Identification of Novel Regulatory Genes in Development of the Avian Reproductive Tracts

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Abstract

The chicken reproductive system is unique in maintaining its functions including production of eggs or sperm, fertilization of the egg by sperm maintained in sperm nests, production of hormones regulating its growth, development and function, and reproduction. Development of the reproductive organs is a highly regulated process that results in differentiation and proliferation of germ cells in response to predominant regulatory factors such as hormones and transcription factors. However, only a few genes are known to determine morphogenesis of the chicken reproductive tract and their mechanisms are unknown. Therefore, in the present study, we investigated the expression patterns of four genes including SNCA, TOM1L1, TTR and ZEB1 in the gonads at embryonic days 14 and 18, and in immature (12-week-old) and mature (50-week-old) chickens, as well as the reproductive tract including ovary, oviduct and testes of the respective sexes by qRT-PCR, in situ hybridization and immunofluorescence analyses. The expression of SNCA, TOM1L1 and ZEB1 genes was higher in immature and mature female reproductive tracts than expression of TTR. In addition, different temporal and spatial patterns of expression of the four genes were observed during maturation of testis in chickens. Specifically, SNCA, TOM1L1 and TTR were highly expressed in testes of 12-week-old chickens. Moreover, several chicken specific microRNAs (miRs) were demonstrated to affect expression of target gene mRNAs by directly binding to the 3’-UTR of their target genes through actions at the post-transcriptional level as follows: miR-153 and miR-1643 for SNCA; miR-1680 for TTR; and miR-200b and miR-1786 for ZEB1. These results suggest that four-selected genes play an important role in development of the male and female reproductive tract in chickens and expression of most candidate genes is regulated at the post-transcriptional level through specific microRNAs.

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Introduction

The chicken is an invaluable animal model for research on embryology and reproductive developmental biology. In avian models, sexual differentiation of the reproductive system is initiated in the embryonic gonads from embryonic day 6.5 (E6.5, HH30) to be either ovaries or testes and this occurs asymmetrically in females and symmetrically in males. It depends on which component of the embryonic gonad, cortex or medullary, is colonized by germ cells that migrate there through bloodstream [1,2,3]. In the female (ZW), germ cells asymmetrically populate the left and right gonads. The thickened outer cortex only develops in the left gonad while medulla cords form lacunae. However, the right gonad regresses and fails to develop the cortex. Female germ cells enter meiosis that commences between E14 and E18 in the left gonad [4,5,6]. Then, by beginning folliculogenesis, granulosa and theca cells surrounding the oocyte are formed from cells in the cortex of the left functional gonad. There are several candidate genes, forkhead box L2 (FOXL2), wingless-related MMTV integration site 4 (WNT4), proprotein convertase subtilisin/kexin type 6 (PCSK6) and bone morphogenetic protein 6 (BMP6) known to regulate follicular development [3,7,8].

The chicken oviduct has a major role in that it is a reproductive tract that produces eggs. The oviduct derives from the Mullerian duct that develops only on the left side in female chicks whereas the right Mullerian duct degenerates in female chicks and both of the Mullerian ducts degenerate in male chicks. The immature oviduct develops rapidly after 16 weeks of age and egg laying begins when chickens are 16- to 20-weeks-old [9,10]. The oviduct of laying hens consists of four specific segments which are the infundibulum (fertilization), magnum (formation of the soft shell membrane) and shell gland (formation of the outer egg shell) [11]. Development of the oviduct is stimulated in response to estrogen and previous studies have shown the regulatory genes, serpin peptidase inhibitor, clade B (ovalbumin), member 3 (SERPINB3) [12], SERPINB11 [15], adenosylhomocysteinase-like 1 (AHCYL1) [14], alpha 2 macroglobulin (A2M) [15] and pleiotrophin (PTN) [16] are highly expressed during development of the immature oviduct in chickens.

In contrast to female reproductive organs, gonadal morphogenesis leading to a mature testis is symmetrical between left and
right gonads and originates in thickened medullary cords via proliferation of Sertoli cells within the cords that are anlage of the seminiferous tubules of male embryos (ZZ). Testes of 6-week-old chicken have seminiferous tubules that include a simple layer of spermatogonia, Sertoli cells, basal lamina and myoid cells. In testes of 50-week-old chickens all stage of spermatogenesis from spermatogonia to spermatozoa are found along with Sertoli cells surrounded by basal lamina and myoid cells [3,17]. In development of testis, sex-determining genes such as doublesex and mab-3 related transcription factor 1 (DMRT1) (Z-linked gene) and sex determining region Y-box 9 (SOX9) (Sertoli cell differentiation factor) participate in testicular morphogenesis [18]. However, cellular and tissue-specific regulation for spermatogenesis remains unknown.

In a previous study, we identified several novel genes based on significant changes in their expression and functional categorization of genes changed between left and right gonads at embryonic days 6 and 9 through microarray analysis that may regulate gonadal morphogenesis in the both sexes of chicken embryos. We focused on four genes including synuclein alpha (SNCA), target of myb 1 (chicken) like 1 (TOM1L1), transthyretin (TTR) and zinc finger E-box binding homeobox 1 (ZEB1) that are associated with cellular proliferation and embryonic development and regulated by FSH and LH for E6 gonadal cells. However, these genes have not been investigated with respect to their influence on development of chicken reproductive tract. Therefore, we hypothesized that these selected genes effect changes in morphogenesis of reproductive organs in chickens. Accordingly, we determined differential patterns of mRNA expression and verified cell- and tissue-specific localization of mRNAs and proteins encoded by the four genes of interesting during development of female and male reproductive tracts in chickens. Moreover, we investigated post-transcriptional regulation of expression of three of the genes (SNCA, TTR and ZEB1) using a miRNA target validation assay. Results of present study provide novel insights into SNCA, TOM1L1, TTR and ZEB1 genes with respect to their tissue-specific expression during differentiation of germ cells into mature reproductive organs and post-transcriptional regulation of their expression by specific miRNAs in chickens.

Results

Comparative Expression of SNCA during Development of Reproductive Organs in both Sexes of Chickens

As illustrated in Figure 1A, the results from quantitative RT-PCR analyses indicated that expression of SNCA mRNA decreased 80% in left gonads at E18, 20% ($P<0.05$) in oviducts of 12 week old chicks and 40% and 76% ($P<0.05$) in the adult ovary and oviduct at 50 weeks, respectively as compared with SNCA expression in the gonads at E14. On the other hand, SNCA mRNA expression increased 11.2-fold ($P<0.001$) in the ovaries of 12-week-old chickens. Moreover, expression of SNCA decreased 70% and 90% in the gonads at E18 and testis of 50-week-old chickens, respectively as compared with SNCA expression in the gonads at E14 (Figure 1B). However, SNCA increased 26.3-fold ($P<0.001$) in the testis of 12-week-old chickens as compared with expression of SNCA in E14 gonads. In situ hybridization and immunofluorescence analyses detected SNCA mRNA and protein localized mainly in the cortex region of embryonic gonads and both were highly expressed in ovarian follicles of the immature chicken oviduct (Figure 2A and 2B). In males, in accordance with mRNA expression, SNCA protein was abundant in the seminiferous tubules of 12-week-old pups and weakly expressed in Sertoli cells of 50-week-old testes (Figure 3A and 3B).

Comparative Expression of TOM1L1 during Development of Reproductive Organs in both Sexes of Chickens

Tissue specific expression of TOM1L1 mRNA was found to increase 9.4- ($P<0.001$) and 4.9- ($P<0.01$) fold in oviducts at 12-weeks and ovaries at 50 weeks, respectively and decrease 70% in oviducts at 50 weeks as compared with TOM1L1 expression in the female gonads at E14 by quantitative RT-PCR (Figure 4A). In the reproductive tract of male chickens, TOM1L1 expression increased 5.2-fold ($P<0.001$) in testes at 12weeks and decreased 70% ($P<0.05$) in testes of at 50weeks in male chickens as compared with TOM1L1 expression in the gonads at E14 (Figure 4B). In addition, TOM1L1 expression was strong in the oviduct of 12-week-old chickens and moderately expressed in ovarian follicles of 12- and 50-week-old female chickens (Figure 5A and 5B). In male chickens, TOM1L1 mRNA and protein were most abundant in the seminiferous cord of embryonic gonads at E14 and E18,
seminiferous tubules of immature testis (12 wks) and spermatogonia of testes at 50 weeks (Figures 6A and 6B).

**Comparative Expression of TTR during Development of Reproductive Organs in both Sexes of Chickens**

Chicken TTR gene expression was demonstrated in the reproductive tract during development of ovaries, oviduct and testes. In females, TTR was expressed weakly during development of the ovary and oviduct. The expression levels indicated 0.01- (P<0.001), 0.08- (P<0.01), 0.02- 0.3- (P<0.001) and 0.02- fold changes in TTR mRNA in the embryonic gonads at E14, 12-week-old ovaries and oviducts and 50-week-old ovaries and oviducts as compared with TTR expression in the embryonic gonads at E14 (Figure 7A). Next, TTR mRNA was evaluated during testis development in chickens. The results showed that TTR expression decreased 92% in the embryonic gonads at E18 and increased 5.1-
Figure 3. Cell-specific localization of mRNA and protein for SNCA in male reproductive tracts during their development. Localization of SNCA expression was analyzed in the male reproductive tract of chickens during their development by in situ hybridization (A) and immunofluorescence analyses (B). Cell nuclei were stained with DAPI (blue). Legend: S, Sertoli cell; Sc, seminiferous cord; St, seminiferous tubule. Scale bar represents 100 μm and 20 μm for first and second horizontal panels of (A) and 50 μm for (B). See Materials and Methods for a complete description of the methods.

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chickens.

participates in development of both the oviduct and testis in (Figure 12A and 12B). These results suggest that ZEB1
gonads and Sertoli cells of testes from 50-week-old male chickens
were localized predominantly to seminiferous cords of E14
expressed in the cortex and medullary region of gonads at E14
and in ovarian follicles of ovaries from 12-week-old female
in luminal epithelium of the oviduct at 12- and 50-weeks of age

Asterisks denote statistically significant differences (** p<0.01 and * p<0.05).

Figure 4. Quantitative analysis of TOM1L1 mRNA expression in female and male reproductive tracts during their development. Quantitative RT-PCR was conducted using cDNA templates from female (A) and male (B) gonads at embryonic days 14 and 18, 12-week-old ovary and oviduct and 50-week-old ovary and oviduct. The asterisks denote statistically significant differences (*** p<0.001, ** p<0.01 and * p<0.05).

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Comparative Expression of ZEB1 during Development of Reproductive Organs in Female and Male Chickens

Tissue-specific expression of ZEB1 in the female and male reproductive tracts of chickens during development was demonstrated using quantitative RT-PCR, in situ hybridization and immunofluorescence analyses. As illustrated in Figure 10A, ZEB1 mRNA expression increased in the reproductive tracts of 12- and 50-week-old female chickens as compared to embryonic gonads. Its expression increased 11.9- (P<0.001), 13- (P<0.0001), 2.2- (P< 0.05) and 7.6- (P<0.001) fold in 12-week-old ovaries and oviducts and 50-week-old ovaries and oviducts, respectively as compared with ZEB1 expression in the gonads at E14. In male chickens, ZEB1 expression increased gradually from E18 gonads to adult testes. Expression of ZEB1 mRNA increased 0.2- (P<0.001), 0.6- (P<0.01) and 1.3- (P<0.01) fold in the gonads at E14 and E18 and testis of 12- and 50-week-old male chickens, respectively as compared with ZEB1 expression in the gonads at E14 (Figure 10B). ZEB1 mRNA and protein were highly expressed in luminal epithelium of the oviduct at 12- and 50-weeks of age and in ovarian follicles of ovaries from 12-week-old female chickens (Figure 11A and 11B). In addition, ZEB1 was weakly expressed in the cortex and medullary region of gonads at E14 and E18. In male reproductive organs, ZEB1 mRNA and protein were localized predominantly to seminiferous cords of E14 gonads and Sertoli cells of testes from 50-week-old male chickens (Figure 12A and 12B). These results suggest that ZEB1 participates in development of both the oviduct and testis in chickens.

Post-transcriptional Regulation of Genital Ridge Development Regulatory Genes by Chicken microRNAs

We next investigated the possibility that expression of SNCA, TTR and ZEB1 is regulated at the post-transcriptional level by microRNAs (miRNAs) using a miRNA target validation assay. In order to find target miRNAs and their binding sites within the 3'-UTR of SNCA, TTR and ZEB1 genes, the miRNA target prediction database (miRDB: http://mirdb.org/miRDB/) was used. It revealed several putative binding sites for miRNAs including miR-153 and miR-1643 for SNCA, miR-1680* for TTR and miR-200b and miR-1786 for ZEB1 (Figures 13, 14 and 15). However, no specific target miRNA was detected for TOM1L1. Thus, we determined if these specific miRNAs influence expression of SNCA, TTR and ZEB1 via their 3'-UTR. A fragment of each 3'-UTR with binding sites for the miRNAs was cloned downstream of the green fluorescent protein (GFP) reading frame, thereby creating a fluorescent reporter for function of the 3'-UTR region (Figure 13B, 14B and 15B). After co-transfection of eGFP-3'-UTR and DsRed-miRNA, analyses for intensity of GFP expression and percentage of GFP-expressing cells were conducted using FACS and fluorescence microscopy. In the presence of miR-153 and miR-1643 decreased the intensity and percentage of GFP-SNCA-expressing cells 58% and 61% (Figure 13). In addition, miR-1680* decreased the intensity and percentage of cells expressing TTR by 58% (Figure 14). Furthermore, miR-200b and miR-1786 decreased the intensity and percentage of GFP-ZEB1-expressing-cells by 63% and 66%, respectively (Figure 15). These results indicate that specific miRNAs associated with target transcripts may be involved in development of reproductive organs in chickens and regulate their expression at the post-transcriptional level during morphogenesis of the oviduct, oviduct and testis.

Discussion

Results of the current study revealed differential temporal and spatial expression patterns for key genes, SNCA, TOM1L1, TTR and ZEB1 that are important for development and differentiation of chicken reproductive tract in both sexes. In addition, the results indicate that among the four selected genes, expression of SNCA, TTR and ZEB1 is post-transcriptionally regulated via specific miRNAs binding directly the 3'-UTR of these target genes. These results support our hypothesis that molecular patterning of the
The chicken is a firmly entrenched animal model for research in embryology and reproductive developmental biology, but little is known about regulatory genes that control development of the reproductive tract in female and male chickens. In our previous study (Lim and Song, 2014, in submission), we reported novel genes and hormonal regulation of gonad morphogenesis in chicken embryos. To demonstrate the differential patterns of expression of mRNAs and proteins of SNCA, TOM1L1, TTR and ZEB1 during development and differentiation of germ cells and primordial tissues to mature reproductive organs, we performed quantitative RT-PCR, in situ hybridization and immunofluorescence analyses.

SNCA belongs to a family of small and highly conserved proteins in vertebrates including alpha-, beta- and gamma-

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**Figure 5. Cell-specific localization of mRNA and protein for TOM1L1 in female reproductive tracts during their development.** Cell-specific expression of TOM1L1 mRNA and protein in development of the female reproductive tract was demonstrated by in situ hybridization (A) and immunofluorescence analyses (B). Cell nuclei were stained with DAPI (blue). Legend: C, cortex; F, follicle; L, lacunae; LE, luminal epithelium; M, medullar. Scale bar represents 100 μm and 20 μm for first and second horizontal panels of (A) and 50 μm for (B). See Materials and Methods for a complete description of the methods.
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Figure 6. Cell-specific localization of mRNA and protein for TOM1L1 in male reproductive tracts during their development. Localization of TOM1L1 expression was analyzed in the male reproductive tract of chickens during their development by in situ hybridization (A) and immunofluorescence analyses (B). Cell nuclei were stained with DAPI (blue). Legend: S, Sertoli cell; Sc, seminiferous cord; Sg, spermatogonia; St, seminiferous tubule. Scale bar represents 100 μm and 20 μm for first and second horizontal panels of (A) and 50 μm for (B). See Materials and Methods for a complete description of the methods. doi:10.1371/journal.pone.0096175.g006
synuclein. The SNCA gene has 7 exons (5 protein-coding) and is expressed mainly in the brain, particularly in the hippocampus, caudate nucleus, amygdala, substantia nigra and thalamus in adult humans [19]. The SCNA gene has been specifically related to several neurodegenerative diseases such as Parkinson’s disease via three point mutations (A53T, A30P and E46K) [20,21] and Alzheimer’s disease through accumulation of the gene product in humans [22]. In addition, testosterone increases SNCA in the majority of neurons in brain and spinal cord during embryogenesis [23]. However, there are no published results on SNCA and development of reproductive organs of any animal or human model. We reported that SNCA is expressed in both male and female embryonic gonads in chickens. Therefore, we investigated expression of SNCA during development of the reproductive organs from embryonic gonads to adult male and female reproductive tracts. SNCA was detected predominantly in ovary and testis of 12-week-old chickens which suggests that SNCA might have an important role in morphogenesis of ovary and testis.

TOM1L1 is also known as Src-activating and signaling molecule (SRCASM). This gene is an activator for Src family tyrosine kinases (SFKs) that include nine members that have significant roles in mitogenesis and morphological alterations via induction of growth factors [25]. TOM1L1 is tyrosine-phosphorylated in response to EGFR ligand as a SFK substrate downstream of EGFR. Increased expression of TOM1L1 activates endogenous SFKs preferably for phosphorylating Fyn and Src. Therefore, TOM1L1 links with EGFR and SFK-dependent signaling in differentiation of keratinocytes [26,27]. In addition, TOM1L1 has a role as a regulatory adaptor bridging activated EGFR in endocytosis by EGF stimulus [28]. In the present study, we determined that TOM1L1 was expressed strongly in luminal epithelium of the immature oviduct and follicles of adult ovaries in female chickens and in testes of 12-week-old male chickens. These results show that TOM1L1 likely has a role in regulating development of the immature oviduct, ovarian folliculogenesis and seminiferous tubules in chickens.

TTR (also called prealbumin) is one of the transporters of thyroid hormones and cooperates with retinol-binding protein (RBP) and vitamin A (retinol). TTR directly binds the thyroid hormones (T3 and T4) in the central channel constituted by tetrameric assembly of the monomers [29,30,31], and it indirectly provides vitamin A as retinol bound to RBP [32]. TTR has a well-established role in regulating spermatogenesis through effects on retinol metabolism in the adult testis of rats. Circulating retinol binds to a complex of RBP and TTR which is present in the peritubular cells associated with intracellular CRBP which has a high affinity for binding retinol [33]. The peritubular cells secrete retinol as a complex form to the Sertoli cells that oxidize retinol into retinoic acid which stimulates differentiation of germ cells [34]. Therefore, TTR assists in development of germ cells within the developing seminiferous tubules. In females, it is not known if TTR regulates oogenesis or embryogenesis. However, thyroid hormones transported by TTR from serum into the oocyte play a crucial role in embryogenesis in various species, especially as it is accumulates in the yolk of oocytes during oogenesis in chickens [35]. In our study, TTR was highly expressed during testes development, predominantly in the seminiferous tubules of immature testes. Otherwise, in the female reproductive tract, expression of TTR was weak in immature and mature ovaries. These results indicate that the TTR gene might have an important role in development and maturation of the postnatal testis in chickens.

ZEB1 (also known as EF1, TCF8, AREB6, Nil-2-a) is a transcription factor binding to DNA via two zinc finger clusters, one at the N-terminus and one at the C-terminus, and it can modulate transcription of target genes by binding directly to 5'-CACCT sequences in their promoter regions [36]. ZEB1 plays an important role in development, cell proliferation, differentiation, migration and reproduction [37,38]. In addition, ZEB1 protein induces cell migration during development and cancer progression by repressing expression of E-cadherin in epithelial cells [39,40,41,42]. ZEB1 is regulated by steroid hormones, estrogen [43], progesterone [44] and androgen [45]. In chickens, estrogen induces proliferation and differentiation of tubular gland cells associated with production of egg white proteins and stimulates ZEB1 expression leading to activation of transcription of downstream targets in the chick oviduct [43,46]. In this study, ZEB1 was highly expressed in both the immature and mature female reproductive tract as compared to the embryonic gonads suggesting a key role in development of the oviduct of adult female chickens. Moreover, in male chickens, ZEB1 expression increased gradually with progressive development of the testes from an E18 gonad to an adult testis. Thus, ZEB1 may play a crucial role in egg development.
production through effects on development of the oviduct, as well as all stages of development of the testis in male chickens.

Based on results from validation of gene expression during development of chicken reproductive organs, we next investigated whether target genes undergo post-transcriptional regulation by specific microRNAs. MicroRNAs (miRNAs) are small non-coding single stranded RNAs of 18–23 nucleotides that play a role as post-transcriptional regulators and transformers of cell fate through modulation of target-mRNA translation in various cells and tissues. In other words, miRNAs have crucial regulatory effects in a variety of biological events including growth, development, differentiation and control of cell cycle by modulating gene expression [47,48,49]. For example, expression of miRNAs during gonadal development in chickens and mammals has been reported [50,51,52]. In addition, several miRNAs regulate mechanisms required for development and differentiation of the oviduct and ovarian cancer in female chickens [14,16,53,54]. Moreover, miR-34c down-regulates genes related to germ cell differentiation and
Localization of TTR expression was analyzed in the male reproductive tract of chickens during their development by in situ hybridization (A) and immunofluorescence analyses (B). Cell nuclei were stained with DAPI (blue). Legend: S, Sertoli cell; Sc, seminiferous cord; St, seminiferous tubule. Scale bar represents 100 μm and 20 μm for first and second horizontal panels of (A) and 50 μm for (B). See Materials and Methods for a complete description of the methods.
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Figure 9. Cell-specific localization of mRNA and protein for TTR in male reproductive tracts during their development.
its expression was detected mainly in the later stages of meiosis in spermatogenesis in chickens [55]. Based on previous reports, miRNAs might play a role during morphogenesis of the ovary, oviduct and testis in chickens. However, few miRNAs have been investigated with respect to their regulation of target genes and mechanisms whereby they act remain unknown. In this study, we performed an in vitro target assay of miRNAs to determine if SNCA, TTR and ZEB1 transcripts are regulated at the post-transcriptional level by target miRNAs. As illustrated in Figures 13, 14, and 15, specific target miRNAs of chickens attenuate intensity of GFP-transcripts are regulated at the post-transcriptional level by target miRNAs. As illustrated in Figures 13, 14, and 15, specific target miRNAs of chickens attenuate intensity of GFP expressing cells. These results indicate that at least one to two miRNAs directly bind to the development-regulatory genes of reproductive organs and post-transcriptionally regulate their expression during development of the male and female reproductive tracts of chickens.

In conclusion, our results provide evidence for temporal and spatial expression of five genes that influence development of reproductive organs of chickens from the embryonic stage to the immature and mature stages of development. Expression of SNCA, TTR and ZEB1 are modulated via post-transcriptional regulation by specific target miRNAs which warrant further study. These results suggest roles for four important genes that likely regulate development of reproductive organs in chickens.

Materials and Methods

Experimental Animals and Animal Care

The experimental use of chickens for this study was approved by the Animal Care and Use Committee of Korea University. All chickens were exposed to a light regimen of 15 h light and 9 h dark with ad libitum access to feed and water, and subjected to standard poultry husbandry guidelines.

Tissue Samples

The left and right gonads were collected separately from the mesonephric kidney of chicken embryos at E14 and E18 in a 1.5 ml tube containing diethylpyrocarbonate treated PBS (DEPC-PBS). Then we centrifuged the sample at 1,080 x g for 5 min to allow collection of each gonad from the bottom of the tubes. After removal of the DEPC-PBS, the gonads were stored at −80°C until RNA was extracted. Also we collected whole embryos and fixed them in freshly prepared 4% paraformaldehyde in PBS (pH 7.4). After 24 h, the samples fixed in 4% paraformaldehyde were changed to 70% ethanol for 24 h and then dehydrated in a graded series of increasing concentrations of ethanol. Embryos were then incubated in xylene for 3 h and embedded in Paraplast-Plus. Paraffin-embedded tissues were sectioned at 5 μm.

RNA Isolation

Total cellular RNA was isolated from frozen tissues using Trizol reagent according to manufacturer’s recommendations. The quantity and quality of total RNA was determined by spectrometry and denaturing agarose gel electrophoresis, respectively.

Quantitative RT-PCR Analysis

Total RNA was extracted from gonads on embryonic day 14 and 18 from both sexes and ovaries, oviducts and testes from 12- and 50-week-old females and males using TRIzol and purified using an RNeasy Mini Kit. Complementary DNA was synthesized using a Superscript III First-Strand Synthesis System. Gene expression levels were measured using SYBR Green and a StepOnePlus Real-Time PCR System. The glyceraldehydes 3-phosphate dehydrogenase (GAPDH) gene was analyzed simultaneously as a control and used for normalization of data. GAPDH expression is most stable among other housekeeping genes and it is used commonly for normalizing for variations in loading. Each target gene and GAPDH were analyzed in triplicate. Using the standard curve method, we determined expression of the examined genes using the standard curves and Ct values, and normalized them using GAPDH expression. The PCR conditions were 95°C for 3 min, followed by 40 cycles at 95°C for 20 sec, 60°C for 40 sec, and 72°C for 1 min using a melting curve program (increasing the temperature from 55°C to 95°C at 0.5°C per 10 sec) and continuous fluorescence measurement. ROX dye was used as a negative control for the fluorescence measurements. Sequence-specific products were identified by generating a melting curve in which the Ct value represented the cycle number at which a fluorescent signal was statistically greater than background, and relative gene expression was quantified using the 2−ΔΔCt method [56]. For the control, the relative quantification of gene expression was normalized to the Ct value for the control oviduct.

Figure 10. Quantitative analysis of ZEB1 mRNA expression in female and male reproductive tracts during their development. Quantitative RT-PCR was conducted using cDNA templates from female (A) and male (B) gonads at embryonic days 14 and 18, 12-week-old ovary and oviduct and 50-week-old ovary and oviduct. The asterisks denote statistically significant differences (*** p<0.001, ** p<0.01 and * p<0.05).

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In Situ Hybridization Analysis

For hybridization probes, PCR products were generated from cDNA with the primers used for RT-PCR analysis. The products were extracted from the gel and cloned into TOPO TA cloning vector. After verification of the sequences, plasmids containing gene sequences were linearized and transcribed using a DIG RNA labeling kit with T7 or SP6 polymerase. Information on the probes has been published (Lim and Song, 2014, in submission). Tissues were collected and fixed in freshly prepared 4% paraformaldehyde, embedded in paraffin and sectioned at 5 µm on APES-treated (silanized) slides. The sections were then deparaffinized in xylene and rehydrated to diethylpyrocarbonate (DEPC)-treated water through a graded series of alcohol. The sections were treated with 1% Triton X-100 in PBS for 20 min and washed two times in DEPC-treated PBS. After washing in DEPC-treated PBS, the
Figure 12. Cell-specific localization of mRNA and protein for ZEB1 in male reproductive tracts during their development. Localization of ZEB1 expression was analyzed in the male reproductive tract of chickens during their development by in situ hybridization (A) and immunofluorescence analyses (B). Cell nuclei were stained with DAPI (blue). Legend: S, Sertoli cell; Sc, seminiferous cord; St, seminiferous tubule. Scale bar represents 100 μm and 20 μm for first and second horizontal panels of (A) and 50 μm for (B). See Materials and Methods for a complete description of the methods. doi:10.1371/journal.pone.0096175.g012
sections were digested with 5 μg/ml Proteinase K in TE buffer (100 mM Tris-HCl, 50 mM EDTA, pH 8.0) at 37°C. After postfixation in 4% paraformaldehyde, sections were incubated twice for 5 min each in DEPC-treated PBS and incubated in TAE buffer (0.1M triethanolamine) containing 0.25% (v/v) acetic anhydride. The sections were incubated in a prehybridization mixture containing 50% formamide and 4X standard saline citrate (SSC) for at least 10 min at room temperature. After prehybridization, the sections were incubated overnight at 42°C in a humidified chamber in a hybridization mixture containing 40% formamide, 4X SSC, 10% dextran sulfate sodium salt, 10mM DTT, 1 mg/ml yeast tRNA, 1mg/ml salmon sperm DNA, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.2mg/ml RNase-free bovine serum albumin and denatured DIG-labeled cRNA probe. After hybridization, sections were washed for 15 min in 2X SSC at 37°C, 15min in 1X SSC at 37°C, 30 min in NTE buffer (10mM Tris, 500mM NaCl and 1mM EDTA) at 37°C and 30 min in 0.1X SSC at 37°C. After blocking with 2% normal sheep serum, the sections were incubated overnight with sheep anti-DIG antibody conjugated to alkaline phosphatase. The signal was visualized following exposure to a solution containing 0.4 mM 5-bromo-4-chloro-3-indolyl phosphate, 0.4 mM nitroblue tetrazolium, and 2 mM levamisole.

Immunofluorescence Analysis

The localization of four proteins in the reproductive tract of both sexes during their development was evaluated by immunofluorescence (IF) using anti-human SNCA polyclonal antibody (ab21975), anti-human TOM1L1 polyclonal antibody (ab126972), anti-human TTR polyclonal antibody (ab9015) and anti-human ZEB1 polyclonal antibody (ab1972). Antigen retrieval was performed using boiling 10mM sodium citrate buffer pH 6.0 for 10 min after which the slides were cooled on the bench top for 20 min. After antigen retrieval the slides were washed three times in 1X PBS for 5 min. Slides were incubated in blocking buffer (10% normal serum from the same species as the secondary antibody in 1X PBS) for 1 h. After the blocking solution was aspirated, slides were incubated overnight at 4°C with primary antibody. The slides were then rinsed three times in 1X PBS for 5 min each. Slides were then incubated with Alexa Fluor 488 rabbit anti-goat IgG secondary antibody for ZEB1, goat anti-rabbit IgG secondary antibody for TOM1L1 and donkey anti-sheep IgG secondary antibody for SNCA and TTR at a 1:200 dilution for 1 h at room temperature in the dark. Slides were then washed and overlaid with Prolong Gold Antifade with DAPI. For primary antibody, images were captured using a Zeiss confocal microscope.
Figure 14. In vitro target assay for miR-1680* on the TTR transcript. (A) Diagram showing miR-1680* binding sites in TTR 3’-UTR. (B) Schematic expression of vector maps for eGFP with TTR 3’-UTR and DsRed with each miRNA. (C and D) The fluorescence signals of GFP and DsRed were detected using FACS (C) and fluorescent microscopy (D) after co-transfection of pcDNA-eGFP-3’-UTR for the TTR transcript and pcDNA-DsRed-miRNA for miR-1680*.

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Figure 15. In vitro target assay for miR-200b and miR-1786 on the ZEB1 transcript. (A) Diagram showing miR-200b and miR-1786 binding sites in ZEB1 3’-UTR. (B) Schematic expression of vector maps for eGFP with ZEB1 3’-UTR and DsRed with each miRNA. (C and D) The fluorescence signals of GFP and DsRed were detected using FACS (C) and fluorescent microscopy (D) after co-transfection of pcDNA-eGFP-3’-UTR for the ZEB1 transcript and pcDNA-DsRed-miRNA for the miR-200b and miR-1786.

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Statistical Analyses
All quantitative data were subjected to analysis of variance (ANOVA) according to the general linear model (PROC-GLM) of the SAS program. All tests of significance were performed using the appropriate error terms according to the expectation of the mean square for error. Data are presented as mean ± SEM unless otherwise stated. Differences with a probability value of P<0.05 were considered statistically significant.

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Author Contributions
Conceived and designed the experiments: WL GS. Performed the experiments: WL GS. Analyzed the data: WL GS. Contributed reagents/materials/analysis tools: GS. Wrote the paper: WL GS.

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