Expressional changes of AMH signaling system in the quail testis induced by photoperiod

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Abstract

Gonadal sex differentiation proceeds by the interplay of various genes including the transcription factors and secretory factors in a complex network. The sex-differentiating genes are expressed not only during early sex differentiation but also throughout the gonadal development and even in the adult gonads. In addition, the evidence that they actually function in the adult gonads have been accumulated from the studies using the conditional knockout mice. However, many previous studies were focused on one single gene though those genes function in a network. In this study, the expressions of various sex-differentiating genes were analyzed simultaneously in the adult testis of the Japanese quail (*Coturnix japonica*), whose testicular functions are dramatically changed by altering the photoperiod, to elucidate the roles of them in the adult gonad. Anti-Müllerian hormone (AMH) was significantly upregulated in the regressed testis induced by the short-day condition. The expressions of the transcription factors that promote AMH expression in mammals (*SF1*, *SOX9*, *WT1* and *GATA4*) were also increased in the regressed testis. Moreover, AMH receptor (*AMHR2*) showed similar expression pattern to its ligand. We also analyzed the expressions of other transforming growth factor beta (TGFB) superfamily members and their receptors. The expressions of the ligands and receptors of TGFB family, and follistatin and betaglycan in addition to inhibin subunits were increased in the regressed testis. These results suggest that AMH is involved in the adult testicular functions of the Japanese quail together with other TGFB superfamily members.

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Introduction

During early development, a bipotential gonad differentiates into a testis or ovary, and many genes are involved in this process. Sex-differentiating genes have been identified by the mutation analysis in mice and humans (reviews: [Wilhelm et al. 2007](#), [Eggers et al. 2014](#)). Most of them encode the transcription factors, such as Wilms tumor 1 (*WT1*), steroidogenic factor 1 (*SF1* also known as *NR5A1*), SRY-box 9 (*SOX9*), GATA-binding protein 4 (*GATA4*), DSS-AHC critical region on the X chromosome protein 1 (*DAX1* also known as *NR0B1*), doublesex- and mab-3 related transcription factor 1 (*DMRT1*) and forkhead box L2 (*FOXL2*). Moreover, the secretory factors are also involved in the gonadal sex differentiation, for example, fibroblast growth factor 9 (*FGF9*), prostaglandin D₂ synthase (*PTGDS*), wingless-type MMTV integration site family member 4 (*WNT4*) and R-spondin 1 (*RSPO1*).

Gonadal sex differentiation proceeds by the interplay of these factors in a complex network (reviews: [Wilhelm et al. 2007](#), [Cutting et al. 2013](#), [Eggers et al. 2014](#)). The gene networks are well understood in the mouse early gonadal sex differentiation. For example, *Sox9* expression in male is maintained by *Fgf9* and *Ptgds* by establishing

feed-forward loops ([Wilhelm et al. 2007](#), [Eggers et al. 2014](#)). *SOX9* subsequently 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](#)). During ovarian differentiation, *Rspo1* is required for *Wnt4* expression, and it functions via the activation of beta-catenin ([Eggers et al. 2014](#)).

These genes are also expressed during gonadal sex differentiation in other vertebrates, whereas the timing of their expressions and the roles vary among species (reviews: [Morrish & Sinclair 2002](#), [Cutting et al. 2013](#)). For example, *DMRT1* and its paralogs are essential for primary sex determination in the chicken, medaka and *Xenopus laevis*, whereas *Dmrt1* is not required for early testicular differentiation in the mouse ([Cutting et al. 2013](#)). Similarly, *FOXL2* ablation led to female-to-male sex reversal in the goat though it is not required for early ovary formation in the mouse ([Eggers et al. 2014](#)). *Amh* was expressed in the differentiated testis in the mouse, whereas it was expressed from early stages of sex differentiation in the chicken ([Morrish & Sinclair 2002](#)). The early expression of AMH in the chicken is required for the urogenital system growth ([Lambeth et al. 2015](#)).

The sex-differentiating genes are expressed not only during early sex differentiation but also throughout the postnatal gonadal development and even in the adult gonads. Their expressions have been examined mainly in the testis of mice, rats and humans. For example, *SOX9* was expressed in rat Sertoli cells throughout testicular development, and it showed spermatogenic cycle-dependent expression in the adult testis (Fröjdman *et al.* 2000). *GATA4* was expressed in Sertoli and Leydig cells from fetal to adult testis in the mouse (Ketola *et al.* 2002). *SF1* and *DAX1* were expressed throughout testicular development in rats and humans, and *DAX1* expression in the adult rat Sertoli cells showed spermatogenic cycle-specific pattern (Kojima *et al.* 2006). *DMRT1* was expressed during postnatal testicular development and in the adult testis in the mouse (Lei *et al.* 2007).

Moreover, the expressional changes of sex-differentiating genes in the adult testis have been reported in seasonal breeders. In the Iberian mole, the expression of *SOX9* was increased in the inactive testis at the nonbreeding season, whereas those of *WT1*, *SF1* and *DMRT1* were decreased (Dadhich *et al.* 2011). High expressions of *sox9* and *dmrt1* were observed when spermatogenesis was active in the catfish (Raghuveer & Senthilkumaran 2009, 2010) and lambari fish (Adolfi *et al.* 2015).

In addition, the studies using the conditional knockout mice provide direct evidence that sex-differentiating genes actually function in the adult gonads. For example, *Wt1* is critical for spermatogenesis as it regulates the polarity of Sertoli cells (Wang *et al.* 2013) and steroidogenesis as it regulates the expression of paracrine factors (Chen *et al.* 2014). *Dmrt1* (Matson *et al.* 2011) and *Foxl2* (Uhlenhaut *et al.* 2009) are essential for the sex maintenance of the adult testis and ovary respectively.

These studies described previously strongly suggest that sex-differentiating genes play a role in the adult gonadal functions. However, many previous studies were focused on one single gene though gonadal sex differentiation proceeds by the interplay of various factors. Namely, few studies have examined the network of the genes including the transcription factors and secretory factors. From this situation, we realized the necessity of analyzing the expression profiles of various sex-differentiating genes simultaneously in terms of the gene network to understand their significances.

The Japanese quail (*Coturnix japonica*) is a long-day breeder, and its testicular morphology and functions are dramatically changed by altering the photoperiod. For example, the testicular weight can change about a 100-fold within 30 days (Follett & Farner 1966). This dynamic change accompanies tissue regression and reconstruction. Histological changes of the testis were reported (Eroschenko & Wilson 1974), but few studies have been conducted at the molecular (gene expression) level. Study on the sex-differentiating genes

during the dynamic testicular changes will lead to the understanding of their roles in the adult gonad.

From these backgrounds, this study aimed to clarify the expressional changes of various sex-differentiating genes associated with the testicular changes of the quail as a first step to understand their significances in the adult gonad. As a result, we found that *AMH* was highly expressed and significantly upregulated in the regressed testis induced by the short-day condition. The expressions of the transcription factors that promote *AMH* expression in mammals (*SF1*, *SOX9*, *WT1* and *GATA4*) were also increased in the regressed testis. Moreover, *AMH* receptor (*AMHR2*) showed a similar expression pattern as its ligand depending on the testicular changes, suggesting their involvement in spermatogenesis. *AMH* belongs to the transforming growth factor beta (TGFB) superfamily, most of which are known to be involved in spermatogenesis (review: Itman *et al.* 2006). The ligands of this superfamily bind to two different serine/threonine kinase receptors referred to as type I and type II. On ligand binding, type I receptors specifically activate the intracellular signaling molecules (SMADs). There are complex signaling cross-talks in this superfamily because their receptors and SMADs are shared among different kinds of the ligands (reviews: Miyazawa *et al.* 2002, Shi & Massagué 2003). Therefore, it is important to examine the possibility of the interaction between *AMH* and other members of the superfamily for understanding the roles of *AMH*. However, few studies have analyzed their signaling system in the testis. Therefore, we also analyzed the expressions of other TGFB superfamily members and their receptors that are involved in spermatogenesis. The results strongly suggest that *AMH* is involved in the adult testicular functions together with other members of the superfamily.

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 darkness (lights on at 0600 h, off at 2200 h) 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 darkness (lights on at 1000 h, off at 1800 h) for 4 weeks, and then killed.

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 killed 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% (w/v) 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 to $5\mu\text{m}$ thickness. The paraffin sections were used for hematoxylin and eosin staining, *in situ* hybridization and immunohistochemistry.

Classification of testicular stages

Testicular morphology was 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 described in the following (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 germ cell markers, *DMC1* (spermatocyte marker) and *ZFAND3* (spermatid marker).

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 $3\mu\text{g}$ denatured total RNA using $5\mu\text{M}$ oligo (dT) primer and 200 U M-MLV Reverse Transcriptase (Promega) in a $20\mu\text{L}$ 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 $3\mu\text{g}$ denatured total RNA using $5\mu\text{M}$ oligo (dT) primer and 200 U of PrimeScript II Reverse transcriptase (TaKaRa), adding 1 M betaine (Sigma-Aldrich) for improving GC-rich amplification, in a $20\mu\text{L}$ 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 5'-terminus, they were incubated with 7 U of Terminal Deoxynucleotidyl Transferase (TaKaRa) and $200\mu\text{M}$ 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 $20\mu\text{L}$ reaction mixture containing an adaptor primer at $1\mu\text{M}$, 0.25 U of TaKaRa Ex Taq (TaKaRa), each dNTP at $250\mu\text{M}$, 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 ([Supplementary Table 1](#), see section on [supplementary data](#) given at the end of this article). The primers were designed to span at least one exon–intron boundary based on the homologies of cDNA sequences from the chicken and other vertebrates. PCR amplifications were performed in a $20\mu\text{L}$ reaction mixture containing each primer at $1\mu\text{M}$, 0.25 U of TaKaRa Ex Taq, each dNTP at $250\mu\text{M}$ and Ex Taq Buffer. The PCR conditions were 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% (w/v) agarose gel and stained with ethidium bromide. The specificity of the PCR was confirmed by sequence analysis.

Real-time PCR

Five microliters of each 120-fold-diluted cDNA from testis samples were amplified in a $20\mu\text{L}$ reaction mixture containing each primer at $0.3\mu\text{M}$ and LightCycler 480 SYBR Green I Master (Roche Diagnostic) using LightCycler 480 Instrument II (Roche Diagnostic). The primers were designed to span at least one exon–intron boundary based on the sequencing results of the conventional RT-PCR products and sequences of the Japanese quail on the NCBI database ([Supplementary Table 1](#)). For negative control, PCR was also conducted using RNA sample without the RT reaction. The PCR condition was as follows: 95°C for 5 min, 45 cycles of incubation at 95°C for 10 s, 60°C for 10 s and 72°C 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 expressions of three housekeeping genes – *GAPDH*, beta-actin (*ACTB*) and peptidylprolyl isomerase A (*PPIA*) – were 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

The DNA sequences from stop codon of *SF3A2* to start codon of *AMH* were obtained from the quail and chicken genome data using NCBI database (GenBank accession number: quail, NC_029543.1; chicken, NC_006115.4). The putative transcription factor binding sites were predicted by MatInspector software in Genomatrix Software Suite v3.6 (Genomatrix 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 as described previously (Otake *et al.* 2011) with some modifications as follows. The paraffin sections were treated with Proteinase K (1 µg/mL, 30 min, 37°C) and hybridized with 1 µg/mL sense and antisense RNA probes. For the probe synthesis, the ORF sequence of *DMC1* was identified from the quail by RT-PCR using *DMC1*-SE01 and

AS01. For the templates of RNA probes, the testicular cDNA was amplified using each primer set (Supplementary Table 1).

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 the 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 were characterized by the previous study (Tsunekawa *et al.* 2000). For antigen retrieval, the sections 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% (v/v) H₂O₂ for 30 min at room temperature, and then blocked with 2% (v/v) 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% (v/v) Tween 20 in PBS (PBST), the sections were incubated with biotinylated goat anti-rabbit IgG antibody (1:200; Vector, Burlingame, CA, USA) 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.

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

Figure 1 Expression analysis of the sex-differentiating genes and steroidogenic genes in the adult testis of the Japanese quail reared under the long-day (LD) or short-day (SD) conditions. Expressions of (A) the transcription factors and (B) the secretory factors involved in sex differentiation were analyzed by RT-PCR. The numbers of each lane indicate individual number, and those in parenthesis show the number of the PCR cycle. (C) Expressions of the secretory factors were quantified by real-time PCR. The relative expressions of the genes were normalized to the geometric mean of *GAPDH* and *PPIA*. Results are shown as mean $\pm$ S.E.M. ($n=6$ /group), and the expression level of the LD group is expressed as 1. ** $P<0.01$ (Mann–Whitney 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.

PCR from the testicular cDNA using *AMHR2*-dSE01 and dAS01 (Supplementary 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 (Supplementary Table 1) respectively. Sense and antisense primers for confirming the sequence were designed based on the results of 3' and 5' RACE. For confirmation, RT-PCR using *AMHR2*-SE03 and AS03, 3' RACE using *AMHR2*-SE02 and 5' RACE using *AMHR2*-AS02 were performed (Supplementary Table 1). All the PCR amplifications were performed in a 20- μ L reaction mixture containing each primer at 1 μ M, 0.25 U of TaKaRa Ex Taq, each dNTP at 250 μ M, 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 a 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 (BioDynamics, Tokyo, Japan) and sequenced. The sequencing of the partial ORF was conducted independently three times to avoid potential PCR amplification errors.

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 (Supplementary Table 1). The primers were designed to span at least one exon–intron boundary. PCR amplifications were performed as described previously. For negative control, PCR was also conducted using RNA sample without the RT reaction. The PCR conditions were 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

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

and stained with ethidium bromide. The specificity of the PCR was confirmed by sequence analysis. The typical data from three independent analyses were shown as a result.

Statistical analysis

All data are expressed as mean $\pm$ S.E.M. The expression levels of the genes between the active and regressed testis were analyzed by Mann–Whitney *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, Japan).

Results

Expression analysis of the sex-differentiating genes and steroidogenic genes in the testis of the long-day and short-day conditions

We analyzed the expressions of various sex-differentiating genes (the transcription factors and secretory factors) in the adult testis of the Japanese quail at the long-day and short-day conditions. Among the transcription factors, the expressions of *SF1*, *WT1*, *SOX9*, *GATA4* and *DAX1* were increased in the regressed testis induced by the short-day condition, whereas that of *FOXL2* was decreased and *DMRT1* expression was not changed (Fig. 1A). Among the secretory factors, the expressions of *AMH*, *PTGDS* and *WNT4* were increased in the regressed testis, whereas those of *FGF9* and *RSPO1* were not changed (Fig. 1B). The expressional change of *AMH* was the largest among the secretory factors when the expressions were quantified by real-time PCR analysis (Fig. 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 those of *HSD3B* and *CYP17A1* (*P450C17*) were not changed (Fig. 1D). The expressions of *LHCGR*, *STAR* and *CYP11A1* (*P450SCC*) in the regressed testis showed individual differences (Fig. 1E analyzed by increasing the number of samples).

Prediction of the putative transcription factor-binding sites in the AMH promoter

To examine whether the binding sites of the transcription factors involved in sex differentiation are present in the quail *AMH* promoter, we analyzed the promoter sequence using MatInspector software. In the quail *AMH* promoter, there were several putative binding sites for them, such as one SF1 site, three SOX9 sites, two WT1 sites and one GATA4 site (Fig. 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. We also analyzed the promoter sequence of the chicken for the comparison with that of the quail. The chicken *AMH* promoter sequence was about 500 bp longer than that of the quail and 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. 2, lower panel).

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)

Figure 3 *In situ* hybridization analysis of *AMH* and germ cell markers in the quail testis of the long-day and short-day conditions. (A, E) The results of *in situ* hybridization for *AMH* and immunohistochemistry for DDX4 (also known as VASA). Blue signal: *AMH*, Brown signal: DDX4. (B, F) Hematoxylin and eosin staining. (C, G) The results of *in situ* hybridization for *DMC1*. (D, H) The results of *in situ* hybridization for *ZFAND3*. The germ cell types in the testis of the LD condition were spermatogonia, spermatocytes, spermatids and spermatozoa (Stage V), whereas those in the testis of the SD condition were only spermatogonia (Stage II). All scale bars indicate 50 μ m. Dotted lines indicate the outline of seminiferous tubules.

Figure 4 Expression analysis of *AMHR2* in the quail. (A) Expression analysis of *AMH* and *AMHR2* in various tissues of the adult Japanese quail. RT – represents the negative control using testis total RNA without reverse transcription. The numbers in parenthesis show the number of the PCR cycles. (B) Expression analysis of *AMHR2* in the quail testis of the long-day and short-day conditions 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 $\pm$ S.E.M. ($n=6$ /group), and the expression level of the LD group is expressed as 1. ** $P<0.01$ (Mann–Whitney *U* test).

was performed in the same section to identify the germ cells in the seminiferous tubules. The expressions of the spermatocyte marker *DMC1* (Yoshida *et al.* 1998) and spermatid marker *ZFAND3* (also known as *TEX27*, Otake *et al.* 2011) were also analyzed to examine the germ cell types in the regressed testis. The ORF sequence of *DMC1* was identified from the quail for probe synthesis (GenBank accession number: KU975604). *AMH* mRNA was strongly expressed in Sertoli cells (*DDX4* negative) in the regressed testis, whereas the signal was weakly detected in the active testis. *DMC1* and *ZFAND3* were expressed in the active testis, whereas they were not in the regressed testis (Fig. 3). No signal was detected when each sense probe was used as a negative control.

Identification and expression of *AMHR2*

To examine the expression of AMH receptor (*AMHR2*), its partial cDNA sequence was identified from the Japanese quail by RT-PCR and RACE (GenBank accession number: KU715092). The deduced amino acid sequence contained

a transmembrane domain and protein kinase domain (predicted using TMHMM Server and Pfam database respectively) (Supplementary Fig. 1). The amino acid sequence of the quail *AMHR2* was compared with those of other amniotes (Supplementary Fig. 2). 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 (version 2.7). The protein kinase domain of the quail *AMHR2* showed 84–94% identity (89–97% similarity) with that of other birds, 56–67% identity (70–74% similarity) with that of reptiles and 54–55% identity (70–71% similarity) with that of mammals. In addition, 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 was clustered with avian *AMHR2* sequences (data not shown). We performed tissue distribution analysis of *AMHR2* together with *AMH* to examine where they are expressed in the adult quail. *AMH* and

Figure 5 Real-time PCR analysis of *AMH*, *AMHR2* and germ cell markers during the process of testicular changes induced by photoperiod in the quail. The relative expressions of the genes were normalized to the geometric mean of *GAPDH* and *PPIA*. Results are shown as mean $\pm$ S.E.M. and the expression level of the lowest group is expressed as 1. (A) Expressional changes by the days after the long-day treatment ($n=5$ /group). (B) Expressional changes by the testicular stages. Stage II: spermatogonia ($n=14$), Stage III: spermatogonia and spermatocytes ($n=11$), Stage V: spermatogonia, spermatocytes, spermatids and spermatozoa ($n=5$). * $P<0.05$, ** $P<0.01$, *** $P<0.001$ (Steel–Dwass test).

AMHR2 were expressed primarily in the testis and ovary. Their expressions were detected in the brain and slightly in the thyroid gland, adrenal gland, kidney and heart at 35 cycles (Fig. 4A). The expression of *AMHR2* was also examined in the testis of the long-day and short-day conditions. *AMHR2* expression was increased in the regressed testis (Fig. 4B).

Expression analysis of *AMH* and *AMHR2* during the process of testicular changes induced by photoperiod

The expressions of *AMH*, *AMHR2* and germ cell markers were analyzed during the process of testicular

changes induced by photoperiod. The expressions of *AMH* and *AMHR2* were decreased gradually after transferring to the long-day condition. The expressions of *DMC1* and *ZFAND3* were increased from 7 days to 15 days after the long-day treatment respectively (Fig. 5A). The data were analyzed by the testicular stages to examine their association with spermatogenesis. The expressions of *AMH* and *AMHR2* were 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. 5B).

Figure 6 Expression analysis of other TGF β superfamily members and their receptors in the quail testis of the long-day and short-day conditions by RT-PCR and real-time PCR. (A) The ligands and receptors of BMP family. (B) The ligands and receptors of TGF β family. (C) The ligands and receptors of activin family and their related genes. The numbers of each lane indicate individual number and those in parenthesis show the number of the PCR cycles. Some selected genes were also analyzed by real-time PCR. The relative expressions of the genes were normalized to the geometric mean of *GAPDH* and *PPIA*. Results are shown as mean $\pm$ S.E.M. ($n=6$ /group), and the expression level of the LD group is expressed as 1. ** $P<0.01$ (Mann–Whitney U test).

Figure 7 Real-time PCR analysis of other TGFB superfamily members and their receptors during the process of testicular changes induced by photoperiod in the quail. (A) The ligands and receptors of TGFB family. (B) Activin family and its related genes. The relative expressions of the genes were normalized to the geometric mean of *GAPDH* and *PPIA*. Results are shown as mean $\pm$ S.E.M. 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).

Expression analysis of other TGFB superfamily members and their receptors in the testis of the long-day and short-day conditions

The expressions of other TGFB superfamily members and their receptors were analyzed. Among the receptors of bone morphogenetic protein (BMP) family, the expressions of the type II receptor *BMFR2* and three type I receptors, activin receptor-like kinase 2 (*ALK2* also known as *ACVR1*), *ALK3* (*BMFR1A*) and *ALK6* (*BMFR1B*) were increased in the regressed testis. However, the expressions of BMPs were low and not changed between the active and regressed testis (Fig. 6A). Among the ligands of TGFB family, the expressions of *TGFB2* and *TGFB3* were increased in the regressed testis whereas that of *TGFB1* was hardly detected. Among their receptors, the expressions of the type II receptor *TGFB2R*, two type I receptors, *ALK1* (*ACVRL1*) and *ALK5* (*TGFB1R*), and endoglin (*ENG*: accessory receptor essential for *ALK1* signaling) were increased in the regressed testis (Fig. 6B). In activin family, the expressions of inhibin alpha (*INHA*), inhibin beta A (*INHBA*) and inhibin beta B (*INHBB*) were increased in the regressed testis. The expression of follistatin (*FST*: activin-binding protein) was also increased in the regressed testis. In the two type II receptors, *ACVR2A* was mainly expressed but not changed between the active and regressed testis. In the two type I receptors, *ALK7* (*ACVR1C*) expression was decreased in the regressed testis, whereas that of *ALK4* (*ACVR1B*) was not changed. *BETAGLYCAN* (inhibin co-receptor also known as *TGFB3R*) expression was increased in the regressed testis (Fig. 6C).

Expression analysis of other TGFB superfamily members and their receptors during the process of testicular changes induced by photoperiod

We then analyzed the expressions of other TGFB superfamily members and their receptors during the process of testicular changes induced by photoperiod to examine their association with spermatogenesis. Among the ligands and receptors of TGFB family, the expressions of *TGFB2*, *ALK1* and *ENG* were decreased at Stage III and those of *TGFB2* and *ALK1* were further decreased at Stage V. *TGFB3* expression was decreased at Stage V and that of *ALK5* was not different among the stages (Fig. 7A). Among activin family and its related genes, the expressions of *INHBA*, *INHBB*, *FST* and *BETAGLYCAN* were decreased at Stage III and those of *INHBA*, *INHBB*, and *FST* were further decreased at Stage V. *INHA* expression was decreased at Stage V (Fig. 7B).

Discussion

The genes involved in early sex differentiation have been reported to be expressed throughout gonadal development, and even in the adult gonads. In addition, gonadal dysfunctions have been observed in adult conditional knockout mice of those genes, suggesting their roles in the adult gonads. However, their physiological significances still remain largely unknown because many previous studies have focused on one single gene, especially one transcription factor, though sex-differentiating genes work in a network. In this study, we for the first time analyzed the expressions of various

sex-differentiating genes including the transcription factors and secretory factors simultaneously in the adult testis of the Japanese quail whose testicular functions are dramatically changed by altering photoperiod (Follett & Farner 1966). 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 with that of the long-day control (data not shown), and this result is consistent with the previous report (Follett & Farner 1966).

The expressions of most of the transcription factors (*SF1*, *WT1*, *SOX9*, *GATA4* and *DAX1*) were increased in the regressed testis (Fig. 1A). Among the secretory factors, the expressions of *AMH*, *PTGDS* and *WNT4* were increased in the regressed testis. In particular, *AMH* was expressed at high level and significantly upregulated in the regressed testis (Figs 1B, C, and 3). These results are consistent with the findings about the regulation of *Amh* expression during early sex differentiation period. *SF1*, *WT1*, *GATA4* and *SOX9* are known to upregulate *Amh* expression synergistically by binding to its promoter in mammalian fetal testis (Lasala et al. 2004). In addition, *WT1* and *GATA4* can upregulate *Amh* expression by physical interactions with *SF1*.

To examine whether the binding sites of these transcription factors are present in the quail *AMH* promoter, we analyzed the promoter sequence using MatInspector software. In the quail *AMH* promoter, there were several putative binding sites for the transcription factors involved in sex differentiation, such as one *SF1* site, three *SOX9* sites, two *WT1* sites and one *GATA4* site (Fig. 2, upper panel). We also analyzed the promoter sequence of the chicken for the comparison with that of the quail. The chicken *AMH* promoter sequence was about 500bp longer than that of the quail, and contained one *SF1* site, four *SOX9* sites, two *WT1* sites and one *GATA4* site (Fig. 2, lower panel). The presence of the binding sites for *SF1*, *SOX9*, *WT1* and *GATA4* strongly suggests that these transcription factors contribute to the increased expression of *AMH* in the regressed quail testis. Among these 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 is suggested that *SOX9* contributes to subsequent upregulation of *AMH* because it coincides with the onset of *SOX9* expression (Morrish & Sinclair 2002). In future study, it is necessary to examine whether *SOX9*, *WT1* and *GATA4* bind to their putative binding sites and activate *AMH* expression.

On the other hand, androgens are thought to be responsible for the downregulation of *AMH* expression because of their negative correlation in serum level (Rey et al. 1993). 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 (Fig. 2, upper panel). In the chicken *AMH* promoter, there were one PR site and one common site for AR, GR and PR (Fig. 2, lower panel). The presence of AR-binding site suggests the direct inhibitory effect of androgens on *AMH* expression.

Interestingly, the quail and chicken *AMH* promoters contained a binding site for CLOCK/BMAL1 heterodimer that is involved in the regulation of circadian rhythm (Bell-Pedersen et al. 2005) (Fig. 2). In the chicken, there were additional three CLOCK/BMAL1 sites that formed tandem repeats. In some teleost species like medaka, *amh* gene lays 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, we performed synteny analysis using NCBI database. There was a conserved synteny of *LINGO3*, *OAZ1*, *AMH* and *DOT1L* on chromosome 28 (Supplementary Fig. 3). However, *CLOCK* and *KIT* were on the different chromosome (Chr 4) like in the mouse and zebrafish. *DDX59*, *KIF14*, *NR5A2* and *LHX9* were also on the different chromosome (Chr 8) like in the mouse. Daily rhythms of the expressions of the genes involved in reproduction including *amh* were reported from the zebrafish (Di Rosa et al. 2016) though *amh* and *clock* are not on the same chromosome. Therefore, in future study, it will be interesting to examine whether the expression of *AMH* is regulated by CLOCK in relation to photoperiodism in the quail and chicken.

Another possible interaction is expected between *PTGDS* and *SOX9*. *Sox9* is upregulated by prostaglandin D₂ (converted from prostaglandin H₂ by *PTGDS*) in the mouse (Wilhelm et al. 2007) and the chicken (Moniot et al. 2008) during 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 D₂ signaling.

DAX1 expression was also increased in the regressed testis (Fig. 1A), whereas it is known to repress *Amh* expression in mammals (Lasala et al. 2004). *DAX1* may be upregulated by *WNT4* that was also increased in the regressed quail testis (Fig. 1B and C) as reported in mammals (Jordan et al. 2001). *DAX1* is also known to repress the expressions of the genes involved in steroidogenesis in mammals (Lalli et al. 1998), but the expressions of most of the steroidogenic genes were not changed or showed individual differences in the regressed quail testis (Fig. 1D and E). These results suggest that the roles of *DAX1* in birds may be different from those reported in mammals. The expression patterns of *DAX1* during sex differentiation support this idea. *Dax1* expression declined during mouse testicular differentiation (Morrish & Sinclair 2002); however, it was maintained in the chicken, suggesting

that it would not inhibit the male pathway in birds (Smith *et al.* 1999).

Among the genes involved in steroidogenesis, the decreased expression of *CYP19A1* in the regressed testis is consistent with the inverse relation with *AMH* reported in the chicken during sex differentiation (Nishikimi *et al.* 2000). In contrast, the expressions of the other genes in the regressed testis were inconsistent with the inhibitory effects of *AMH* on the steroidogenic gene expressions, such as *Cyp17a1*, *Cyp11a1* and *Hsd3B* reported in the mouse (Racine *et al.* 1998). The effects of *AMH* on the expressions of steroidogenic genes may differ among species. Further studies are needed to examine the expressional regulation of steroidogenic enzymes in the quail.

There are a few reports about the expressions of sex-differentiating genes in the adult testis of seasonal breeders. In the Iberian mole, *SOX9* expression was increased in the inactive testis, and this is consistent with our result (Fig. 1A). However, the expressions of *WT1*, *SF1* and *DMRT1* were decreased, and *AMH* was not expressed (Dadhich *et al.* 2011). One of the reasons for these expressional differences would be the degree of testicular regression. In the mole testis, primary spermatocytes were continuously present though they were absent (not expressed *DMC1*) in the regressed testis of the quail under the short-day condition (Fig. 3). In contrast to the quail and mole, *sox9* was abundantly expressed during active spermatogenesis in the catfish (Raghuveer & Senthilkumaran 2010) and lambari fish (Adolfi *et al.* 2015). More studies using other seasonal breeders are necessary to reveal the similarities and differences of the expression patterns of sex-differentiating genes in the adult gonads.

In summary for the expressions of sex-differentiating genes, *AMH* was significantly upregulated in the regressed testis induced by the short-day condition. The expressions of the transcription factors that promote *AMH* expression in mammals (*SF1*, *SOX9*, *WT1* and *GATA4*) were also increased in the regressed testis. Moreover, there were putative binding sites for these transcription factors in the quail *AMH* promoter. These results suggest that *AMH* contributes to the adult testicular functions in the quail. Therefore, we then analyzed the expression of *AMH* receptor (*AMHR2*).

To examine the expression, we identified the partial *AMHR2* cDNA sequence from the Japanese quail because its nucleotide sequence in avian species was not available on genome database. The deduced amino acid sequence contained a transmembrane domain and protein kinase domain (Supplementary Fig. 1). The protein kinase domain was well conserved among amniotes (Supplementary Fig. 2). However, we could not identify the full ORF sequence probably because of the high GC content (about 70%), which might have affected the reverse transcription and PCR reaction. Recently, the partial cDNA sequence of the chicken

AMHR2 was identified, 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 (Fig. 4B). The transcription factors increased in the regressed testis (*SF1*, *WT1* and *GATA4*) may also contribute to the increased expression of *AMHR2* because the binding sites for these factors are present in mammalian *Amhr2* promoter (Klattig *et al.* 2007). In future studies, it is necessary to identify the promoter sequence of avian *AMHR2* to investigate its expressional regulation.

AMH and *AMHR2* were expressed primarily in the adult testis and ovary. Their expressions were detected in the brain and slightly in the thyroid gland, adrenal gland, kidney and heart (Fig. 4A). 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 expressions of *Amh* and *Amhr2* in the brain were reported in the mouse (Lebeurrier *et al.* 2008) and Nile tilapia (Pfennig *et al.* 2015). The expressions 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* were conducted during the process of testicular changes induced by photoperiod and analyzed by the testicular stages to examine their association with spermatogenesis. The expressions of *AMH* and *AMHR2* were decreased at Stage III when spermatocytes appeared in the seminiferous tubules (Fig. 5B). 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) and mouse (Al-Attar *et al.* 1997). In the quail, *AMHR2* expression was also decreased at Stage III. Similar results were reported in the black porgy. The expressions of *amh* and *amhr2* were increased during pre-meiotic period and declined in the mature testis during the spawning season (Wu *et al.* 2010). Increased expressions of *AMH* and *AMHR2* in the regressed quail testis and their decreased expressions at Stage III suggest that *AMH* is involved in the regulation of meiosis and/or spermatogonial proliferation and differentiation. The expression pattern in the adult rat testis supports the role of *AMH* in the germ cell proliferation. *Amh* and *Amhr2* mRNA were expressed at a maximal level in the seminiferous tubule segment where mitotic divisions of spermatogonia are minimum (Baarends *et al.* 1995). In addition, *Amh* exerted the inhibitory effects on spermatogonial proliferation and differentiation in the zebrafish testis tissue culture (Skarr *et al.* 2011).

AMH belongs to the TGFB superfamily, and most of the members are known to be involved in spermatogenesis (review: Itman *et al.* 2006). There are complex signaling cross-talks in this superfamily because their receptors and intracellular signaling molecules (SMADs) are shared among various kinds of the ligands (reviews: Miyazawa *et al.* 2002, Shi & Massagué 2003). Thus, we also

analyzed the expressions of other TGFB superfamily members that are involved in spermatogenesis and their receptors. Three BMP type I receptors – ALK2, ALK3 and ALK6 – and their downstream factors SMAD1/5/8 are shared with AMH (Miyazawa *et al.* 2002, Shi & Massagué 2003). The expressions of BMP type II and type I receptors, *BMPR2*, *ALK2*, *ALK3* and *ALK6* were increased in the regressed testis (Fig. 6A). Increased expressions of the three type I receptors further support the activation of AMH signaling in the regressed testis. However, the expressions of BMPs were low and not changed between the active and regressed testis (Fig. 6A). Therefore, it is suggested that BMPs are involved in the maintenance of testicular functions in both the active and regressed testis.

TGFBs activate ALK5-SMAD2/3 pathway in almost all TGFB responsive cells. In addition, they also activate ALK1 and SMAD1/5/8 (SMADs that are activated by AMH) in the endothelial cells (Miyazawa *et al.* 2002, Shi & Massagué 2003). The expressions of *TGFB2*, *TGFB3* and their type II and type I receptors were increased in the regressed testis (Fig. 6B). The expressions of both *ALK1* and *ENG*, an accessory receptor essential for ALK1 signaling (Lebrin *et al.* 2005), were increased in the regressed testis (Fig. 6B). *ALK5* expression was also increased in the regressed testis. However, it is important to recruit ALK1 into TGFB receptor complex, and ALK5 kinase activity is required for optimal ALK1 activation (Goumans *et al.* 2003). Therefore, these results suggest that TGFBs activate ALK1–SMAD1/5/8 rather than ALK5–SMAD2/3 pathway in the regressed testis and may function cooperatively with AMH via SMAD1/5/8. In the testicular stages, the expressions of *TGFB2*, *ALK1* and *ENG* were decreased at Stage III. *ALK5* expression was not different among the stages (Fig. 7A) though it was increased in the regressed testis. This discrepancy of *ALK5* expression might have reflected the difference of photoperiod manipulation. Increased expressions of *ALK1* and *ENG* in the regressed testis and decrease of them at Stage III suggest that ALK1 pathway is involved in the inhibition of cell proliferation. Some previous studies support this idea. For example, constitutively active form of ALK1 expression in cultured human endothelial cells inhibited the cell proliferation (Lamouille *et al.* 2002). Moreover, *Alk1* deficiency resulted in the increased proliferation of endothelial cells in the mouse (Oh *et al.* 2000) and zebrafish (Roman *et al.* 2002). However, the stimulatory effects of ALK1 on cell proliferation were also reported from other *in vitro* studies (review: Lebrin *et al.* 2005). These discrepancies of the roles of ALK1 are not clearly understood though it is considered that they may be caused by different cell lines used and culture conditions (Lebrin *et al.* 2005). Further studies are necessary to reveal the functional differences of the two TGFB signaling pathways.

Activins activate SMAD2/3, the different group from SMAD1/5/8 (Miyazawa *et al.* 2002, Shi & Massagué

2003). Therefore, no cooperative functions with AMH are expected. However, the mutually antagonistic functions of the two groups of SMADs were reported in endothelial cells (Lebrin *et al.* 2005) and osteoblast (Matsumoto *et al.* 2012). The expressions of inhibin subunits, *INH A*, *INH B A* and *INH B B*, were increased in the regressed testis (Fig. 6C). These results are consistent with the results reported in other seasonal breeding mammals, such as the Siberian hamster (Rao *et al.* 1995) and bank vole (Tähkä *et al.* 1998). Increased expressions of the alpha and beta subunits suggest that the production of both inhibins (composed of alpha and beta subunits) and activins (composed of two beta subunits) are increased in the regressed testis. In the regressed testis, the expressions of *FST* (activin-binding protein) and *BETAGLYCAN* (inhibin co-receptor) were also increased (Fig. 6C). Betaglycan acts as an inhibin co-receptor with activin type II receptor and facilitates inhibin antagonism on activin signaling (Shi & Massagué 2003). These results suggest that activin signaling is inhibited by follistatin and inhibins in the regressed quail testis. Increased expression of *BETAGLYCAN* in the regressed testis may also contribute to the activation of TGFB signaling because it mediates the binding of TGFBs to the type II receptor, particularly important for *TGFB2* (Shi & Massagué 2003). In contrast, it is suggested that activin signaling is activated in the active testis because the expression of *ALK7*, a type I receptor involved in the signal transduction of activin AB and activin B (Tsuchida *et al.* 2004), was increased, and those of *FST* and *BETAGLYCAN* were decreased (Fig. 6C). In the testicular stages, the expressions of *INH B A*, *INH B B*, *FST* and *BETAGLYCAN* were decreased at Stage III (Fig. 7B). These results suggest that the balance between activins and inhibins regulates the germ cell proliferation and differentiation. Previous studies in mammals support this idea. Inhibin reduced the number of differentiating spermatogonia in the adult testis of the Chinese hamster (van Dissel-Emiliani *et al.* 1989). Moreover, in 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). The synergistic effect of inhibin and AMH on tumor development was reported in the double-deficient mouse (Matzuk *et al.* 1995). Since inhibins inhibit activin signaling, it is suggested that AMH acts against activins.

In conclusion, we for the first time analyzed the expressions of various sex-differentiating genes in the adult testis of the Japanese quail. *AMH* was highly expressed and significantly upregulated in the regressed testis induced by the short-day condition. The expressions of the transcription factors that promote *AMH* expression in mammals (*SF1*, *SOX9*, *WT1* and *GATA4*) were also increased in the regressed testis. Moreover, *AMHR2* showed the similar expression pattern to its ligand. We also analyzed the expressions

of other TGFB superfamily members and their receptors. The expressions of the ligands and receptors of TGFB family and *FST* and *BETAGLYCAN* in addition to inhibin subunits were increased in the regressed testis. These results suggest that AMH contributes to the alteration of the testicular functions such as spermatogonial proliferation and differentiation in the Japanese quail induced by photoperiod, together with other members of TGFB superfamily. In this study, we analyzed the expression of various genes and showed the results as relative expression level when normalized to reference genes. As the testis tissue was changed dramatically, we cannot exclude the possibility that the changes of cell type composition affected gene expression level. In future studies, it is necessary to examine the localization of the genes differentially expressed between the long-day and short-day conditions in the testis to elucidate their expressional changes at cellular level and the relationships between AMH and the other genes.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-16-0175>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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