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**HOKKAIDO UNIVERSITY**
Studies on \textit{cHEMGN} gene specifically involved in early gonadal differentiation in chicken

(ニワトリ特異的に初期生殖腺分化に機能する \textit{cHEMGN} 遺伝子の研究)

A DISSERTATION
submitted to the Graduate School of Life Science,
Hokkaido University
in partial fulfillment of the requirements for the degree
DOCTOR OF LIFE SCIENCE
by

Tomohiro Nakata

March 2014
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General Introduction

The sex of birds, which is constituted by a ZZ/ZW sex chromosome, is genetically determined as either homogametic ZZ for males or heterogametic ZW for females (Clinton, 1998; Smith and Sinclair, 2004). A master gene that regulates sex determination in birds remains to be identified, and the molecular mechanisms of sex determination and gonadal differentiation are unclear. By contrast, mammals have a male heterogametic sex chromosome constitution, XX/XY (XX for females and XY for males), and the molecular mechanisms of sex determination have been studied well.

The molecular switch that controls sex determination is the SRY (sex-determining region Y) gene linked to the Y chromosome (Koopman et al., 1991) in eutherian mammals. SRY is expressed by pre-Sertoli cells of undifferentiated gonads of XY embryos at 10.5 days post coitum (dpc) (Koopman et al., 1990). SRY and NR5A1 (nuclear receptor subfamily 5, group A, member 1; also known as Ad4BP/SF1) bind to the enhancer regions of Sox9 (SRY-box 9) and synergistically activate Sox9 transcription (Sekido and Lovell-Badge, 2008). SOX9 functions as a transcriptional activator of Amh (anti-Müllerian hormone), and AMH activation leads to the degeneration of the Müllerian duct in XY embryos (Behringer, 1994).

Although the SRY homologue is absent in birds (Mizuno et al., 2002), many chicken homologues of genes important for mammalian gonadal differentiation have been identified. Many of these genes are also expressed by early embryonic gonads of the chicken (Smith and Sinclair, 2004). A strong candidate for male sex determination is DMRT1 (doublesex and mab-3-related transcription factor 1), which is indispensable for male differentiation in both chickens and mammals (Raymond et al., 2000; Smith et al., 2009a). DMRT1 is linked to the Z
chromosome in the chicken (Nanda et al., 1999). Furthermore, DMRT1 knockdown by RNA interference (RNAi) shows partial male-to-female sex reversal (Smith et al., 2009a).

Both SOX9 and AMH are necessary for testis differentiation after sex determination and development of other genitalia in the chicken; however, these genes are regulated differently in mammals. The expression of SOX9 is preceded by AMH expression in the chicken (Smith et al., 1999a; Kamata et al., 2004), and it is unclear how SOX9 and AMH expression is induced. DMRT1 can activate SOX9 indirectly because there is a lag between the induction of DMRT1 and SOX9 expression on days 4.5 and 6.5, respectively (Smith et al., 1999a; Kamata et al., 2004). From this finding, intervening factors are suggested which involved in SOX9 regulation.

To better understand the molecular mechanism of avian sex determination, I focused on the chicken Hemogen homologue gene (cHEMGN) previously identified as a novel gene in our laboratory. I firstly performed expression analysis of cHEMGN in early embryonic gonads and hematopoietic tissues of the chicken (see Chapter I). I next performed functional analyses by virus-mediated overexpression and knockdown of cHEMGN (see Chapters II and III, respectively). From the results of these experiments, I conclude that cHEMGN is a transcription factor in the nucleus of (pre-)Sertoli cells after sex determination and that it directly or indirectly triggers SOX9 expression, indicating that this gene is specifically involved in sex determination in the chicken.
Chapter I

The expression pattern of $cHMG\text{N}$ in early embryos

Abstract

An avian sex-determining gene has not yet been identified, and the molecular mechanisms of sex determination and gonadal differentiation are unclear. In a previous study from our laboratory, a comprehensive transcriptome analysis was performed in embryonic gonads to identify genes involved in chicken-specific sex determination, and the $cHMG\text{N}$ gene was identified. A mammalian hemogen is a transcription factor expressed exclusively by hematopoietic tissues, indicating that this protein regulates the proliferation and differentiation of hematopoietic cells. I performed a detailed expression analysis of $cHMG\text{N}$ in embryonic gonads and hematopoietic tissues of the chicken. The expression of $cHMG\text{N}$ commenced on day 5.5, prior to $SOX9$ expression. $cHMG\text{N}$ expression drastically increased, peaked on day 8.5, and then disappeared before hatching. Dual-label in situ hybridization studies showed $cHMG\text{N}$ to colocalize with $SOX9$, illustrating its expression in (pre-)Sertoli cells. The expression pattern of $cHMG\text{N}$ in hematopoietic tissues in the chicken was consistent with that of the mouse, as previously reported. These results demonstrate that $cHMG\text{N}$ is specifically involved in early gonadal differentiation in birds and that its function is either acquired only in the avian lineage or lost in the mammalian lineage.
Introduction

To identify novel genes involved in gonadal differentiation in the chicken, our laboratory conducted a comprehensive transcriptome analysis, namely, high coverage expression profiling (HiCEP; Fukumura et al., 2003), which compared transcripts from male and female gonads between days 5.5 and 6.5 (Kuroiwa et al., unpublished data). From 33,962 transcripts identified, 18 transcripts were either exclusively expressed in male gonads or expressed at levels that were more than 5-fold higher in male gonads than in female gonads. Sequence analysis revealed that cHEMGN was among these transcripts.

Hemogen [Hemgn in mice, EDAG (erythroid differentiation-associated gene protein) in humans] is a recently identified nuclear protein exclusively expressed by hematopoietic tissues, namely, the blood islands of the yolk sac, primordial blood cells, the fetal liver during embryogenesis, the adult spleen, and bone marrow (BM) (Yang et al., 2001). In the adult BM, this gene is predominantly expressed in primitive progenitor and stem cell populations, but not in terminally differentiated mature blood cells. Transgenic mice overexpressing Hemgn show stimulation of myelopoiesis and suppression of lymphopoiesis, suggesting its regulatory role in hematopoiesis (Li et al., 2007). Although nuclear factor-κ B can regulate this gene (Li et al., 2004), the involvement of Hemgn in gonadal development during embryogenesis has not been reported.

Based on results from Southern blot analysis, fluorescence in situ hybridization (FISH) mapping, and chicken genome database searches (Ishiguro, 2010), cHEMGN is a single-copy gene located on the long arm of the Z chromosome (Zq21). cHEMGN is specifically expressed in early embryonic gonads, and its mRNA level in males is higher than in females (Sakamoto,
The cHEMGN protein is also expressed in early embryonic male gonads at days 6.5–8.5 (Ishiguro, 2010). Southern blot analysis indicates that cHEMGN is conserved among several avian species (Ishiguro, 2010). However, its expression patterns in embryonic gonads and hemotopoietic tissues are not known. Therefore, I firstly performed expression analysis of cHEMGN in early embryonic gonads and hemotopoietic tissues of the chicken.

I determined the localization of cHEMGN mRNA and protein in early embryonic gonads and conclude that cHEMGN is specifically expressed in the nucleus of (pre-)Sertoli cells of early embryonic gonads. In the chicken, cHEMGN was expressed not only in hemotopoietic tissues but also in early embryonic male gonads. A significantly higher level of cHEMGN expression in male gonads than in female gonads suggests the existence of a specific enhancer for cHEMGN expression in male gonads. These results raise the possibility that cHEMGN is specifically involved in the gonadal differentiation of birds.
Materials and Methods

Animals

Fertilized eggs of the Hy-Line Maria strain of chicken (*Gallus gallus domesticus*) were purchased from Takeuchi hatchery (Nara, Japan) and incubated at 37.8°C. Embryos were sampled at objective developmental stages which were complied with the report of Hamburger and Hamilton (1951).

Genomic DNA extraction and sexing by PCR

Genomic DNA was extracted from bloods of chicken embryos using Dr. GenTLE (Takara) according to the manufacturer’s protocol. Sexing was performed as described by Fridolfsson and Ellegren (1999), using *CDH1* primers shown in Table 1. The PCR reactions were carried out in 10 µl of 1×Ex *Taq* buffer containing 0.5 µl genomic DNA, 2 mM MgCl2, 0.2 mM each of four deoxynucleoside triphosphates (dNTPs), 10 mM of each primer, and 0.25 U of Ex *Taq*.

Total RNA isolation from early chicken embryos

Total RNAs were isolated from chicken gonads by RNasy (QIAGEN) and from chicken hematopoietic tissues by TRIZOL Reagent (Invitrogen), respectively, according to the manufacturer’s protocol.

Northern blot analysis

The *cHEMGN* and 18S rRNA genes were cloned by PCR, using the primers shown in Table 1. The PCR conditions were 94°C for 2 min, then 35 cycles of 94°C for 30 sec, 60°C for 30 sec,
and 72°C for 30 sec, and finally 72°C for 5 min. The PCR products for the \textit{cHEMGN} and 18S rRNA genes were 640 and 103 bp, respectively. The PCR products were subcloned using the pGEM T-Easy vector system (Promega). The cDNA clones were labeled using a digoxigenin (DIG) RNA labeling mix (Roche) and T7 or SP6 RNA polymerase (Ambion: MAXIscript). Twenty micrograms of total RNA were electrophoresed on a formaldehyde/agarose gel and transferred to a positively charged nylon membrane. DIG-labeled probes were diluted in DIG easy Hyb (100 ng/ml) and, after pre-hybridization, added to the membrane for 18 h to hybridize.

\textbf{RT-PCR and quantitative RT-PCR (qRT-PCR)}

Total RNA was treated with DNase I and then reverse transcribed using SuperScript III reverse transcriptase (Invitrogen) and oligo(dT). The primers used for amplification of \textit{cHEMGN}, \textit{AMH}, \textit{SOX9}, and \textit{GAPDH} are shown in Table 1. The PCR conditions were 94°C for 2 min, then 35 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec, and finally 72°C for 5 min.

For qRT-PCR, RNA extraction and cDNA synthesis were carried out according to the methods described above. The qRT-PCR reactions were performed according to the manufacturer’s instructions, using Power SYBR Green PCR Master Mix (Applied Biosystems) and an ABI 7300 Fast Real-time PCR System (Applied Biosystems). To achieve maximum efficiency in the qRT-PCR, the amplicon size was kept small (90–101 bp). The primers used are presented in Table 1. The qRT-PCR reactions were performed in triplicate using 96-well plates and a 10-µl reaction volume. The data were analyzed using the ΔΔCt method and normalized by \textit{ACTB} (Beta-actin) expression levels.
Whole-Embryo in Situ Hybridization (WISH) and in situ hybridization

Chicken embryos were fixed in 4% (wt/vol) paf formaldehyde and processed for WISH according to a previous report (Yoshioka et al., 1998). The urogenital tissue of chicken embryos was slowly frozen in tissue-tek (Sakura Finetek), kept at −80°C until use and processed for in situ hybridization, as previously reported (Matsubara et al., 2010). For WISH and in situ hybridization, chicken cDNA and DIG-labeled probes were prepared as described above for northern blot analysis. The cDNA clone of SOX9 in pBluescript SK was a kind gift of Professor Hidefumi Yoshioka (Department of Natural Sciences, Hyogo University of Teacher Education). For double-label in situ hybridization, probe labeling and slide preparation were performed as described above. The sections were incubated at 60°C for 18 h in the same buffer containing 100–200 ng/ml DIG-labeled cRNA probes and 100–200 ng/ml biotin-labeled cRNA probes. The slides were treated with 1:500 diluted anti-DIG-rhodamine (Roche) and avidin-FITC in 1% BSA in 4×SSC at 37°C for 1 h.

Immunohistochemistry (IHC)

Urogenital tissues from chicken embryos were fixed for 1 h in formalin at room temperature. Paraffin sections (10-µm thickness) were cut and thaw-mounted onto Matsunami adhesive silane (MAS) coated glass. The sections were covered with primary antibodies diluted in Block-Ace (Dainippon), and incubated overnight at 4°C. The primary antibody, rabbit anti-cHEMGN, was used at dilutions of 1:200. The sections were washed in PBS for 5 min twice and treated with the secondary antibody (AlexFluor 488-labeled goat anti-rabbit IgG, Invitrogen, 1:400) in PBS overnight at 4°C.
Protein extraction from early chicken embryos

Tissue samples were homogenized in ice-cold PBS including 1×proteinase inhibitor (Roche) (3 ml per 1 mg of tissue) by using pestle. Homogenized samples were mixed with 2×lysis buffer (0.3 M NaCl, 0.1 M Tris-HCl pH 7.5, 0.5% sodium deoxycholate, 2% NP-40 [Nacalite], 2 mM EDTA) including 1×proteinase inhibitor. Samples were incubated on ice for 20 min, and sonicated twice to shear nucleic acids (TOMY UD201 output=1, 10 s). Samples were centrifuged at 15,000 rpm for 10 min at 4°C, and the supernatant was collected into a new tube, and stored at -80°C.

Western blot analysis

Proteins were electrophoresed in 10% acrylamide gel. Proteins were transferred to Amersham Hybond-P (GE Healthcare) at 25 V for 1.5 h using a Semi Dry transfer instrument (BIO CRAFT). The membrane was blocked for 2 h at room temperature in 5% skimmed milk (Wako)/TBS-T (TBS + 0.1% Triton X-100). Rabbit anti-cHEMGN antibody (Ishiguro 2010) was diluted at 1:5000 in TBS-T and added to membrane. After 1.5 h incubation at room temperature, membrane was washed four times in TBS-T for 10 min each time. As the secondary antibody, peroxidase-labeled anti-rabbit antibody (GE Healthcare) was diluted in TBS-T and added in PBS. After 1 h incubation at room temperature, membrane was washed four times in TBS-T for 10 min each time, and treated with ECL Plus Western blotting Detection Reagent for 5 min at room temperature. Signal was detected by using an LAS-3000 imager (FUJIFILM).
Results

Expression pattern of the cHEMGN mRNA in early embryonic gonads

Northern blot analysis demonstrated that cHEMGN expression in male gonads was higher than in female gonads at day 7.5 (Fig. 1). qRT-PCR analysis revealed that cHEMGN was expressed in male gonads at day 5.5 onwards, with its expression dramatically increasing and peaking at day 8.5. Its expression was then lost before hatching (Fig. 2). By contrast, cHEMGN expression in female gonads was very low throughout embryogenesis. cHEMGN expression in male gonads at day 8.5 was more than 10-fold higher than in female gonads. RT-PCR results showed that AMH expression was upregulated between days 5.5 and 6.5, similar to cHEMGN expression (Fig. 3). SOX9 was expressed at day 6.5 onwards and upregulated at day 8.5 (Fig. 3). These results are consistent with previous findings that demonstrate AMH expression in male gonads prior to SOX9 expression (Smith et al., 1999a; Oreal et al., 1998).

Localization of cHEMGN mRNA and protein in early embryonic gonads

The localization of the cHEMGN mRNA in early embryonic gonads was determined by WISH and in situ hybridization. cHEMGN expression was observed in the entire male gonad at day 7.5 (Fig. 4). In frozen cross-sections prepared from days 6.5, 7.5, and 8.5 embryos, cHEMGN expression was restricted to the gonadal medulla of males (Fig. 5).

To identify the cells that expressed cHEMGN, dual-label in situ hybridization was performed using mRNA probes against SOX9 and chicken vasa homologue (CVH, also known as DDX4) as markers for Sertoli cells and germ cells, respectively (Fig. 6). Dual-label in situ hybridization showed the cHEMGN signal to colocalize with the SOX9 signal in the gonadal
medulla (Fig. 7a); however, by contrast, the cHEMGN signal did not colocalize with that of CVH (Fig. 7b).

The cHEMGN protein localized to the nuclei of male gonadal cells by immunohistochemistry (IHC) (Fig. 8).

**Expression pattern of cHEMGN in hematopoietic tissues**

cHEMGN expression in hematopoietic tissues of the chicken was examined by northern blot analysis (Fig. 9). High cHEMGN expression was detected in the blood of both sexes, with a higher level in males than in females, whereas low expression was detected in the spleen and BM of both sexes. The expression levels were compared between sexes by qRT-PCR, and results showed cHEMGN levels in the spleen, BM, and blood were 2- to 3-fold higher in males than in females (Fig. 10).

The expression of cHEMGN protein in hematopoietic tissues was examined by western blot analysis and IHC. High expression was detected in the blood of males, whereas low expression was detected in the spleen of males and the blood of females (Fig. 11). The cHEMGN protein localized to the nuclei of blood cells (Fig. 12).
Discussion

The fate of undifferentiated gonads is determined around day 5.5 in the chicken, after which the gonads rapidly develop and differentiate (Ebensperger et al., 1988). qRT-PCR results showed cHEMGN expression in the male gonads at day 5.5 onwards, which dramatically increased and peaked at day 8.5. Expression was then lost before hatching (Fig. 2). These results indicate that cHEMGN functions at an extremely early stage in testis differentiation in the chicken.

The mammalian hemogen gene encodes a nuclear protein that functions as a transcription factor with two diagnostic motifs (i.e., a coiled-coil domain and a bipartite nuclear localization signal) at the N-terminus (Yang et al., 2001). While a comparison of mouse and chicken amino acids sequences showed a low identity (26%), the two motifs are conserved in both species (Ishiguro, 2010). The cHEMGN protein also localized to the nuclei of Sertoli (or pre-Sertoli) cells (Fig. 8), and its expression was observed prior to SOX9 expression (Fig. 3), illustrating that cHEMGN is a transcriptional factor upstream of SOX9 in the chicken.

While cHEMGN expression in male gonads at day 8.5 was more than 10-fold higher than in female gonads (Fig. 2), its expression was only 2- to 3-fold higher in hematopoietic tissues of the male than those of the female (Fig. 10). The cHEMGN gene is located on the Z chromosome; therefore, its gene dosage is different between sexes, namely, ZZ males and ZW females (Ishiguro, 2010). By contrast, the chicken Z chromosome lacks a gene dosage compensation system such as X chromosome inactivation in mammals. Therefore, the expression of many genes on the Z chromosome in males (ZZ) is higher than in females (ZW). Previous studies have measured the male-to-female (M:F) ratio of Z-linked genes in the chicken.
and have reported M:F expression ratios ranging from 0.4 to 2.7 for Z genes (McQueen et al., 2001; Kuroiwa et al., 2002; Itoh et al., 2007); thus differences in cHEMGN expression between sexes in hematopoietic tissues may reflect the Z-linked gene dosage of cHEMGN. Furthermore, the 10-fold higher expression in male embryonic gonads than in female gonads (Fig. 2) suggests the existence of a specific enhancer for cHEMGN expression in male gonads.

cHEMGN expression in hematopoietic tissues was observed in mammals and chickens (Figs. 9–12). By contrast, expression in gonadal tissues was only observed in chicken embryos, demonstrating that cHEMGN function is either acquired only in the avian lineage or lost in the mammalian lineage.
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*F, forward; R, reverse.
Figures

Figure 1

Northern blot analysis of \textit{cHEMGN} in the brain, heart, liver, forelimb, hindlimb, mesonephros and gonads of male and female chickens at day 7.5. 18S rRNA was used as a loading control.
**Figure 2**

qRT-PCR analysis of *cHEMGN* in early embryonic gonads of male (blue line) and female (pink line) chickens at days 5.5, 6.5, 7.5, 8.5, 10, 15, and 20. The *cHEMGN* expression level in female gonads at day 5.5 was arbitrarily set at 1.0, and relative expression levels are shown. Data are presented as mean ± SEM; n ≥ 3.
Figure 3

RT-PCR analysis of *cHEMGN, AMH*, and *SOX9* in early embryonic gonads of male chickens at days 5.5, 6.5, 7.5, and 8.5. *GAPDH* was used as a loading control.
20

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days
Figure 4

WISH analysis of \textit{cHEMGN} in male and female chicken embryos at day 7.5. Scale bars are 300 µm.
Figure 5

*In situ* hybridization analysis of *cHEMGN* in gonads of male and female chickens at day 6.5 (a), 7.5 (b), and 8.5 (c). A sense probe was used as a negative control for hybridization. Scale bars are 100 µm.
In *situ* hybridization analysis of *SOX9* (a) and *CVH* (b) in gonads of male chickens at day 8.5. A sense probe was used as a negative control for hybridization. Scale bars are 100 µm.
Figure 7

Dual-label *in situ* hybridization of *cHEMGN*, *SOX9* and *CVH* in gonads of male chickens at day 8.5. Scale bars are 100 µm.
Figure 8

IHC of cHEMGN in gonads of male chickens at day 8.5. Scale bars are 100 μm. The area indicated by the box is shown at a higher magnification.
Figure 9

Northern blot analysis of \textit{cHEMGN} in the spleen, BM, and blood of male and female chicken embryos at day 8.5. 18S rRNA was used as a loading control.
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32
Figure 10

qRT-PCR analysis of cHEMGN in the spleen, BM, and blood of male (black bars) and female (white bars) chickens at day 8.5. The cHEMGN expression level in female gonads was arbitrarily set at 1.0, and relative expression levels are shown. Data are presented as mean ± SEM; n ≥ 3.
Figure 11

Western blot analysis of cHEMGN in the spleen, BM, and blood of male and female chickens at day 8.5.
**Figure 12**

Immunohistochemistry of cHEMGN in blood cells of the chicken at day 8.5. Scale bar is 10 μm.
Chapter II

Functional analysis of cHEMGN in early testis development by gain-of-function

Abstract

cHEMGN was expressed in the nuclei of (pre-)Sertoli cells of early embryonic male gonads during gonadal differentiation, and its expression level increased in the gonads of aromatase inhibitor-induced masculinized ZW embryos, illustrating that cHEMGN is involved in testis differentiation. Therefore, I examined the function of cHEMGN by infecting embryos with a retrovirus that carried cHEMGN. ZW embryos overexpressing cHEMGN showed masculinized gonads with a male-like morphology, characterized by a dense medulla and a male-like (interior) distribution of germ cells. The expression levels of male markers, SOX9 and DMRT1, increased in the gonads of ZW embryos overexpressing cHEMGN compared to controls. By contrast, the expression levels of female markers, CYP19A1 (cytochrome P450, family 19, subfamily A, polypeptide 1; also known as aromatase) and FOXL2 (forkhead box L2), decreased in the gonads of ZW embryos. The expression of SOX9 protein was also induced in gonads compared to controls. These findings indicate that cHEMGN functions as a transcription factor upstream of SOX9 in (pre-)Sertoli cells after sex determination.
**Introduction**

In Chapter I, I showed the chicken-specific expression of *cHEMGN* in the nuclei of (pre-)Sertoli cells in early embryonic gonads, indicating that *cHEMGN* functions in early testis differentiation as a transcription factor. However, there was the possibility that *cHEMGN* does not function in testis differentiation because the mammalian hemogen gene has no role in testis differentiation in humans and mice. Masculinized ZW embryos can be produced by treatment with an aromatase inhibitor, fadrozole (Elbrecht and Smith, 1992; Abinawanto et al., 1996, 1998). In our previous study, *cHEMGN* expression in masculinized ZW embryos was examined to correlate changes in its level with testis differentiation (Aduma, 2011). Paraffin cross-sections from the left gonad of a masculinized ZW embryo at day 10.5 exhibited a male-like morphology with a dense medulla and a reduced cortex, although the medulla was slightly fragmented. *CYP19A1* expression was detected in ZW female embryos but not in ZZ male or masculinized ZW embryos by *in situ* hybridization. *SOX9* and *cHEMGN* expression was observed in the gonads of ZZ male and masculinized ZW embryos. qRT-PCR analysis of *cHEMGN* expression in the gonads of ZW female, ZZ male, and masculinized ZW embryos revealed an increase in its level in masculinized ZW embryos at day 8.5. Although these findings indicate the involvement of *cHEMGN* in testis differentiation, further studies are necessary to expand the role of *cHEMGN* in early gonadal differentiation in the chicken.

Mouse and medaka transgenic animals are commonly used to determine the functions of genes that are thought to be involved in sex determination. In these studies, transgenic gonads overexpressing or repressing a target gene are histologically and molecularly analyzed. Functional genomics in the avian model, however, has lagged behind that in the mouse and
medaka, and the production of transgenic birds is technically difficult (Mozdziak and Petitte, 2004) due to the features of avian reproductive physiology. During fertilization in the chicken, it is difficult to detect pronuclei in the large yolky oocyte. On the germinal disc, approximately 30 male pronuclei are present, and the ovum is surrounded by a large volume of albumen, a shell membrane and an egg shell. These features make the production of transgenic birds difficult. To circumvent these technical difficulties, several studies have attempted to isolate and genetically modify primordial germ cells (Naito et al., 1998, 1999; Mozdziak et al., 2005; Chang et al., 1997; Park and Han, 2000; Park et al., 2003a, b; Hong et al., 1998; van de Lavoir et al., 2006). However, the transmission rate of the germ line is quite low, requiring further breeding of chimeric birds.

A more direct approach involves the direct delivery of viral vectors into early blastoderms. The RCAS (replication-competent avian leucosis sarcoma virus long terminal repeat with splice acceptor) virus has been widely used to misexpress genes in specific embryonic structures such as the limb (Logan and Tabin, 1998). The advantage of using RCAS is that it is replication competent, spreading horizontally to neighboring cells after infection, as well as vertically to daughter cells, resulting in global transgene expression in a high percentage of directly infected embryos (higher than 80%), without the need to breed chimeric birds. In fact, Smith and colleagues have shown overexpression of HINTW [a histidine triad nucleotide binding protein, -W linked; also known as WPKCI and ASW (Smith et al., 2009b)] and CYP19A1 (Lambeth et al., 2013) by using the retroviral vector RCAS in ovo. Furthermore, CYP19A1 overexpression in the male chicken induces gonadal sex reversal (Lambeth et al., 2013).
I produced transgenic embryos overexpressing cHEMGN by infecting chicken embryos with RCAS.A (RCAS, subgroup A env gene) that carried the cHEMGN gene (RCAS.A.cHEMGN). ZW gonads overexpressing cHEMGN showed a male-like morphology with a male-like distribution of primordial germ cells. The expression levels of SOX9 and DMRT1 increased, while those of CYP19A1 and FOXL2 decreased. These results illustrate that cHEMGN functions as a transcription factor in the DMRT1 and SOX9 molecular cascade in (pre-)Sertoli cells.
Materials and Methods

Animals, genomic DNA extraction and sexing

Chicken strain, identification of developmental stages, genomic DNA extraction and sexing by PCR are described in Chapter I.

Preparation of RCAS.A.cHEMGN.

The cHEMGN transgenic chicken embryos were produced by infection with RCAS.A. RCAS.A proviral DNA was the kind gift of Dr. Hughes (Hughes et al., 1987). The specific primers with ClaI site were designed to amplify the cHEMGN ORF (Table 2). The products were subcloned using the pGEM T-easy Vector System. After confirmation of the insert by sequencing, the pGEM T-easy-cHEMGN plasmid DNA was subsequently digested with ClaI, and cloned into ClaI-digested RCAS.A.

Endotoxin-free proviral DNA was prepared using the PureYield™ Plasmid Miniprep System (Promega). The DNA was transfected into DF1 cells using Lipofectoamine 2000 (Invitrogen) according to the manufacturer’s protocol. Cells were then transferred to 10 dishes (78.5 cm²) and cultured in DMEM+10% FBS to superconfluence. Active virus was concentrated from the pooled medium. Briefly, 100 ml of medium was centrifuged at 6,000 g overnight. The supernatant was then carefully poured off, leaving a small pellet in 200 µl of medium. The resuspended viral solution was aliquoted and stored at -80°C until use. Viral titer was determined as described previously (Smith et al., 2009c).
**Injection of embryos with RCAS.A.cHEMGN.**

Concentrated virus containing 0.025% Fast Green tracking dye was injected into the subgerminal cavity of day 0 (stage X) blastderms. Approximately 3 µl was injected per embryo and a total of 173 eggs were injected. A negative control comprised embryos that were not infected with virus. Eggs were sealed and incubated until day 8–9.

**RNA extraction and qRT-PCR**

RNA extraction and qRT-PCR were performed as described in Chapter I. Primers for *DMRT1, SOX9, CYP19A1, FOXL2* and *ACTIN-B* are shown in Table 2.

**IHC**

IHC was carried out according to the method described in Chapter I. Rabbit anti-SOX9 and rat anti-CVH antibodies were kindly gifted from Dr. Lovell-Badge and Dr. Hattori, respectively. The primary antibodies, rabbit anti-cHEMGN, rabbit anti-SOX9, and rat anti-CVH, were used at dilutions of 1:200, 1:500, and 1:300, respectively. The sections were washed in PBS for 5 min twice and treated with the secondary antibody (AlexFluor 488-labeled goat anti-rabbit IgG, 1:400, for cHEMGN and SOX9; AlexFluor 488-labeled donkey anti-rat IgG, Invitrogen, 1:250, for CVH) in PBS overnight at 4°C.
Results

**Histological analysis of ZW gonads overexpressing cHEMGN**

The number of experimental embryos used is shown in Table 3. Uninfected embryos were used as negative controls.

The gonads of wild-type ZW embryos showed asymmetric development. The right gonad regressed, while the left gonad showed hypertrophic development of the cortex and the presence of a cavity in the medulla (Fig. 13). However, the gonads of ZW embryos overexpressing cHEMGN showed bilateral development characteristic of the male morphology (Fig. 13). I performed histological analysis of gonad cross-sections from male, female, and ZW embryos overexpressing cHEMGN at day 8.5 by hematoxylin and eosin staining (Fig. 14). ZW gonads overexpressing cHEMGN showed a male-like morphology characterized by a dense medulla with seminiferous cords (Fig. 14).

**Molecular analysis of ZW gonads overexpressing cHEMGN**

The expression of male and female markers in the gonads of embryos overexpressing cHEMGN was examined. Key markers of testicular differentiation are DMRT1 and SOX9, while markers of ovarian development are FOXL2 and CYP19A1. At the mRNA level, DMRT1 and SOX9 expression increased, while FOXL2 and CYP19A1 expression decreased in ZW gonads overexpressing cHEMGN compared to controls (Fig. 15).

The expression of cHEMGN and SOX9 proteins was examined by IHC in paraffin cross-sections prepared from control male, control female, and ZW gonads overexpressing cHEMGN (Fig. 16). In control male embryos at day 8.5, cHEMGN and SOX9 proteins were
expressed normally in the nuclei of (pre-)Sertoli cells, but control female gonads lacked the expression of these proteins (Fig. 16). The cHEMGN protein was high throughout the gonads of ZW embryos overexpressing cHEMGN, whereas SOX9 protein was observed only in the medullary region of gonads (Fig. 16).

The distribution of germ cells was examined by IHC using an anti-CVH antibody on paraffin cross-sections prepared from control male, control female, and ZW gonads overexpressing cHEMGN (Fig. 16). CVH-positive germ cells were distributed throughout the interior of control male gonads, but control female gonads exhibited a cortical distribution of germ cells. ZW gonads overexpressing cHEMGN showed a male-like (i.e., interior) distribution of germ cells (Fig. 16).
Discussion

DMRT1 expression commences on day 4.5 (Raymond et al., 1999, Smith et al., 1999b) prior to cHEMGN expression (Chapter I), suggesting that cHEMGN functions downstream of DMRT1. DMRT1 expression is maintained in Sertoli cells after sex determination; however, expression in germ cells is activated during gonadal development (Smith et al., 2003). These results indicate that DMRT1 has at least two functions in (pre-)Sertoli cells of embryonic gonads, namely, the activation of testis differentiation and testis development after sex determination. They also illustrate that expression of each gene was independently regulated because the masculinization by overexpression of cHEMGN induced DMRT1 expression in gonads of ZW embryos.

A high expression level of cHEMGN protein was found in ZW gonads overexpressing cHEMGN. However, the SOX9 protein was expressed only in the medulla of ZW gonads overexpressing cHEMGN at day 8.5. These results indicate that the target of cHEMGN is SOX9 in (pre-)Sertoli cells and that the target in Sertoli cells and hematopoietic cells is different.

ZW gonads overexpressing cHEMGN showed a male-like morphology and germ cell distribution, with high expression of male markers but low expression of female markers (Figs. 15, 16), illustrating that the cHEMGN-overexpressing ZW gonads are masculinized. Based on the results presented in this chapter, I conclude that cHEMGN is specifically involved in the early events of chicken sex determination and that cHEMGN functions as a transcription factor in the DMRT1 and SOX9 molecular cascade in (pre-)Sertoli cells.
<table>
<thead>
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<th>Experiment</th>
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<th>Direction of primer</th>
<th>Primer sequence</th>
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<td></td>
<td></td>
<td>R</td>
<td>5'-ATC GAT TTA AAA TAG GCT TGA TGA-3'</td>
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<tr>
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<td>DMRT1</td>
<td>F</td>
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<td>SOX9</td>
<td>F</td>
<td>5'-CGG ATC CAG CAA GAA CAA A-3'</td>
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<td></td>
<td></td>
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<td></td>
<td>ACTIN-B</td>
<td>F</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>5'-TCT CCT GCT CGA AAT CCA GT-3'</td>
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<sup>a</sup>CDS, full-length coding sequence

<sup>b</sup>F, forward; R, reverse.
Table 3. The number of embryos using experiments.

<table>
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<tr>
<th>Embryos treated with RCAS.A.cHEMGN</th>
<th>Survived embryos until day 8.5</th>
<th>Genotype of embryos treated with RCAS.A.cHEMGN (63 embryos)</th>
<th>qRT-PCR (^a)</th>
<th>HE-staining and IHC (^b)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>ZZ</td>
<td>ZW</td>
<td>N.D.</td>
</tr>
<tr>
<td>Number of embryos</td>
<td>173</td>
<td>63</td>
<td>22</td>
<td>33</td>
</tr>
</tbody>
</table>

\(^a\) Embryos treated with RCAS.A.cHEMGN were used. Mixed mRNAs from gonads of all embryos were used as template.

\(^b\) ZW embryos treated with RCAS.A.cHEMGN were used.

\(^c\) ZW embryos showing clear expression of SOX9 and male-like distribution of germ cells demonstrated by CVH expression.
Figures

Figure 13

Urogenital gonads from male, female and ZW embryos overexpressing *cHEMGN* (RCAS.A.cHEMGN ZW). The ZW gonads overexpressing *cHEMGN* showed bilateral development. Dashed lines encircle gonads from male, female and ZW embryos overexpressing *cHEMGN*. Scale bar is 300 µm.
Male  Female  RCAS.A.cHEMGN (ZW)
Figure 14

Hematoxylin and eosin staining of cross-sections from left and right gonads from male, female, and ZW embryos overexpressing cHEMGN (RCAS.A.cHEMGN ZW). The ZW gonad overexpressing cHEMGN showed a male-like morphology characterized by a dense medulla with seminiferous cords. Scale bar is 100 μm.
Figure 15

qRT-PCR analysis of *DMRT1*, *SOX9*, *CYP19A1*, and *FOXL2* in early embryonic gonads of the control female (black bars), ZW embryos overexpressing *cHEMGN* (RCAS.A.cHEMGN ZW, dark gray bars), control males (light gray bars) and ZZ embryos overexpressing *cHEMGN* (RCAS.A.cHEMGN ZZ, white bars) at day 8.5. Data presented are mean ± SEM; *P < 0.001; n ≥ 3.
The diagram illustrates the expression levels of four genes: DMRT1, SOX9, CYP19A1, and FOXL2, in different conditions:

- **Female (ZW)**
- **RCAS.A.cHEMGN (ZW)**
- **Male (ZZ)**
- **RCAS.A.cHEMGN (ZZ)**

The expression levels are quantified on a logarithmic scale, with the following ranges:

- **DMRT1**: 1 to 6
- **SOX9**: 1 to 20
- **CYP19A1**: 1 to 800
- **FOXL2**: 1 to 100

The asterisk (*) indicates a statistically significant difference between the conditions.
**Figure 16**

Immunohistochemistry of cHEMGN, SOX9, and CVH in cross-sections from gonads of male, female, and ZW embryos overexpressing *cHEMGN* (RCAS.A.cHEMGN ZW) at day 8.5. Scale bar is 100 µm. Male and female embryos uninfected with viruses were used as negative controls.
Male | Female | RCAS.A.cHEMGN (ZW)
---|---|---
cHEMGN
SOX9
CVH
Chapter III

Functional analysis of \textit{CHEMGN} in early testis development by loss-of-function

Abstract

In Chapter II, I proposed that \textit{CHEMGN} functions as a transcription factor upstream of \textit{SOX9} in (pre-)Sertoli cells after sex determination. However, the role of \textit{CHEMGN} in testis differentiation is still unclear. To address this question, I performed a functional analysis by infecting embryos with a retrovirus that carried \textit{CHEMGN}-specific microRNAs (miRNAs). These miRNAs effectively repressed exogenous \textit{CHEMGN-eGFP} expression \textit{in vitro}. In these 	extit{ovo}, the \textit{CHEMGN} expression level in ZZ gonads infected with RCASBP.A.eGFP-miR1-2 decreased from one-third to two-thirds that of the control males; however, the infected ZZ gonads showed normal morphology. These results indicate that RCASBP.A.eGFP-miR1-2-mediated knockdown was not sufficient for repression of testis differentiation. Therefore, a virus was constructed to increase the efficiency of target gene silencing for further analysis.
Introduction

RNA interference (RNAi) provides an effective tool to silence gene expression, and it is widely used to analyze the functions of different genes. *DMRT1*, the most likely candidate for sex determination in birds, was analyzed by RNAi knockdown using RCASBP.B that carried miRNA and short hairpin RNA (Smith *et al.*, 2009a). A decrease in the level of DMRT1 protein *in ovo* leads to partial sex reversal of the embryonic gonads characterized by feminization in genetically male (ZZ) embryos. Germ cells also show a female pattern of distribution in feminized male gonads. These results indicate that *DMRT1* is required for testis determination in the chicken, and also illustrate that RNAi is a valuable approach to determine the function of genes involved in sex determination.

Das and colleagues have designed an effective RNAi vector for optimal gene silencing in chicken cells (Das *et al.*, 2006). A chicken U6 promoter to express RNAs modeled on miRNA30 was used to construct the vector, which is embedded within chicken miRNA operon sequences to ensure optimal Drosha and Dicer processing of transcripts. The chicken U6 promoter works significantly better than promoters of mammalian origin and achieves up to 90% silencing of target genes in combination with a miRNA operon expression cassette (Das *et al.*, 2006). Two types of miRNAs can be inserted into the expression cassette, enabling dual gene silencing from a single vector. The vector is also modified to insert the retrovirus vector RCAS. The use of this RNAi system paves the way for large-scale genetic screening in chicken embryos.

I demonstrated that the gonads of ZW embryos overexpressing *cHEMGN* showed sex reversal in Chapter II. In this chapter, I performed *cHEMGN* knockdown by using the RNAi
vector designed by Das et al. (2006) to show that cHEMGN is necessary and sufficient for testis differentiation. The RNAi vectors constructed for cHEMGN knockdown were highly efficient in vitro. In contrast, sufficient silencing could not be observed in ovo, indicating that the RNAi vector requires further improvement.
Materials and Methods

Animals, genomic DNA extraction and sexing

Chicken strain, genomic DNA extraction and sexing are described in Chapter I.

Preparation of RNAi vectors

The miRNAs to target cHEMGN were designed by BLOCK-iT RNAi Designer (Invitrogen) and scramble miRNAs were designed by siRNA Wizard v3.1 (Invivogen). The miRNA sequences were molecularly cloned by PCR. Takara Ex Taq kit was used in PCR amplification, and four primers were used as template according to a previous report (Das et al., 2006). The primers used as templates are shown in Table 4. Gene specific primers for cHEMGN suitable for 1st and 2nd cloning site were used together with template primers Wand Y for 1st cloning site, and Z and AA for 2nd cloning site, respectively. The PCR conditions were heat denaturation of 94°C for 2 min, then 35 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec, and finally 72°C for 5 min. The PCR product was digested with Nhe I and Mlu I for 1st site of pRFPRNAi.C and Mlu I and Sph I for 2nd site of pRFPRNAi.C, and cloned into Nhe I and Mlu I -digested and Mlu I and Sph I -digested pRFPRNAi.C, respectively.

pRFPRNAi.C containing miRNAs to target cHEMGN or scramble miRNA in 1st and 2nd site was digested with Not I and Cla I, and cloned into Not I and Cla I-digested RCASRNAi.A.eGFP or RCASRNAi.B. Each vector was named as follows: RCASRNAi.A.eGFP.miR1-2, RCASRNAi.A.eGFP.miR1s-2s, RCASRNAi.B.miR1-2, and RCASRNAi.B.miR1s-2s. The method of virus preparation is shown in Chapter II.
**RNAi knockdown *in vitro* and *in ovo***

DF1 cells infected with RCASRNAi.B.miR1-2 were grown in DMEM containing 10% FBS, 0.35% glucose and L-glutamine, and plated at $2 \times 10^4$ cells on 24-well plates 24 h before infection of RCAS.A.cHEMGN-eGFP.

DF1 cells previously infected with RCASRNAi.B.miR1-2 or RCASRNAi.B.miR1s-2s and DF1 cells uninfected with any viruses were infected with RCAS.A.cHEMGN-eGFP diluted to $10^2$. After 48 hours from infection, RNAs and proteins were isolated from these cells to examine the expression of *cHEMGN-eGFP* mRNA and protein by RT-PCR, qRT-PCR and western blot analysis.

The methods for injection of embryos, RNA extraction, RT-PCR, quantitative RT-PCR and western blot analysis are described in Chapter I and Chapter II.
Results

RNAi knockdown in vitro

Two different miRNAs were simultaneously used to knockdown cHEMGN transcripts, and these miRNAs were delivered by an avian retroviral vector RCASRNAi.B (Das et al., 2006). RCASRNAi.B carried the two miRNAs with a chicken U6 RNA polymerase promoter. To confirm gene knockdown with these two miRNAs, a double infection was performed using RCASRNAi.B.miR1-2 or RCASRNAi.B.miR1s-2s, and RCAS.A.cHEMGN-eGFP that overexpressed a cHEMGN-eGFP fusion protein. At 48 h after infection of RCAS.A.cHEMGN-eGFP, eGFP fluorescence was detected in DF1 cells uninfected with the virus or infected with RCASRNAi.B.miR1s-2s but not in those infected with RCASRNAi.B.miR1-2 (Fig. 17). The expression level of exogenous cHEMGN-eGFP was examined in each group of DF1 cells by RT-PCR and qRT-PCR using primers that amplified cHEMGN sequences. cHEMGN-eGFP expression significantly increased in DF1 cells uninfected with the miRNA virus and those infected with RCASRNAi.B.miR1s-2s (Figs. 18, 19). Results from western blot analysis were similar to those from mRNA expression studies (Fig. 20). Only in DF1 cells infected with RCASRNAi.B.miR1-2, cHEMGN-eGFP mRNA and protein were expressed at low levels after 48 h of RCAS.A.cHEMGN-eGFP infection (Figs. 17–20).

RNAi knockdown in ovo

The number of experimental embryos used is shown in Table 5. All embryos were analyzed for knockdown of the target gene at day 8.5. Two males (nos. 21 and 39) and one female (no.
45) showed high expression of eGFP in the gonads (Fig. 21). The gonads of these chickens showed a morphology similar to that of wild-type males or females, namely, the male gonads underwent bilateral development and the female gonad underwent asymmetric development.

*cHEMGN* expression was analyzed in male gonads and the female gonad (nos. 21, 39 and 45) by qRT-PCR using an uninfected ZZ embryo as a negative control. The expression level of *cHEMGN* in male gonads decreased to one-third and two-thirds that of the control males, respectively (Fig. 22).
Discussion

The miRNA vectors designed for cHEMGN knockdown showed efficient gene silencing in vitro (Figs. 17–20), indicating that this RNAi system worked effectively at least in vitro. By contrast, efficient knockdown could not be achieved in ovo. Male no. 21 carrying cHEMGN miRNA showed a statistically significant decrease in expression in the gonads compared to the control male (Fig. 21). However, the gonads of male no. 21 showed normal morphology as evidenced by bilateral development, indicating that the level of knockdown obtained in this embryo was not sufficient.

In a previous study from our laboratory that used masculinized ZW embryos produced by aromatase inhibitor treatment, cHEMGN expression increased to a level that was approximately one half that of the control males (Aduma, 2011). These results indicate that cHEMGN expression at one half of the control level could induce testis differentiation. Furthermore, cHEMGN expression in females was less than one-tenth that of males (Chapter I). Therefore, the level of cHEMGN expression in knockdown embryos must be close to that of females to see the effects on testis differentiation by cHEMGN knockdown.

To further improve the knockdown efficiency, other miRNA sequences should be tested in future studies. In general, the knockdown efficiency depends on the sequence and location of the target RNA. Furthermore, a specific miRNA might be more effective if it is directly transcribed via a long terminal repeat (LTR) promoter, namely, the endogenous promoter of the RCAS virus, since the transcriptional activity of the LTR promoter is relatively high. The construction of a new vector to efficiently knockdown the target gene is needed for further analysis.
### Table 4. Primer list.

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<tr>
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<td>Z</td>
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<td>F</td>
<td></td>
<td>5'- GGC GCC GCT TAC CGT CCT GCC GAC -3'</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td></td>
<td>5'- GCC GCC GCG GTA CCA TAA AGT G -3'</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>cHEMN</td>
<td></td>
<td>5'- GCT GAT ATG TCG TCA AGG AGA -3'</td>
</tr>
<tr>
<td></td>
<td>GAPDH</td>
<td></td>
<td>5'- GCC GTC CTC TCT GGC AAG -3'</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>cHEMN</td>
<td></td>
<td>5'- GCT GAT ATG TCG TCA AGG AGA -3'</td>
</tr>
<tr>
<td></td>
<td>ACTNB</td>
<td></td>
<td>5'- GCC AAG TAT TGC AGG AAC -3'</td>
</tr>
</tbody>
</table>

*F, forward; R, reverse.*
Table 5. The number of embryos using experiments.

<table>
<thead>
<tr>
<th>Embryos treated with RCASBP.A.eGFP.miR1-2</th>
<th>Survived embryos until day 8.5</th>
<th>eGFP positive embryo treated with RCASBP.A.eGFP.miR1-2 (41 embryos)</th>
<th>Genotype of embryos treated with RCASBP.A.eGFP.miR1-2 (16 embryos)</th>
<th>qRT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of embryos</td>
<td>109</td>
<td>41</td>
<td>16</td>
<td>ZZ</td>
</tr>
<tr>
<td></td>
<td></td>
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<td></td>
<td>ZW</td>
</tr>
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<td></td>
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<td></td>
<td></td>
<td>ZZ</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ZW</td>
</tr>
</tbody>
</table>

5 11 2 1
Figures

Figure 17

eGFP expression in uninfected, RCASRNAi.B.miR1-2-infected, and RCASRNAi.B.miR1s-2s-infected DF1 cells before and after infection of RCAS.A.cHEMGN-eGFP. Scale bars are 100 µm.
<table>
<thead>
<tr>
<th></th>
<th>Bright field</th>
<th>eGFP</th>
<th>Bright field</th>
<th>eGFP</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DF1</strong></td>
<td><img src="image1" alt="Bright field" /></td>
<td><img src="image2" alt="eGFP" /></td>
<td><img src="image3" alt="Bright field" /></td>
<td><img src="image4" alt="eGFP" /></td>
</tr>
<tr>
<td><strong>DF1</strong></td>
<td><img src="image5" alt="Bright field" /></td>
<td><img src="image6" alt="eGFP" /></td>
<td><img src="image7" alt="Bright field" /></td>
<td><img src="image8" alt="eGFP" /></td>
</tr>
<tr>
<td><strong>DF1</strong></td>
<td><img src="image9" alt="Bright field" /></td>
<td><img src="image10" alt="eGFP" /></td>
<td><img src="image11" alt="Bright field" /></td>
<td><img src="image12" alt="eGFP" /></td>
</tr>
<tr>
<td><strong>RCASRNAi.B.</strong></td>
<td><img src="image13" alt="Bright field" /></td>
<td><img src="image14" alt="eGFP" /></td>
<td><img src="image15" alt="Bright field" /></td>
<td><img src="image16" alt="eGFP" /></td>
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<tr>
<td><strong>miR1-2</strong></td>
<td><img src="image17" alt="Bright field" /></td>
<td><img src="image18" alt="eGFP" /></td>
<td><img src="image19" alt="Bright field" /></td>
<td><img src="image20" alt="eGFP" /></td>
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<tr>
<td><strong>DF1</strong></td>
<td><img src="image21" alt="Bright field" /></td>
<td><img src="image22" alt="eGFP" /></td>
<td><img src="image23" alt="Bright field" /></td>
<td><img src="image24" alt="eGFP" /></td>
</tr>
<tr>
<td><strong>RCASRNAi.B.</strong></td>
<td><img src="image25" alt="Bright field" /></td>
<td><img src="image26" alt="eGFP" /></td>
<td><img src="image27" alt="Bright field" /></td>
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<td><strong>miR1s-2s</strong></td>
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<td><img src="image30" alt="eGFP" /></td>
<td><img src="image31" alt="Bright field" /></td>
<td><img src="image32" alt="eGFP" /></td>
</tr>
</tbody>
</table>

*After infection of RCAS.A.cHEMGN-eGFP*
Figure 18

RT-PCR analysis of $cHEMGN\text{-}eGFP$ expression in uninfected (I), RCASRNAi.B.miR1-2-infected (II), and RCASRNAi.B.miR1s-2s-infected (III) DF1 cells before (-) and after (+) infection with RCAS.A.cHEMGN-eGFP. $GAPDH$ was used as a positive control.
qRT-PCR analysis of \textit{cHEMGN-eGFP} expression in uninfected (I), RCASRNAi.B.miR1-2-infected (II), and RCASRNAi.B.miR1s-2s-infected (III) DF1 cells before (-) and after (+) infection with RCAS.A.cHEMGN-eGFP. The expression level of \textit{cHEMGN-eGFP} before infection of RCASBP.A.cHEMGN-eGFP was arbitrarily set at 1.0. Data presented are mean ± SEM; $*P < 0.001$; $n \geq 3$. 

\textbf{Figure 19}
Figure 20

Western blot analysis of cHEMGN-eGFP in uninfected (I), RCASRNAi.B.miR1-2-infected (II), and RCASRNAi.B.miR1s-2s-infected (III) DF1 cells before (-) and after (+) infection with RCAS.A.cHEMGN-eGFP.
cHEMGN-eGFP

I  II  III
-  +  -  +  -  +
**Figure 21**

Paired urogenital system and gonads from ZZ embryos infected with RCASBP.A.eGFP-miR1-2.

Dashed lines encircle gonads. Scale bars are 300 μm.
Figure 22

qRT-PCR of \textit{cHEMGN} expression in early embryonic gonads of a control ZZ embryo (black bar), a ZZ embryo carrying RCASBP.A.eGFP-miR1-2 (no. 21, dark gray bar), a ZZ embryo carrying RCASBP.A.eGFP-miR1-2 (no. 39, light gray bars), and a ZW embryo carrying RCASBP.A.eGFP-miR1-2 (no. 45, white bars) at day 8.5. Data presented are mean ± SEM; \(*P < 0.001; n \geq 3.*\)
**Expression level of cHEMGN**

- **Male (ZZ)**
- **RCASBP.A.eGFP-miR1-2: No. 21 (ZZ)**
- **RCASBP.A.eGFP-miR1-2: No. 39 (ZZ)**
- **RCASBP.A.eGFP-miR1-2: No. 45 (ZW)**
General Discussion

In this dissertation, I demonstrated \textit{cHEMGN} expression not only in hematopoietic tissues but also in gonads during differentiation in the chicken. \textit{cHEMGN} was expressed in the nuclei of (pre-)Sertoli cells at an extremely early stage of sex differentiation (Chapter I). ZW gonads overexpressing \textit{cHEMGN} showed masculinization characterized by a male-like morphology, a male-like distribution of germ cells, an increase in the expression levels of male markers (\textit{DMRT1} and \textit{SOX9}), and a decrease in the expression levels of female markers (\textit{CYP19A1} and \textit{FOXL2}) (Chapter II). These results demonstrate that \textit{cHEMGN} specifically functions in the early phase of testis differentiation in the chicken as a transcription factor in the \textit{DMRT1} and \textit{SOX9} molecular cascade in (pre-)Sertoli cells (Fig. 23). These findings also demonstrate the existence of an unknown bird-specific mechanism(s) of sex determination, in which \textit{cHEMGN} is involved. The function of mammalian hemogen is confined to hematopoietic tissues, indicating that its role in sex determination is either acquired only in the avian lineage or lost in the mammalian lineage. This study can provide new insights into the evolutionary process of vertebrate sex determination.

In the chicken, there are few reports that analyze the functions of genes involved in sex determination owing to the difficulty of producing transgenic animals. However, overexpression and/or repression of genes by any transgenic method play an important role in the determination of target gene function. In this dissertation, I overexpressed and repressed \textit{cHEMGN} using the RCAS vector in Chapters II and III, respectively. The gonads of ZW embryos overexpressing \textit{cHEMGN} showed masculinization. This is the first report to show sex reversal in gonads overexpressing a gene involved in sex determination. By contrast, repression of \textit{cHEMGN} using
the RCAS vector carrying *cHEMGN*-specific miRNAs failed to achieve the sex reversal phenotype. The gonads of ZZ embryos carrying miRNAs that target *cHEMGN* showed normal testis morphology characterized by bilateral development. I therefore conclude that silencing of *cHEMGN* expression was efficient *in vitro* but not *in ovo*. To improve knockdown efficiency, construction of a new vector is needed for further analysis.

I propose three subject areas that should be investigated in future studies, as follows: i) target genes that are regulated by *cHEMGN* in Sertoli cells, ii) gonad-specific enhancer regions of *cHEMGN* expression, and iii) transcription factors that mediate the gonadal expression of *cHEMGN*. In my future studies, I hope to better understand the chicken-specific mechanism of sex determination by elucidating the molecular cascade involving *cHEMGN*. 
Figure

Figure 23

Chicken-specific mechanism of sex determination involving $cHEMGN$ in (pre-)Sertoli cells.
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