily members that are involved in spermatogenesis and their receptors was analyzed in the testis of the quail at long-day or short-day condition to examine their association with the AMH signaling. Among the receptors for BMPs, the expression of the type II receptor *BMPR2* and three type I receptors, *ALK2* (also known as *ACVR1*), *ALK3* (*BMPR1A*), and *ALK6* (*BMPR1B*), was increased in the regressed testis induced by the short-day condition. However, the expression of BMPs was low and not different between the active and regressed testis (Fig. R-10). Among the three TGF-β isoforms, the expression of *TGFB2* and *TGFB3* was increased in the regressed testis, whereas that of *TGFB1* was hardly detected. Among their receptors, the expression of the type II receptor *TGFBR2*, two type I receptors, *ALK1* (*ACVRL1*) and *ALK5* (*TGFBR1*), and endoglin (*ENG*: accessory receptor essential for ALK1 signaling) was increased in the regressed testis (Fig. R-11). The expression of three inhibin subunits, inhibin alpha (*INHA*), inhibin beta A (*INHBA*), and inhibin beta B (*INHBB*), was increased in the regressed testis. The expression of follistatin (*FST*: activin-binding protein) was also increased in the regressed testis. Among their receptors, the type II receptor *ACVR2A* was mainly expressed, but its expression was not different between the active and regressed testis. The expression of the type I receptor *ALK7* (*ACVR1C*) was decreased in the regressed testis, while that of *ALK4* (*ACVR1B*) was not changed. *BETAGLYCAN* (inhibin co-receptor also known as *TGFBR3*) expression was increased in the regressed testis (Fig. R-12).

Expression analysis of the ligands and receptors of TGF-β superfamily during the process of testicular changes induced by the long-day condition

I then analyzed the expression of the TGF-β superfamily members and their receptors during the process of testicular changes induced by the long-day condition to examine their association with spermatogenesis. Among TGF-βs and their receptors, the expression of *TGFB2*, *ALK1*, and *ENG* was decreased at Stage III, and that of *TGFB2* and *ALK1* was further decreased at Stage V. *TGFB3* expression was decreased at Stage V, and that of *ALK5* was not different among the stages (Fig. R-13). Among inhibin subunits and their related genes, the expression of *INHBA*, *INHBB*, *FST*, and *BETAGLYCAN* was decreased at Stage III, and that of *INHBA*, *INHBB*, and *FST* was further decreased at Stage V. *INHA* expression was decreased at Stage V (Fig. R-14).

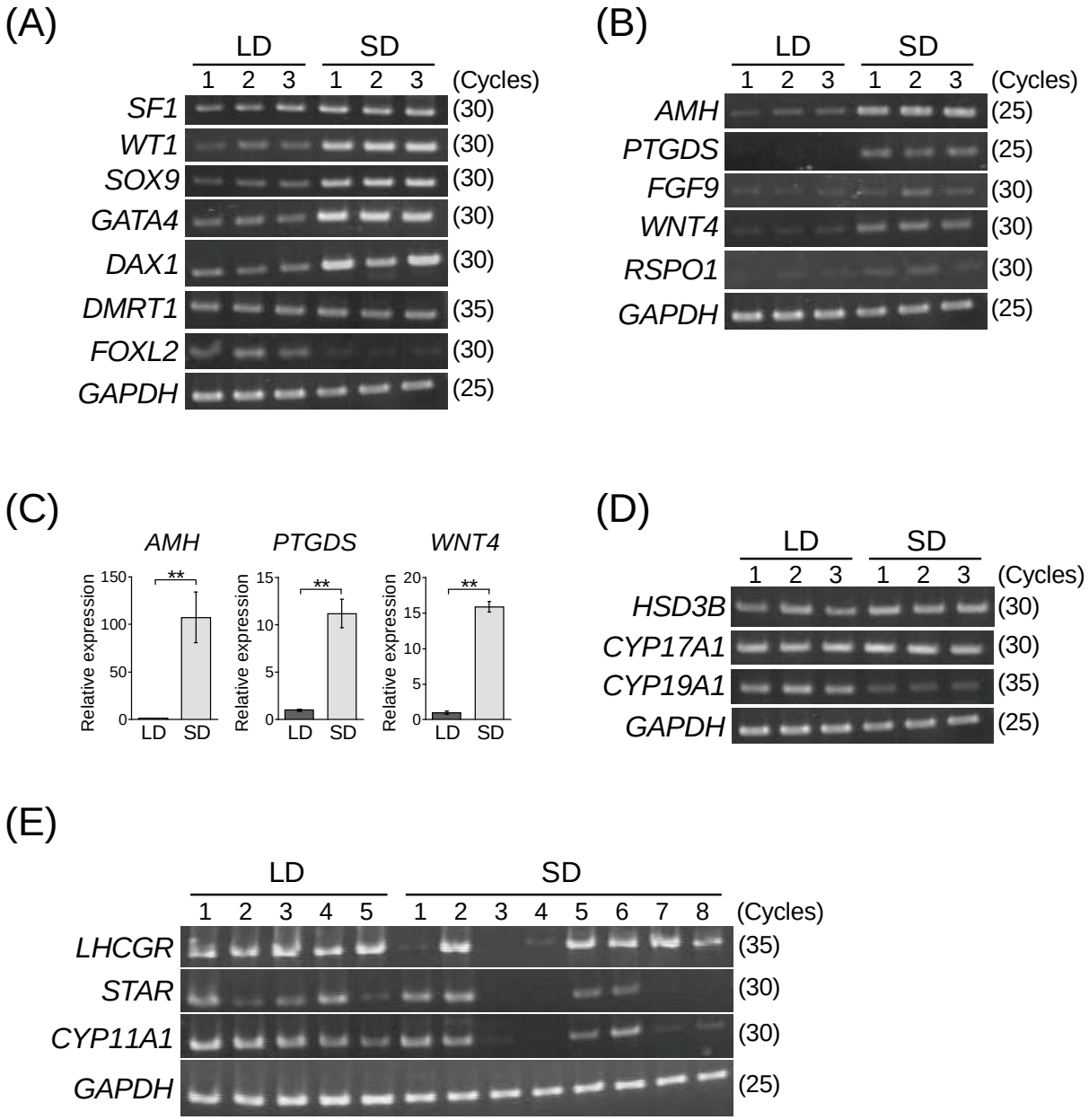


Figure R-1. Expression analysis of the sex differentiation-related genes and steroidogenic genes in the testis of the quail at long-day (LD) or short-day (SD) condition

Expression of (A) the transcription factors and (B) the secretory factors involved in sex differentiation was analyzed by RT-PCR. The numbers of each lane indicate individual number, and those in parenthesis show the number of the PCR cycle. (C) Expression of the secretory factors was quantified by real-time PCR. The relative expression of the genes was normalized to the geometric mean of *GAPDH* and *PPIA*. Results are shown as mean ± SEM. (n=6 / group), and the expression level of the LD group is expressed as 1. ** $p<0.01$ (Mann Whitney's *U* test). (D) The genes involved in steroidogenesis were analyzed by RT-PCR as indicators of adult testicular functions. (E) The steroidogenic genes that showed individual differences were analyzed by increasing the number of samples.

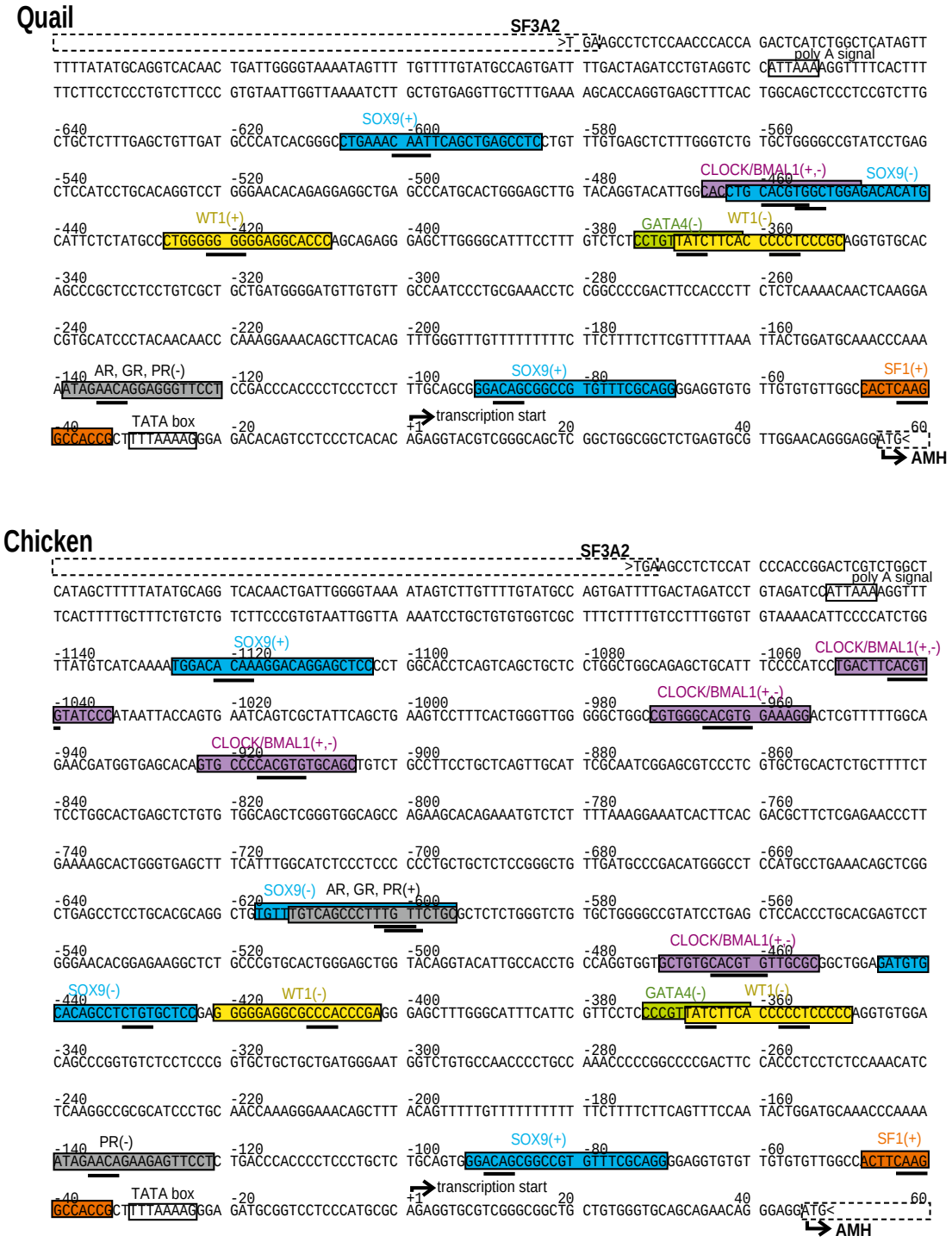


Figure R-2. The putative transcription factor binding sites in the quail and chicken *AMH* promoter region

The DNA sequences from stop codon of *SF3A2* to start codon of *AMH* were analyzed using MatInspector software. The predicted binding sites are indicated by colored boxes, and the core sequences are underlined. (+) or (–) indicates the orientation that the transcription factors recognize (+: sense sequence, -: antisense sequence). Numbers above the sequences indicate the number of nucleotides. The major transcription start site of *AMH* in the chicken (Oreal *et al*., 1998) is indicated by arrow and designated as position +1. The degenerated TATA box (Oreal *et al*., 1998) is indicated by box. The ORF of *SF3A2* and *AMH* are indicated by dotted boxes, and poly A signal of *SF3A2* is indicated by box.

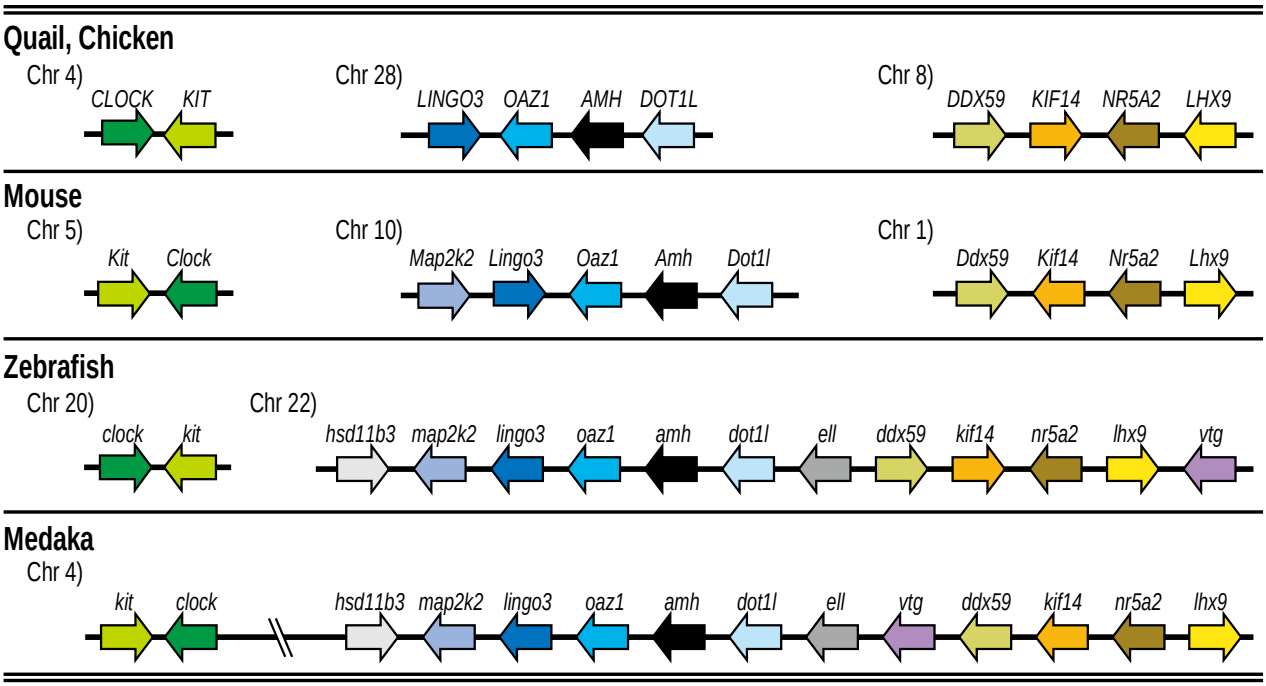


Figure R-3. Schematic diagram of conserved syntenic chromosomal regions surrounding *AMH* in the quail, chicken, mouse, zebrafish, and medaka

Synteny analysis of *AMH* gene was performed using NCBI database. The orientation of the genes is indicated by arrows. The orthologs are indicated by the same colors. The data of the mouse, zebrafish, and medaka are taken from Paibomesai *et al*., 2010 with some modifications.

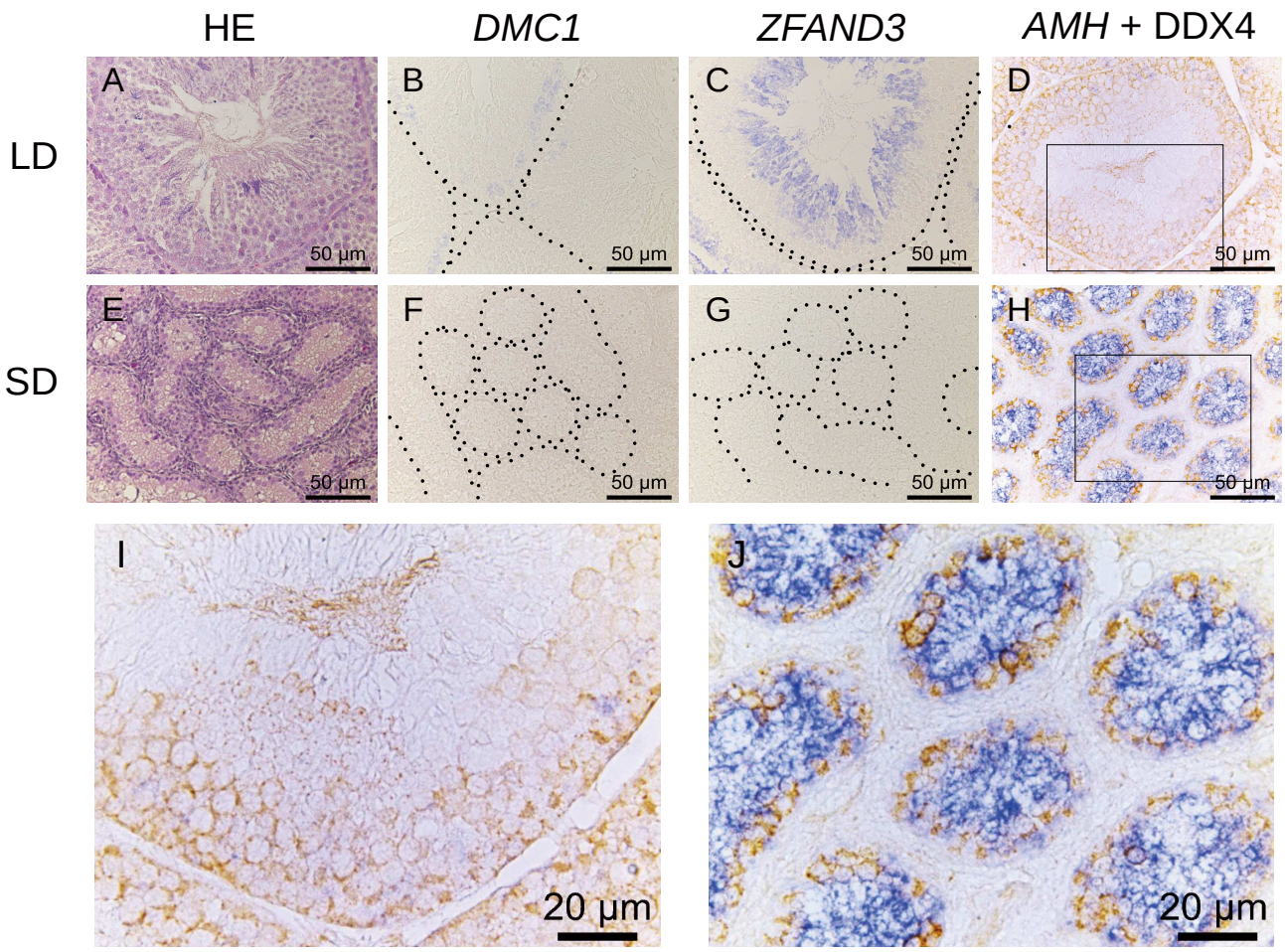


Figure R-4. *In situ* hybridization analysis of *AMH* and germ cell markers in the testis of the quail at long-day (LD) or short-day (SD) condition

(A, E) Hematoxylin and eosin staining. (B, F) The results of *in situ* hybridization for *DMC1*. (C, G) The results of *in situ* hybridization for *ZFAND3*. (D, H) The results of *in situ* hybridization for *AMH* and immunohistochemistry for DDX4 (also known as VASA). Blue signal: *AMH*, Brown signal: DDX4. (I, J) Enlarged view of the region enclosed in the square in (D) and (H), respectively. The germ cell types in the testis of the LD condition were spermatogonia, spermatocytes, spermatids, and spermatozoa (Stage V), while those in the testis of the SD condition were only spermatogonia (Stage II). Dotted lines indicate the outline of seminiferous tubules.

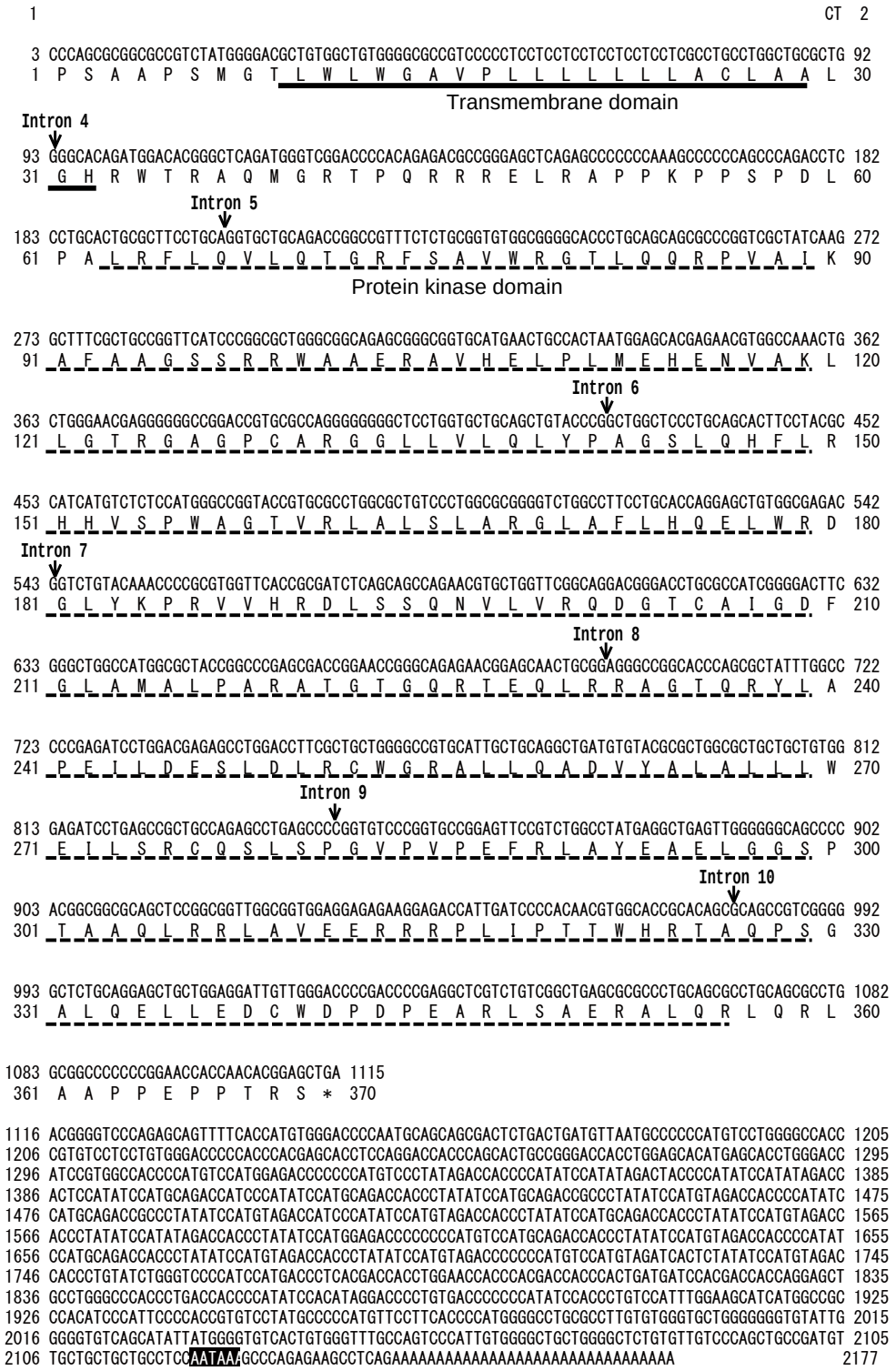


Figure R-5. Nucleotide and deduced amino acid sequences of Japanese quail *AMHR2* cDNA

Numbers on the both sides indicate the number of nucleotides (top) and amino acid residues (bottom). The exon/intron boundaries predicted by the comparison with the chicken genome are indicated by arrows, and the intron number is based on the comparison with the sequences of other vertebrates. The asterisk indicates the stop codon. Polyadenylation signal is indicated by outlined characters. The transmembrane domain predicted by TMHMM Server (version 2.0) is indicated by a line. The protein kinase domain predicted by Pfam database (version 29.0) is indicated by a dotted line.

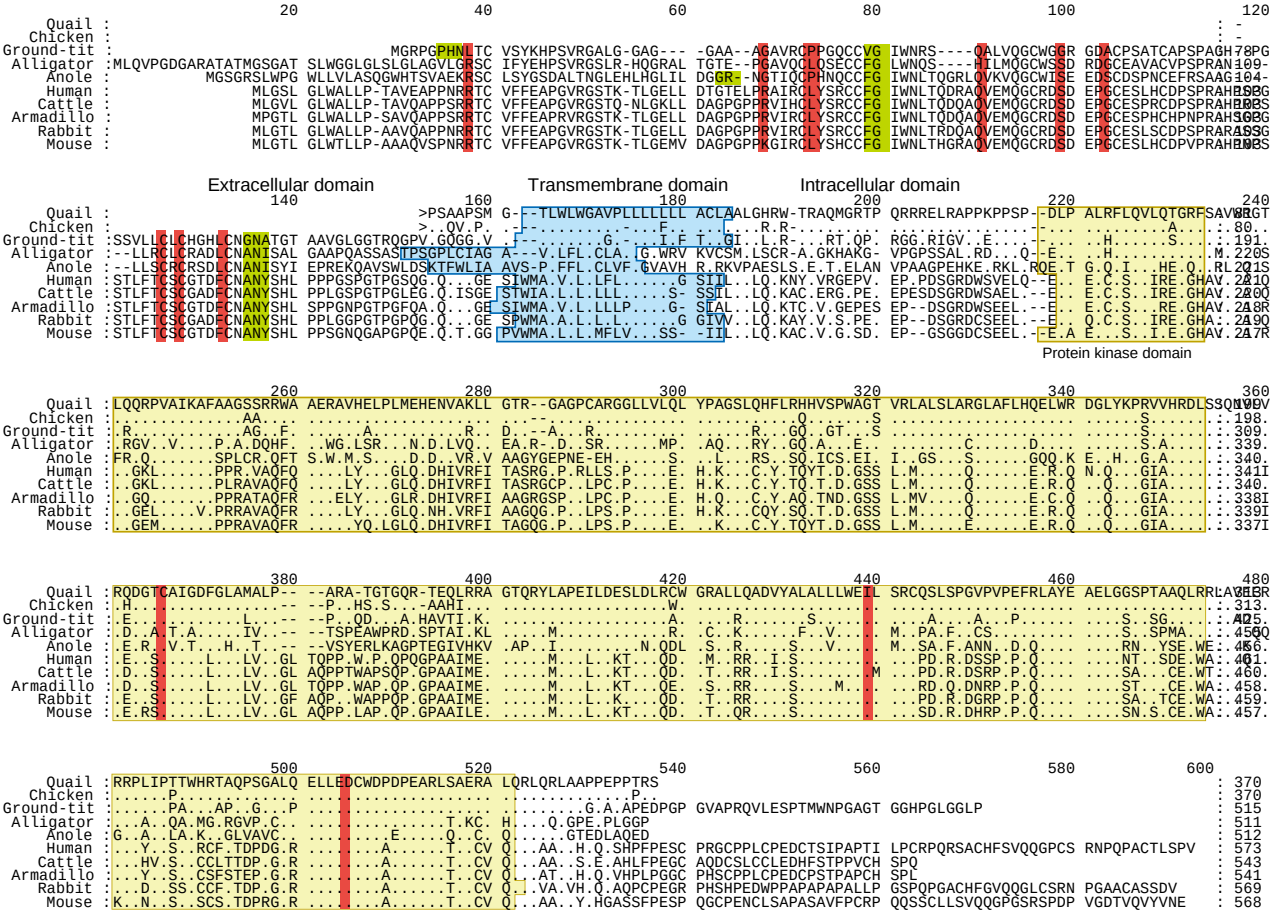


Figure R-6. Alignment of the amino acid sequences of AMHR2 from various amniotes

The CLUSTAL W program (version 2.1) was used with default settings to align the amino acid sequences of AMHR2 from various amniotes. Numbers on the right sides and above the sequences indicate the number of amino acid residues. Dots indicate the identity of amino acid sequences in relation to those of Japanese quail AMHR2. Dashes indicate gaps inserted in the alignment. The transmembrane domains predicted by TMHMM Server (version 2.0) are indicated by blue box. The protein kinase domains predicted by Pfam database (version 29.0) are indicated by yellow box. The conserved cysteine residues are colored in red. The N-glycosylation sites (N-X (any residues except P)-S or T) predicted by NetNGlyc 1.0 Server are colored in green.

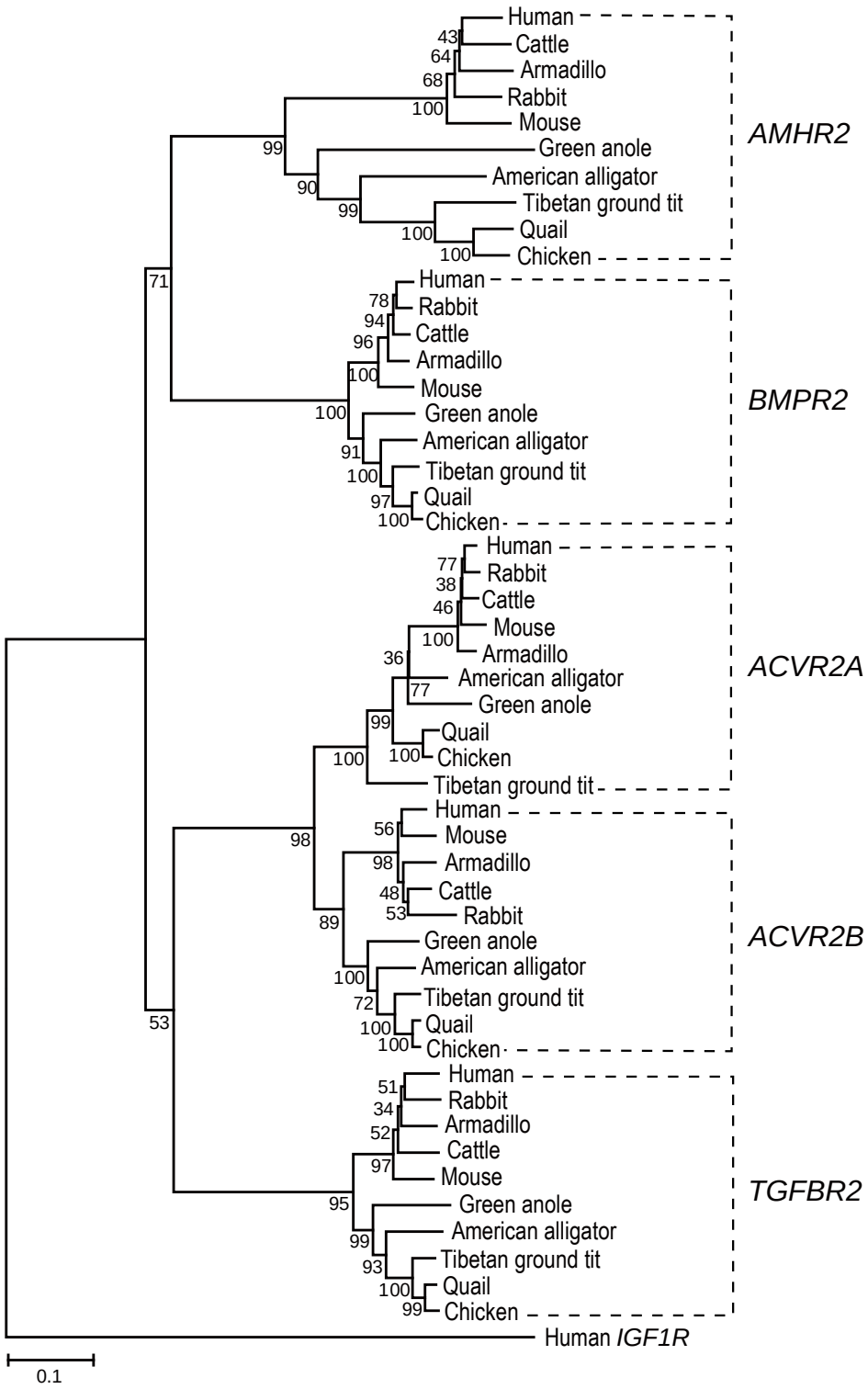


Figure R-7. Phylogenetic tree of *AMHR2* and other TGF-β superfamily type II receptors from various amniotes based on the ORF nucleotide sequences

The tree was constructed using the neighbor-joining method with MEGA software (version 7.0.14). Human *IGF1R* was used as an out group. Bootstrap values of 1000 replications are indicated for all nodes. The scale bar beneath the tree corresponds to estimated evolutionary distance units.

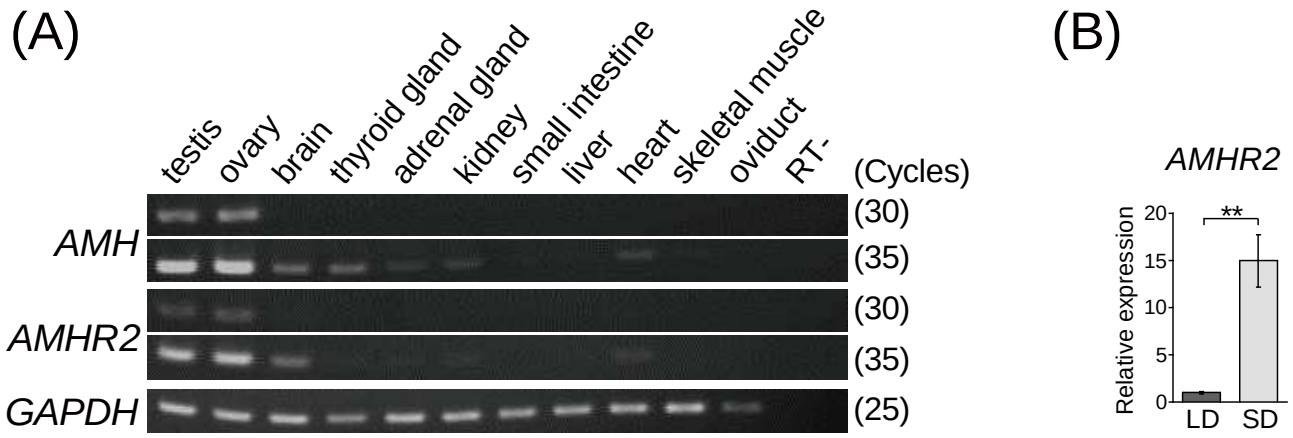


Figure R-8. Expression analysis of *AMHR2* in the adult quail

(A) Expression analysis of *AMH* and *AMHR2* in various tissues of the adult quail. RT− represents the negative control using testis total RNA without reverse transcription. The numbers in parenthesis show the number of the PCR cycle. (B) Expression analysis of *AMHR2* in the testis of the quail at long-day (LD) or short-day (SD) condition by real-time PCR. The relative expression of the gene was normalized to the geometric mean of *GAPDH* and *PPIA*. The result is shown as mean ± SEM. (n=6 / group), and the expression level of the LD group is expressed as 1. ** $p<0.01$ (Mann Whitney's *U* test).

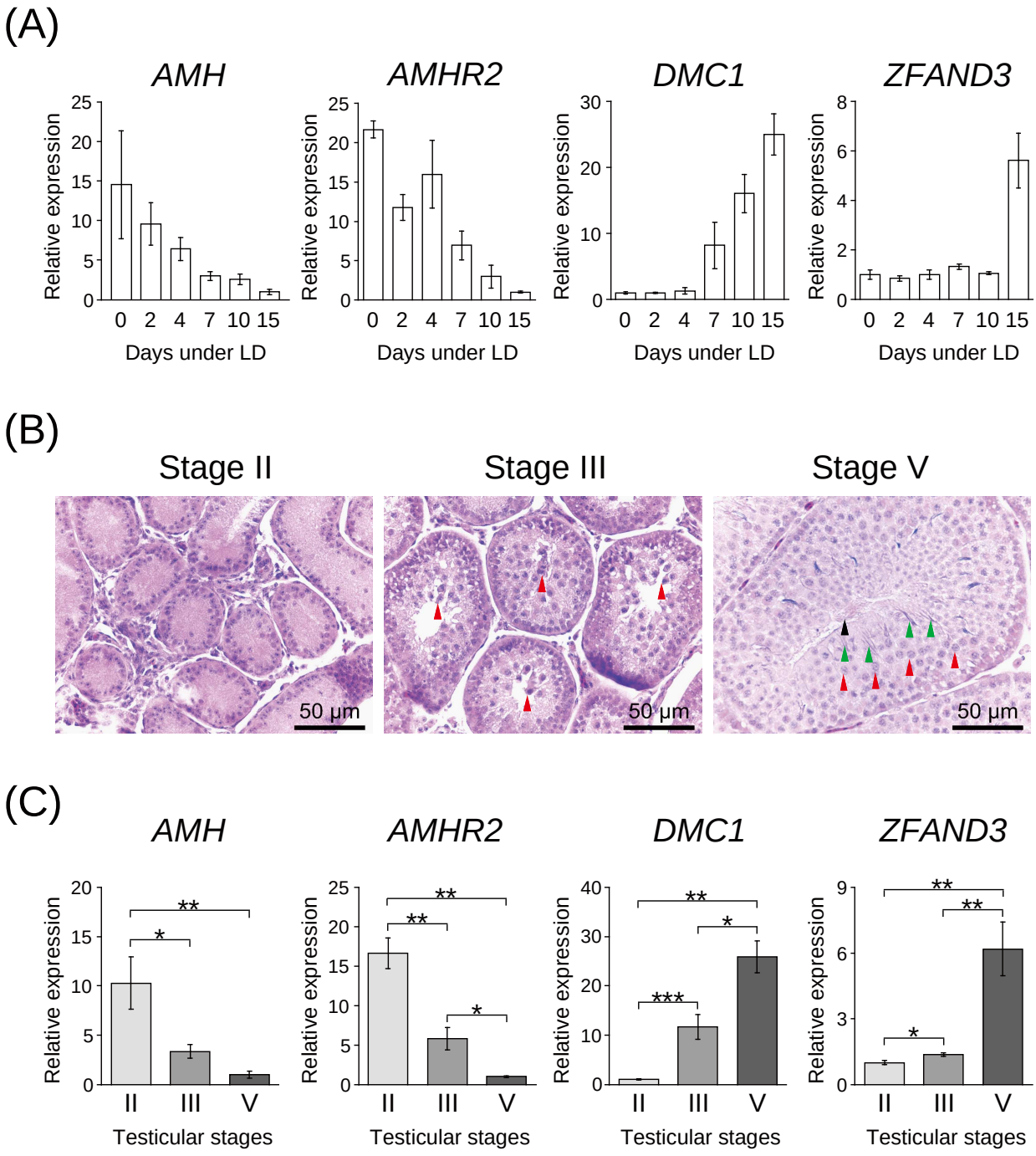


Figure R-9. Expression analysis of *AMH*, *AMHR2*, and germ cell markers during the process of testicular changes induced by the long-day condition

(A) Expressional changes by the days after the long-day treatment (n=5 / group). (B) Representative pictures of the testis at Stage II, Stage III, and Stage V. The testes were stained with hematoxylin and eosin. Stage II: spermatogonia, Stage III: spermatogonia and spermatocytes, Stage V: spermatogonia, spermatocytes, spermatids, and spermatozoa. Red, green, and black arrowheads indicate spermatocytes, spermatids, and spermatozoa, respectively. (C) Expressional changes by the testicular stages. Stage II: n=14, Stage III: n=11, Stage V: n=5. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ (Steel-Dwass test). For the results in (A) and (C), the relative expression of the genes was normalized to the geometric mean of *GAPDH* and *PPIA*. Results are shown as mean ± SEM., and the expression level of the lowest group is expressed as 1.

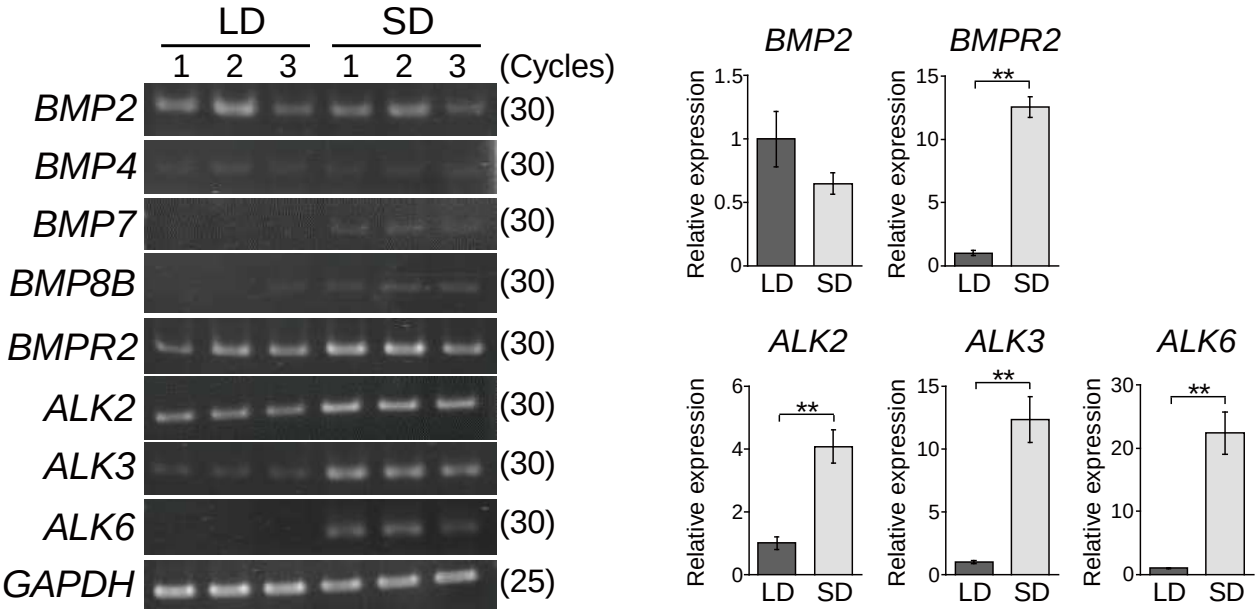


Figure R-10. Expression analysis of BMPs and their receptors in the testis of the quail at long-day (LD) or short-day (SD) condition by RT-PCR and real-time PCR

The numbers of each lane indicate individual number, and those in parenthesis show the number of the PCR cycle. Some selected genes were also analyzed by real-time PCR. The relative expression of the genes was normalized to the geometric mean of *GAPDH* and *PPIA*. Results are shown as mean ± SEM. (n=6 / group), and the expression level of the LD group is expressed as 1. ** $p<0.01$ (Mann Whitney's *U* test).

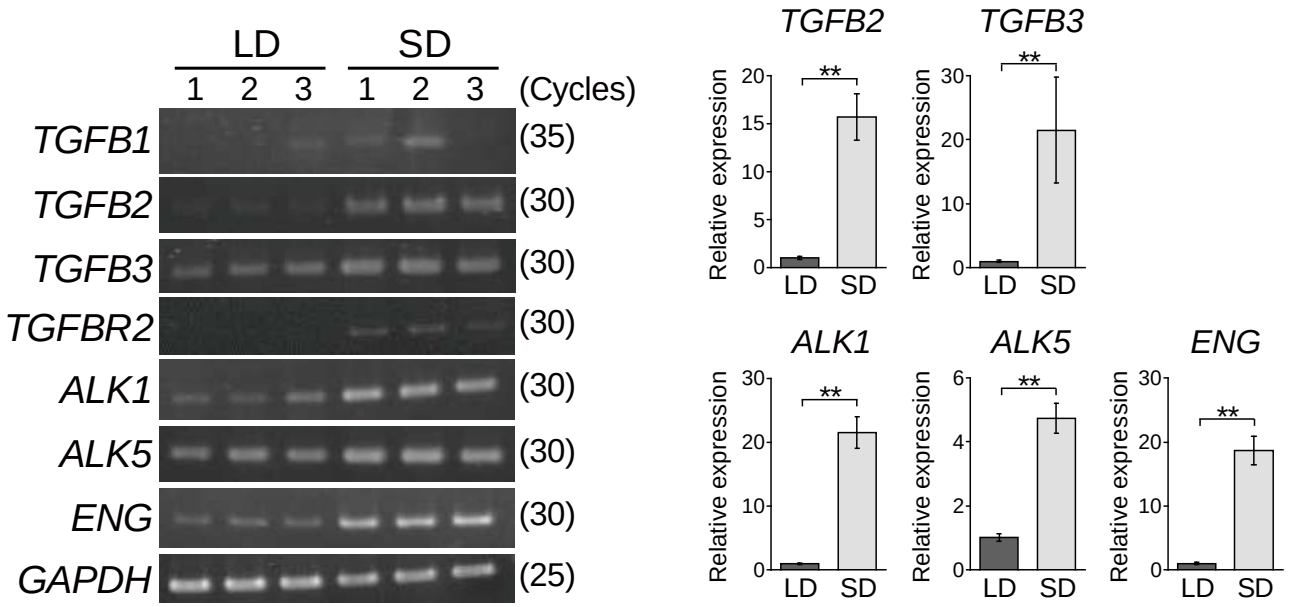


Figure R-11. Expression analysis of TGF-βs and their receptors in the testis of the quail at long-day (LD) or short-day (SD) condition by RT-PCR and real-time PCR

The numbers of each lane indicate individual number, and those in parenthesis show the number of the PCR cycle. Some selected genes were also analyzed by real-time PCR. The relative expression of the genes was normalized to the geometric mean of *GAPDH* and *PPIA*. Results are shown as mean ± SEM. (n=6 / group), and the expression level of the LD group is expressed as 1. ** $p<0.01$ (Mann Whitney's *U* test).

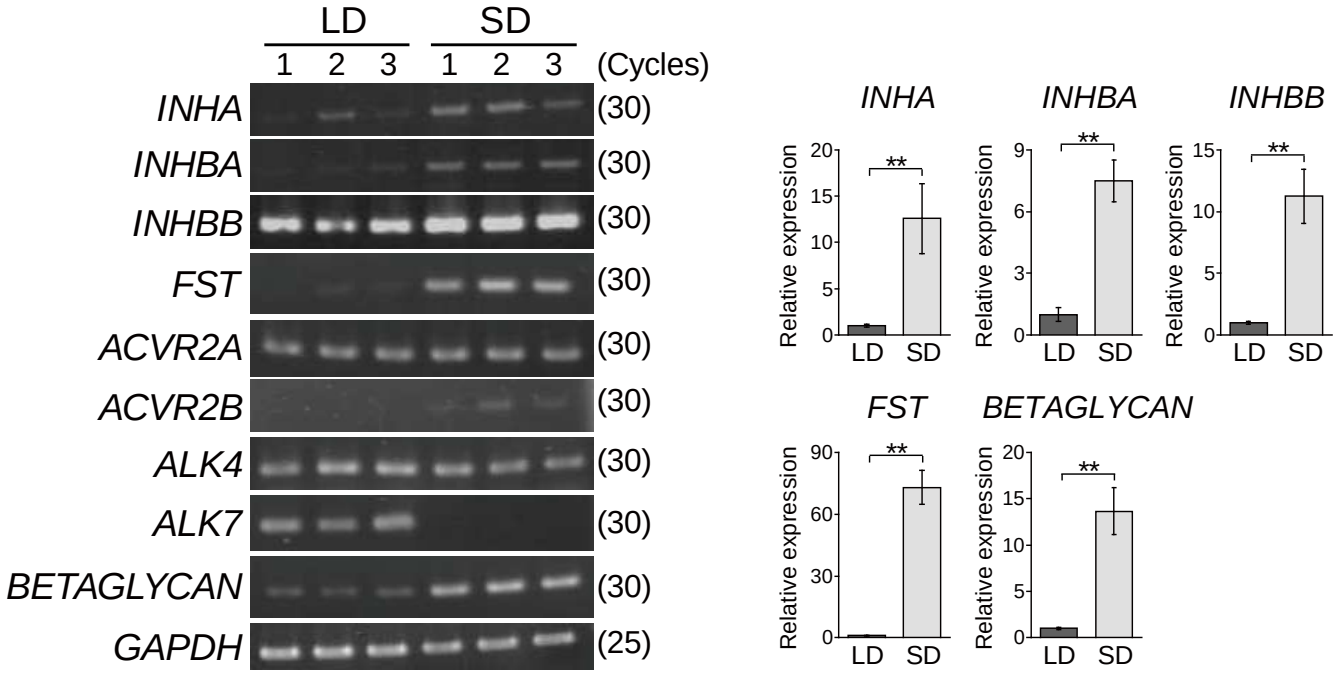


Figure R-12. Expression analysis of inhibin subunits and their related genes in the testis of the quail at long-day (LD) or short-day (SD) condition by RT-PCR and real-time PCR

The numbers of each lane indicate individual number, and those in parenthesis show the number of the PCR cycle. Some selected genes were also analyzed by real-time PCR. The relative expression of the genes was normalized to the geometric mean of *GAPDH* and *PPIA*. Results are shown as mean ± SEM. (n=6 / group), and the expression level of the LD group is expressed as 1. ** $p<0.01$ (Mann Whitney's *U* test).

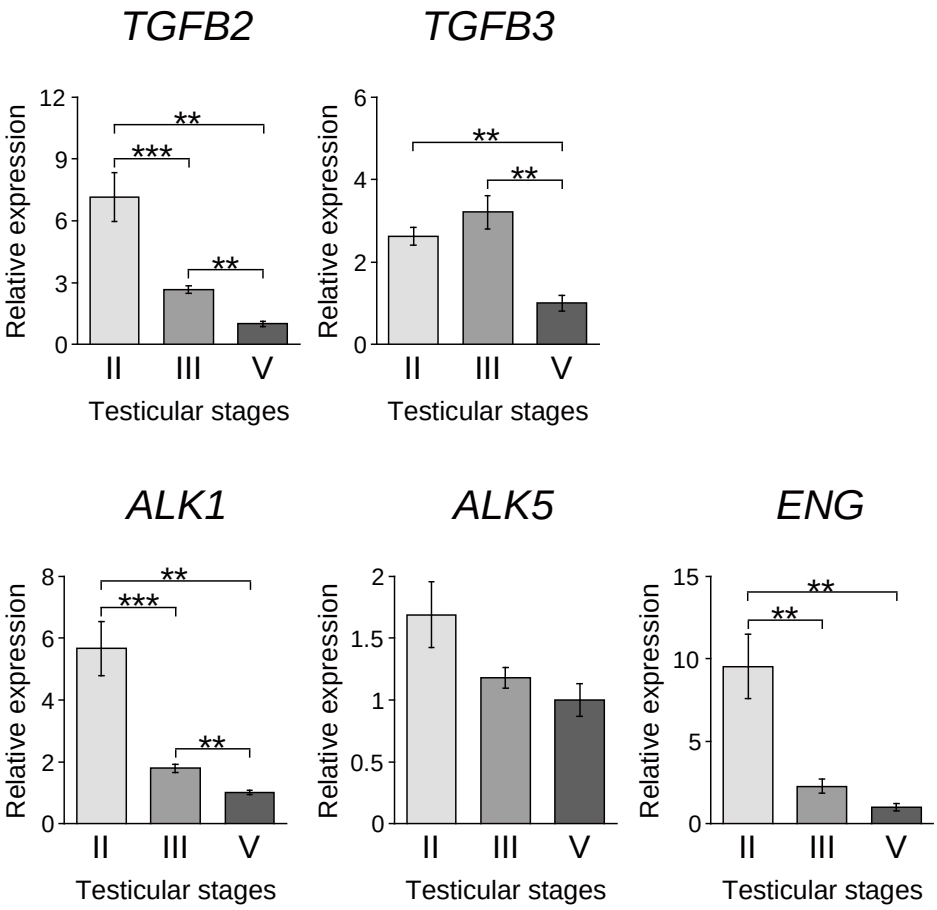


Figure R-13. Expression analysis of TGF-βs and their receptors during the process of testicular changes induced by the long-day condition

The relative expression of the genes was normalized to the geometric mean of *GAPD*H and *PPIA*. Results are shown as mean ± SEM. Stage II: spermatogonia (n=14), Stage III: spermatogonia and spermatocytes (n=11), Stage V: spermatogonia, spermatocytes, spermatids, and spermatozoa (n=5). The expression level of Stage V is expressed as 1. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ (Steel-Dwass test).

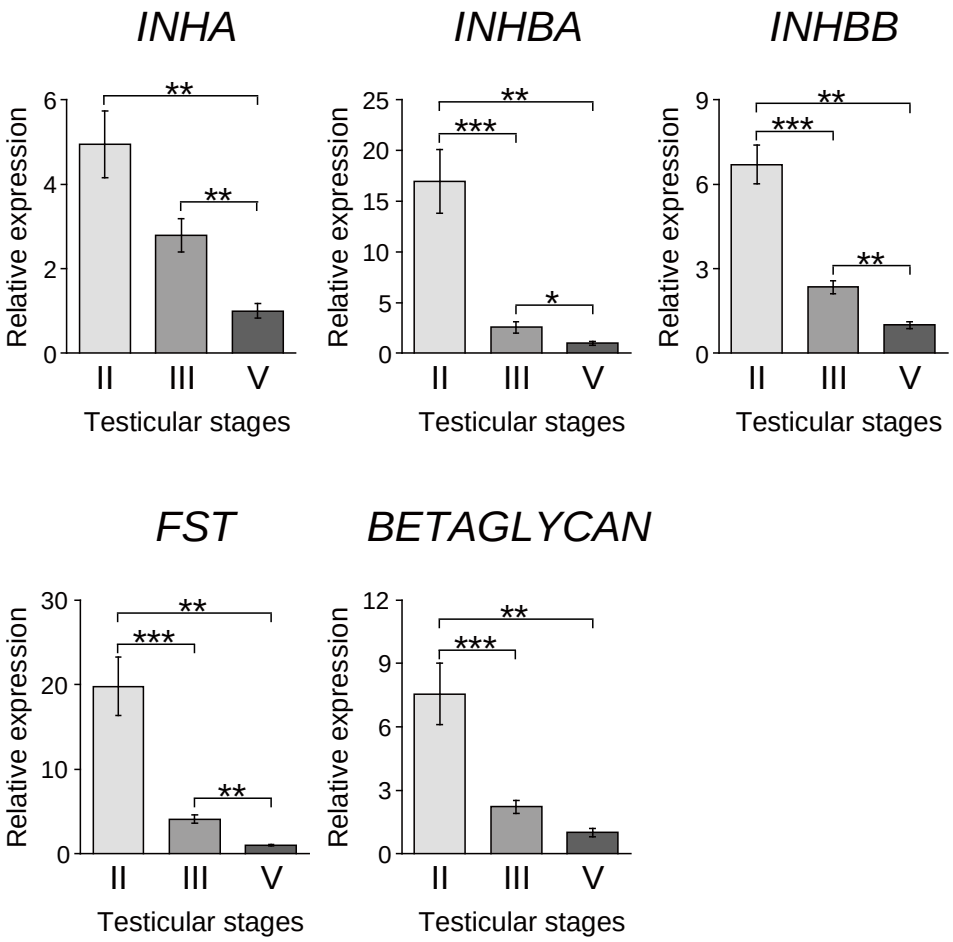


Figure R-14. Expression analysis of inhibin subunits and their related genes during the process of testicular changes induced by the long-day condition

The relative expression of the genes was normalized to the geometric mean of *GAPDH* and *PPIA*. Results are shown as mean ± SEM. Stage II: spermatogonia (n=14), Stage III: spermatogonia and spermatocytes (n=11), Stage V: spermatogonia, spermatocytes, spermatids, and spermatozoa (n=5). The expression level of Stage V is expressed as 1. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ (Steel-Dwass test).

Discussion

Expression of the sex differentiation-related genes in the adult testis

Gonadal sex differentiation proceeds by the interplay of various genes encoding transcription factors and secretory factors in a complex network. Those genes have been reported to be expressed during postnatal gonadal development and even in the adult gonads. Moreover, the studies using conditional knockout mice suggest that they are involved in the adult gonadal functions. On the other hand, few studies have not been conducted from the viewpoint of gene network. In the present study, I analyzed the expression profile of various sex differentiation-related genes encoding the transcription factors and secretory factors associated with the testicular changes in the quail. The adult quail were reared under the long-day or short-day condition for 4 weeks. The average weight of the regressed testis induced by the short-day condition was decreased by about 80-fold compared to that of the long-day control (Fig. I-2). This result is consistent with the previous report (Follett and Farner 1966).

The expression of most of the transcription factors (*SF1*, *WT1*, *SOX9*, *GATA4*, and *DAX1*) was increased in the regressed testis induced by the short-day condition (Fig. R-1A). Among the secretory factors, the expression of *AMH*, *PTGDS*, and *WNT4* was increased in the regressed testis. In particular, *AMH*, which acts on Müllerian ducts during male sex differentiation, was expressed at a high level and significantly upregulated in the regressed testis (Fig. R-1B, C, and Fig. R-4). These results are consistent with the findings about the regulation of *Amh* expression during gonadal sex differentiation period. SF1, WT1, GATA4, and SOX9 are known to upregulate *Amh* expression synergistically by binding to its promoter in mammals (Lasala *et al*., 2004). In addition, WT1 and GATA4 can upregulate *Amh* expression by physical interaction with SF1.

Another possible interaction is expected between *PTGDS* and *SOX9*. *Sox9* is upregulated by prostaglandin D_2 (converted from prostaglandin H_2 by PTGDS) in the mouse (Wilhelm *et al*., 2007) and chicken (Moniot *et al*., 2008) during gonadal sex differentiation. Therefore, it is

suggested that the increased expression of *PTGDS* contributes to the increase of *SOX9* (and *AMH*) expression in the regressed testis of the quail via prostaglandin D_2 signaling.

Unexpectedly, *DAX1* expression was also increased in the regressed testis (Fig. R-1A) though it is known to repress *Amh* expression in mammals (Tremblay *et al*., 2001). *DAX1* may be upregulated by *WNT4*, which was also increased in the regressed quail testis (Fig. R-1B, C), as reported in mammals (Jordan *et al*., 2001, Mizusaki *et al*., 2003). DAX1 is also known to repress the expression of the genes involved in steroidogenesis in mammals (Lalli *et al*., 1998), but most of the steroidogenic genes were not downregulated or showed individual differences in the regressed quail testis (Fig. R-1D, E) though *DAX1* expression was increased. One possible explanation for these discrepancy is that the roles of DAX1 in birds are different from those reported in mammals. Actually, during embryonic development, *Dax1* expression was decreased during testicular differentiation in the mouse (Morrish and Sinclair 2002), while it was maintained in the chicken (Smith *et al*., 1999). This difference in the expression pattern may reflect the functional difference. Although DAX1 was initially thought to have anti-testis functions (Bardoni *et al*., 1994, Swain *et al*., 1998), it was also found to be essential for testis differentiation (Meeks *et al*., 2003a, b). The increased expression of *DAX1* as well as other sex differentiation-related genes in the regressed quail testis may be involved in the maintenance of testicular functions during quiescent period in preparation for the following reactivation by the long-day condition.

To examine the relationship between AMH and the transcription factors that were upregulated by the short-day condition, I analyzed the putative promoter sequence of quail *AMH* gene. In the quail *AMH* promoter region, there were several putative binding sites for the transcription factors, such as one SF1 site, three SOX9 sites, two WT1 sites, and one GATA4 site (Fig. R-2, upper panel). I also analyzed the putative promoter sequence of the chicken, which is a model animal for study on sex differentiation in avian species, for the comparison with that of

the quail. The chicken sequence contained one SF1 site, four SOX9 sites, two WT1 sites, and one GATA4 site (Fig. R-2, lower panel). Among these transcription factors, SF1 was reported to bind to its binding site and activate *AMH* expression in the chicken (Takada *et al*., 2006). In the chicken, *SOX9* is not required for the onset of *AMH* expression because *AMH* expression precedes that of *SOX9* during testicular differentiation. However, it was suggested that *SOX9* contributed to subsequent upregulation of *AMH* because it coincided with the onset of *SOX9* expression (Morrish and Sinclair 2002). The presence of the binding sites for SF1, SOX9, WT1, and GATA4 suggests that these transcription factors contribute to the increased expression of *AMH* in the regressed quail testis induced by the short-day condition. More detailed studies are necessary to examine whether SOX9, WT1, and GATA4 bind to their putative binding sites and activate *AMH* expression.

In relation to sex steroid hormone signaling, there was a common binding site for androgen receptor (AR), glucocorticoid receptor (GR), and progesterone receptor (PR) in the quail *AMH* promoter region (Fig. R-2, upper panel). In the chicken, there were one PR site and one common site for AR, GR, and PR (Fig. R-2, lower panel). Androgens are thought to be responsible for the downregulation of AMH expression because of their negative correlation in serum level (Rey *et al*., 1993). The presence of AR binding site suggests the direct inhibitory effect of androgens on *AMH* expression.

Unexpectedly, the quail and chicken *AMH* promoter regions contained binding sites for CLOCK/BMAL1 heterodimer, which is involved in the regulation of circadian rhythm (Bell-Pedersen *et al*., 2005) (Fig. R-2). In the chicken, there were three additional CLOCK/BMAL1 sites that formed tandem repeats. In some teleost species like medaka, *amh* gene lies in a chromosomal region that contains the *clock* gene and reproductive and cell cycling genes, suggesting the presence of a functional cluster (Paibomesai *et al*., 2010). In the quail and chicken,

there was a conserved synteny of *LINGO3*, *OAZ1*, *AMH*, and *DOT1L* on chromosome 28 (Fig. R-3). However, *CLOCK* and *KIT* were located on the different chromosome (Chr 4) like in the mouse and zebrafish. *DDX59*, *KIF14*, *NR5A2*, and *LHX9* were also located on the different chromosome (Chr 8) like in the mouse. These results indicate that there is no functional cluster including *CLOCK*, *AMH*, and other reproduction-related genes in the quail and chicken. In the zebrafish, the expression of the genes involved in reproduction including *amh* showed daily rhythms (Di Rosa *et al*., 2016) though *amh* and *clock* are not located on the same chromosome. This result suggests that the expression rhythm is not linked to the presence of the functional cluster. In future study, it is important to examine whether the expression of *AMH* is regulated by CLOCK for understanding the molecular mechanism of photoperiod-regulated expression of *AMH* in the quail.

In summary, the expression of many sex differentiation-related genes was increased in the regressed testis induced by the short-day condition. A model of the network of sex differentiation-related genes in the adult quail testis is illustrated in Fig. D-1. It is suggested that this network works in Sertoli cells because the sex differentiation-related genes have been reported to be expressed in Sertoli cells in the mouse (Wilhelm *et al*., 2007) and chicken (Oreal *et al*., 2002). In particular, *AMH* was significantly upregulated in the regressed testis. The expression of the transcription factors that promote *AMH* expression in mammals (*SF1*, *SOX9*, *WT1*, and *GATA4*) was also increased. Moreover, there were putative binding sites for these transcription factors in the quail *AMH* promoter region. These results suggest that the transcription factors involved in sex differentiation play a role in the regulation of *AMH* expression in the adult quail testis, and AMH is involved in the testicular changes. Therefore, I decided to analyze the expression of the AMH-specific type II receptor (*AMHR2*).

AMH signaling in the adult testis

Since the nucleotide sequence of *AMHR2* in the avian species had not been identified and was not available in the genome database, I identified the partial *AMHR2* cDNA sequence from the quail to examine its expression. The deduced amino acid sequence contained a transmembrane domain and protein kinase domain (Fig. R-5). The protein kinase domain was well conserved among amniotes (Fig. R-6). Molecular phylogenetic tree was constructed based on the ORF nucleotide sequences of *AMHR2* and other TGF-β superfamily type II receptors from various vertebrates. The identified sequence from the quail clustered with *AMHR2* sequences from other amniotes (Fig. R-7), showing that it is the quail ortholog of *AMHR2*. I could not identify the full ORF sequence in this study probably because of the high GC content (about 70%), which might have affected the reverse transcription and PCR reaction. The partial cDNA sequence of the chicken *AMHR2* was reported, and the same reason was suggested for the sequencing difficulties in avian species (Cutting *et al*., 2014). *AMHR2* expression was increased in the regressed testis induced by the short-day condition (Fig. R-8B). The transcription factors increased in the regressed testis (*SF1*, *SOX9*, *WT1*, and *GATA4*) may also contribute to the increased expression of *AMHR2* because the binding sites for these factors are present in the mammalian *AMHR2* promoter (de Santa Barbara *et al*., 1998, Klattig *et al*., 2007). In future, it is necessary to identify the promoter sequence of quail *AMHR2* to investigate its expressional regulation.

AMH and *AMHR2* were expressed primarily in the adult ovary in addition to the testis (Fig. R-8A). This indicates that *AMH* expression is not sex-specific in the adult gonads though it is male specific during early gonadal sex differentiation (Morrish and Sinclair 2002, Cutting *et al*., 2013). *AMH* was expressed in the granulosa cells depending on the follicular development in the adult quail ovary (data not shown). Similar expression patterns have been reported in mammals, and AMH is known to inhibit the initial follicle recruitment and FSH-dependent growth and

selection of preantral and small antral follicles (Visser *et al*., 2006). However, the expressional regulation of *AMH* in the ovary still remains unknown. In future, it is important to examine when *AMH* is expressed during ovarian development and its association with the expression of the transcription factors that upregulate *AMH* expression in the testis (*SF1*, *SOX9*, *WT1*, and *GATA4*) to understand the expressional mechanism in the ovary.

AMH and *AMHR2* were also expressed in the brain and slightly in the thyroid gland, adrenal gland, kidney, and heart (Fig. R-8A). Gonad-specific expression of *Amhr2* was reported in the rat (Baarends *et al*., 1994) and medaka (Klüver *et al*., 2007). On the other hand, the expression of *Amh* and *Amhr2* in the brain was also reported in the mouse (Lebeurrier *et al*., 2008) and Nile tilapia (Pfennig *et al*., 2015). The expression of *AMH* and *AMHR2* in the quail brain may be involved in the brain functions, such as neuronal survival reported in the mouse (Lebeurrier *et al*., 2008).

The expression analysis of *AMH* and *AMHR2* was conducted during the process of testicular changes induced by the long-day condition and analyzed by the testicular stages to examine their association with spermatogenesis. The expression of *AMH* and *AMHR2* was decreased at Stage III, when spermatocytes appeared in the seminiferous tubules (Fig. R-9B). The correlation between the decreased expression of *AMH* and the onset of meiosis is consistent with the reports from the human (Rey *et al*., 1996), mouse (Al-Attar *et al*., 1997), and Japanese flounder (Yoshinaga *et al*., 2004). In the quail, *AMHR2* expression was also decreased at Stage III. Similar results were reported in the black porgy. The expression of *amh* and *amhr2* was increased during pre-meiotic period and declined in the mature testis during the spawning season (Wu *et al*., 2010). Increased expression of *AMH* and *AMHR2* in the regressed quail testis and decrease of them at Stage III suggest that AMH is involved in the regulation of meiosis and/or spermatogonial proliferation and differentiation. Previous studies in the rat and fish support the role of AMH in

the germ cell proliferation. In the adult rat testis, *Amh* and *Amhr2* mRNA were expressed at a maximal level in the seminiferous tubule segments where mitotic divisions of spermatogonia are minimum (Baarends *et al*., 1995). The inhibitory effect of Amh on spermatogonial proliferation and differentiation was also demonstrated by using the testis tissue culture from the zebrafish (Skarr *et al*., 2011) and Japanese eel (Miura *et al*., 2002). Moreover, *amhr2* mutation led to the excessive proliferation of germ cells in the medaka *hotei* mutant and caused sex reversal (Morinaga *et al*., 2007). In this context, in several fish species, AMH signaling determines the gonadal sex. A duplicated copy of *amh* on the Y-chromosome (*amhy*) is a male sex-determining gene in the Patagonian pejerrey (*Odontesthes hatcheri*) (Hattori *et al*., 2012) and pejerrey (*Odontesthes bonariensis*) (Yamamoto *et al*., 2014), and *amhy* with a missense SNP is essential for male sex determination in the Nile tilapia (Li *et al*., 2015). In *Takifugu* species, a missense SNP in the *amhr2* gene is associated with phenotypic sex (Kamiya *et al*., 2012). These studies from fish species that have no Müllerian ducts suggest that the functions of AMH in the gonad are evolutionally more ancient than the promotion of Müllerian duct regression.

AMH has also been reported to be involved in steroidogenesis in mammals and fish. For example, overexpression of AMH blocked the differentiation of Leydig cell precursors and decreased the expression of steroidogenic genes, such as *Cyp17a1, Cyp11a1,* and *Hsd3B* in the mouse (Racine *et al*., 1998). In zebrafish testis tissue culture, recombinant Amh inhibited gonadotropin-stimulated androgen production by decreasing the expression of the genes involved in steroidogenesis (Skarr *et al*., 2011). In this study, the expression of *CYP19A1* was decreased in the regressed quail testis induced by the short-day condition (Fig. R-1D). This result is consistent with the inverse relation with *AMH* during chicken gonadal sex differentiation (Nishikimi *et al*., 2000). However, the expression of other steroidogenic genes was not downregulated or showed individual differences in the regressed quail testis (Fig. R-1D, E). These results suggest that the

effects of AMH on the expression of steroidogenic genes differ among species. More detailed studies are needed to verify this possibility.

In summary, the expression of *AMH* and *AMHR2* was increased in the regressed testis, and it was decreased at Stage III, when spermatocytes were observed, during the long-day-induced testicular changes. These results suggest that AMH signaling in the quail is involved in the regulation of spermatogenesis.

TGF-β superfamily signaling system in the adult testis -relationship with AMH signaling-

AMH is a member of the TGF-β superfamily, and most of the members are known to contribute to testicular functions such as spermatogenesis (Itman *et al*., 2006). There are complex signaling cross-talks in this superfamily because their receptors and SMADs are shared among different kinds of ligands (Miyazawa *et al*., 2002, Shi and Massagué 2003). Moreover, mutually antagonistic relationship between the two groups of SMADs (SMAD1/5/8 and SMAD2/3) has been reported. Therefore, it is important to examine the relationship between AMH and other members of the superfamily for understanding the role of AMH in the adult testis. However, few studies have examined their signaling system in the testis. In this study, I also analyzed the expression of the TGF-β superfamily members that are involved in spermatogenesis and their receptors during the testicular changes in the quail to examine their association with the AMH signaling.

BMPs and AMH use the same three BMP type I receptors, ALK2, ALK3, and ALK6, and their downstream factors SMAD1/5/8 (Miyazawa *et al*., 2002, Shi and Massagué 2003). The expression of BMP type II and type I receptors was increased in the regressed testis induced by the short-day condition (Fig. R-10). Increased expression of the type I receptors further supports the activation of AMH signaling in the regressed testis. However, the expression of BMPs was

low and not different between the active and regressed testis (Fig. R-10). In addition, *BETAGLYCAN* (inhibin co-receptor) expression was increased in the regressed testis (Fig. R-12). It is known to enable inhibins to compete with BMPs for binding to BMPR2 (Wiater and Vale 2003). Therefore, it is suggested that BMP signaling is inhibited by inhibins in the regressed testis. These results suggest that there are no cooperative action between AMH and BMPs via type I receptors.

TGF-βs activate two distinct signaling pathways, ALK5-SMAD2/3 and ALK1-SMAD1/5/8 (Miyazawa *et al*., 2002, Shi and Massagué 2003). The expression of *TGFB2*, *TGFB3*, and their receptors was increased in the regressed testis (Fig. R-11). Among the receptors, the expression of both *ALK1* and *ENG*, accessory receptor that facilitates ALK1 signaling (Lebrin *et al*., 2005), was increased in the regressed testis. The expression of *ALK5*, which activates SMAD2/3, was also increased in the regressed testis (Fig. R-11). However, ALK5 is important for recruiting of ALK1 into TGF-β receptor complex, and its kinase activity is required for optimal ALK1 activation (Goumans *et al*., 2003). Therefore, these results suggest that TGF-βs activate ALK1-SMAD1/5/8 rather than ALK5-SMAD2/3 pathway in the regressed testis (Fig. D-2). During the process of testicular changes induced by the long-day condition, the expression of *TGFB2*, *ALK1*, *ENG*, and *BETAGLYCAN* (accessory receptor that mediates the binding of TGF-βs to the type II receptor, particularly important for TGF-β2 signaling) was decreased at Stage III, when spermatocytes appeared in the seminiferous tubules (Fig. R-13 and R-14). These results suggest that TGF-β signaling through ALK1 pathway is involved in the inhibition of germ cell proliferation and differentiation. TGF-β signaling has been reported to affect cell proliferation and differentiation. For example, TGF-β2 is a negative regulator of the fetal and neonatal germ cell proliferation and apoptosis in the mouse, and it is considered to regulate the duration of germ cell quiescence (Moreno *et al*., 2010). Moreover, TGF-β type I receptor ALK1 is known to repress

the proliferation of endothelial cells in mammals and fish (Oh *et al*., 2000, Lamouille *et al*., 2002, Roman *et al*., 2002). The expression of *AMH* and *AMHR2* was also decreased at Stage III (Fig. R-9B). Therefore, it is suggested that TGF-βs are involved in the regulation of spermatogonial proliferation and differentiation together with AMH via SMAD1/5/8 (Fig. D-2).

Activins activate SMAD2/3, the different group from SMAD1/5/8 (Miyazawa *et al*., 2002, Shi and Massagué 2003). The expression of three inhibin subunits, *INHA*, *INHBA*, and *INHBB*, was increased in the regressed testis (Fig. R-12), suggesting that the production of both activins and inhibins are increased. In the regressed testis, the expression of *FST* (activin-binding protein) and *BETAGLYCAN* (inhibin co-receptor), which inhibit activin signaling, was also increased (Fig. R-12). Therefore, it is suggested that activin signaling is inhibited by follistatin and inhibins in the regressed testis. During the process of testicular changes induced by the long-day condition, the expression of *FST* and *BETAGLYCAN* was decreased at Stage III similar to *AMH* and *AMHR2* (Fig. R-14). These results suggest that the balance between the action of activins and inhibins regulates germ cell proliferation and differentiation, and activins function in a manner opposite to AMH. The effects of activins and inhibins on cell proliferation and differentiation have been reported in mammals. For example, inhibin reduced the number of differentiating spermatogonia in the adult testis of the Chinese hamster (van Dissel-Emiliani *et al.*, 1989). In contrast, activin stimulated spermatogonial proliferation and differentiation in germ-Sertoli cell coculture from immature rat testis (Mather *et al*., 1990). Moreover, during the adult rat spermatogenic cycle, activin A stimulated DNA synthesis of intermediate spermatogonia and preleptotene spermatocytes, whereas inhibin A inhibited that of these cells (Hakovirta *et al*., 1993). As described above, Amh is known to inhibit spermatogonial proliferation and differentiation in fish (Miura *et al*., 2002, Skarr *et al.*, 2011). Moreover, the synergistic effect of inhibin and AMH on mouse testicular tumor development was previously reported (Matzuk *et al*., 1995). Therefore, it

is suggested that AMH acts against activins together with inhibins and follistatin in the regressed testis to inhibit spermatogonial proliferation and differentiation (Fig. D-2). The antagonism between AMH and activins may occur at the SMAD level because SMAD1/5/8 and SMAD2/3 are known to be mutually antagonistic (Zeisberg *et al*., 2003, Matsumoto *et al*., 2012).

It is suggested that the signaling cross-talks between AMH and other TGF-β superfamily members occur in Sertoli cells and/or Leydig cells (Fig. D-2) because the expression of AMH type II receptor in those cells has been reported in the rat (Mendis-Handagama *et al*., 2006) and human (Salhi *et al*., 2004). In the rat, TGF-β type I and type II receptors (Lui *et al*., 2003) and activin type II receptor (Kaipia *et al*., 1992, MacConell *et al*., 2002) have been reported to be expressed in Sertoli cells, Leydig cells, and germ cells. However, the localization of these receptors differ among species even in mammals. For example, AMH type II receptor was expressed in Sertoli cells but not in Leydig cells in the mouse (Klattig *et al*., 2007). In the human testis, activin type I and type II receptors were expressed in Sertoli cells and germ cells but not in Leydig cells (Dias *et al*., 2008). Therefore, it is necessary to examine them in the quail testis in future. In addition, it is also needed to analyze the changes in phosphorylation state of SMADs and their localization to examine whether the signaling pathways suggested by the expression analysis are actually activated.

In summary, the expression of the ligands and receptors of TGF-β family was increased in the regressed testis. The expression of follistatin and betaglycan, which inhibit activin signaling, was also increased. In addition, their expression was decreased at Stage III, when spermatocytes were observed in the seminiferous tubules, during the testicular changes induced by the long-day condition. These results suggest that AMH is involved in the regulation of spermatogonial proliferation and differentiation together with other members of the TGF-β superfamily.

Conclusion

This is the first study that analyzed the expression profile of various sex differentiation-related genes associated with the testicular changes in the quail induced by photoperiod. I found that the expression of most of the transcription factors and secretory factors was increased in the regressed testis induced by the short-day condition. Particularly, *AMH* was expressed at a high level and significantly upregulated together with the transcription factors that are known to promote its expression in mammals (*SF1*, *SOX9*, *WT1*, and *GATA4*). Moreover, the putative binding sites for these transcription factors were present in the quail *AMH* promoter region. I also found that *AMHR2* showed an expression pattern similar to *AMH* depending on the photoperiod-induced testicular changes. These results suggest that the sex differentiation-related genes function via AMH in the adult quail testis. In addition, I analyzed the expression of other TGF-β superfamily members and their receptors to examine their association with the AMH signaling. The obtained results suggest that AMH is involved in the regulation of spermatogonial proliferation and differentiation together with other members of the TGF-β superfamily.

A series of the results in this study strongly suggests that the sex differentiation-related genes work in a network that leads to the *AMH* expression and participate in the regulation of spermatogenesis in the testis of the adult quail (Fig. D-3). Although further studies are necessary to elucidate their detailed functions, the findings of the present study should provide important clues to understand the physiological significance of sex differentiation-related genes in the adult gonad.

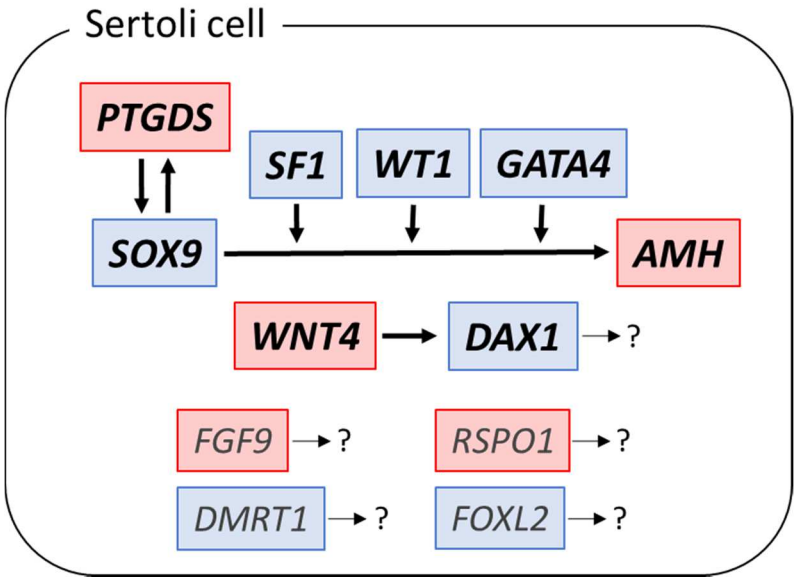


Figure D-1. A model of the network of sex differentiation-related genes in the adult quail testis predicted by the results of the expression analysis

The genes increased in the regressed testis induced by the short-day condition are indicated as bold characters. The genes encoding transcription factors are indicated as blue boxes, and those encoding secretory factors are indicated as red boxes. It is suggested that this network works in Sertoli cells because these sex differentiation-related genes have been reported to be expressed in Sertoli cells in the mouse (Wilhelm *et al*., 2007) and chicken (Oreal *et al*., 2002).

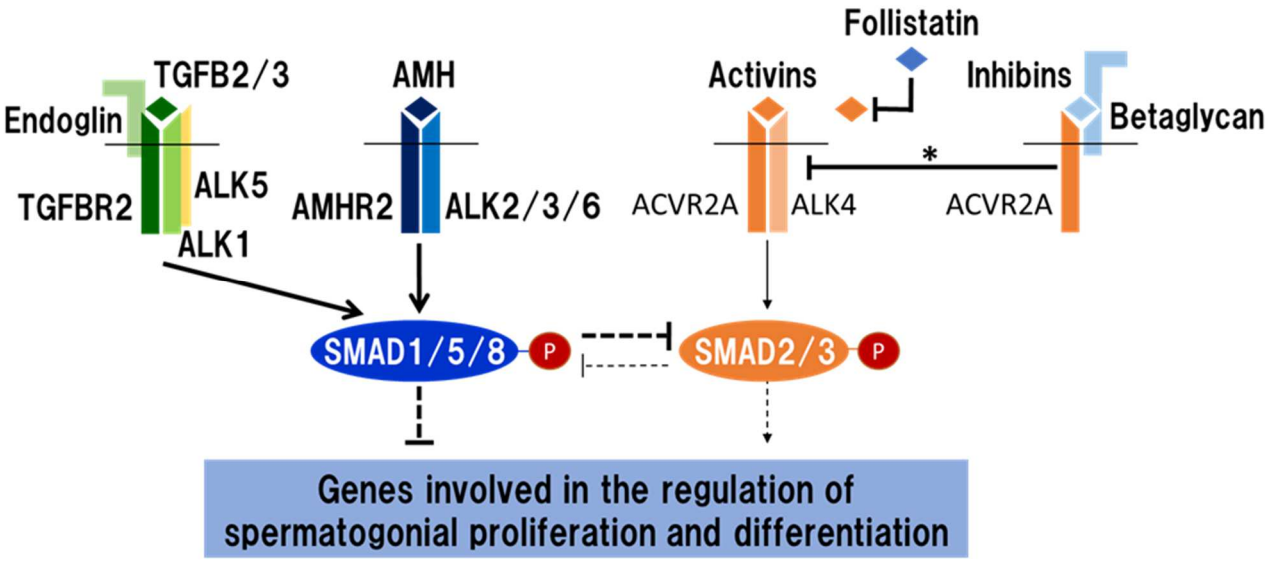


Figure D-2. A putative signaling system of AMH and other members of TGF-β superfamily in the regressed quail testis induced by the short-day condition

The expression of AMH, TGF-βs, and their receptors was increased in the regressed testis. In activin signaling system, the expression of three inhibin subunits was increased, suggesting that the production of both activins and inhibins are increased. The expression of follistatin and betaglycan, which repress activin signal, was also increased (*: Betaglycan promotes inhibin binding to activin type II receptor and thereby prevents activin from binding to the type II receptor). The factors increased in the regressed testis are indicated by bold characters, and the signaling pathways suggested by the expression analysis are indicated by bold lines. The predicted functions of SMADs based on the previous studies described below are indicated by dotted lines. Amh has been reported to inhibit spermatogonial proliferation and differentiation in fish (Miura *et al*., 2002, Skarr *et al*., 2011). TGF-β2 is a negative regulator of germ cell proliferation in the mouse and is considered to regulate the duration of germ cell quiescence (Moreno *et al*., 2010). Moreover, ALK1 signaling is known to repress cell proliferation in mammals and fish (Oh *et al*., 2000, Lamouille *et al*., 2002, Roman *et al*., 2002). The stimulatory effect of activin and inhibitory one of inhibin on spermatogonial proliferation and differentiation have been reported from mammals (van Dissel-Emiliani *et al*., 1989, Mather *et al*., 1990, Hakovirta *et al*., 1993). The two groups of SMADs (SMAD1/5/8 and SMAD2/3) are known to be mutually antagonistic (Zeisberg *et al*., 2003, Matsumoto *et al*., 2012).

It is suggested that the signaling cross-talks occur in Sertoli cells and/or Leydig cells because the expression of AMH type II receptor in those cells has been reported in the rat (Mendis-Handagama *et al*., 2006) and human (Salhi *et al*., 2004). In the rat, TGF-β type I and type II receptors (Lui *et al*., 2003) and activin type II receptor (Kaipia *et al*., 1992, MacConell *et al*., 2002) have been reported to be expressed in Sertoli cells, Leydig cells, and germ cells. However, the localization of these receptors differ among species even in mammals (Klattig *et al*., 2007, Dias *et al*., 2008). Therefore, it is necessary to examine them in the quail testis in future.

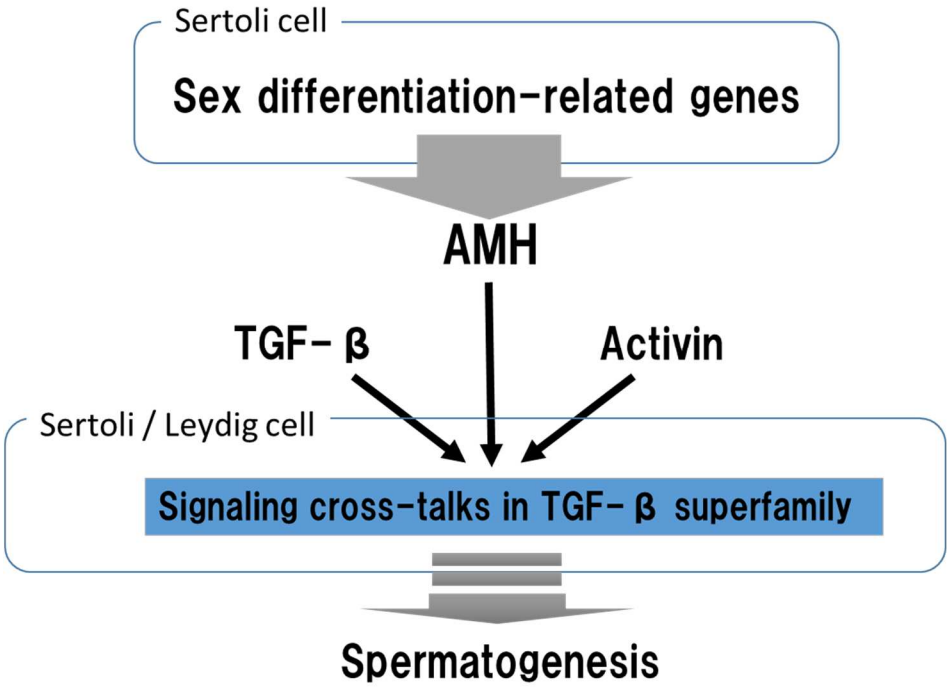


Figure D-3. A Schematic diagram of the expression of sex differentiation-related genes and its association with AMH signaling system in the adult quail testis

AMH was significantly upregulated in the regressed testis induced by the short-day condition. In addition, the expression of the transcription factors that are known to promote *AMH* expression (*SF1*, *SOX9*, *WT1*, and *GATA4*) was also increased. Moreover, the putative binding sites for these transcription factors were present in the quail *AMH* promoter region. These results suggest that the sex differentiation-related genes contribute to the expression of *AMH*.

Among the TGF-β superfamily members that are involved in spermatogenesis (Itman *et al*., 2006), the expression of the ligands and receptors of TGF-β family was increased in the regressed testis. The expression of follistatin and betaglycan, which inhibit activin signaling, was also increased. These results suggest that AMH is involved in the regulation of spermatogenesis together with other members of the TGF-β superfamily.

In conclusion, the results obtained in this study strongly suggest that the sex differentiation-related genes work in a network that leads to the *AMH* expression and participate in the regulation of spermatogenesis in the adult quail testis.

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