Gene Expression Profiling Reveals New Potential Players of Gonad Differentiation in the Chicken Embryo

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Abstract

Background: In birds as in mammals, a genetic switch determines whether the undifferentiated gonad develops into an ovary or a testis. However, understanding of the molecular pathway(s) involved in gonad differentiation is still incomplete.

Methodology/Principal Findings: With the aim of improving characterization of the molecular pathway(s) involved in gonad differentiation in the chicken embryo, we developed a large scale real time reverse transcription polymerase chain reaction approach on 110 selected genes for evaluation of their expression profiles during chicken gonad differentiation between days 5.5 and 19 of incubation. Hierarchical clustering analysis of the resulting datasets discriminated gene clusters expressed preferentially in the ovary or the testis, and/or at early or later periods of embryonic gonad development. Fitting a linear model and testing the comparisons of interest allowed the identification of new potential actors of gonad differentiation, such as Z-linked ADAMTS12, LOC427192 (corresponding to NIM1 protein) and CFC1, that are upregulated in the developing testis, and BMP3 and Z-linked ADAMTS11, that are preferentially expressed in the developing ovary. Interestingly, the expression patterns of several members of the transforming growth factor β family were sexually dimorphic, with inhibin subunits upregulated in the testis, and bone morphogenetic protein subfamily members including BMP2, BMP3, BMP4 and BMP7, upregulated in the ovary. This study also highlighted several genes displaying asymmetric expression profiles such as GREM1 and BMP3 that are potentially involved in different aspects of gonad left-right asymmetry.

Conclusion/Significance: This study supports the overall conservation of vertebrate sex differentiation pathways but also reveals some particular feature of gene expression patterns during gonad development in the chicken. In particular, our study revealed new candidate genes which may be potential actors of chicken gonad differentiation and provides evidence of the preferential expression of BMPs in the developing ovary and Inhibin/Activin subunits in the developing testis.

Introduction

Sex determination in birds is controlled by a genetic ZZ/ZW system in which the female is heterogametic. The avian sex determining mechanism has not been completely elucidated to date, and no homolog of the mammalian sex determining gene, SRY, has been found in birds. However, a recent study suggested that the DMRT1 gene (Doublesex and Mab-3 Related Transcription factor 1), belonging to the zinc finger-like DNA-binding motif (DM) transcription factor family and located on the Z chromosome, acts as a male sex determining factor [1]. Although DMRT1 is essential for testis differentiation, another Z-linked gene may operate upstream of DMRT1 in the avian male determining pathway. It is also possible that a still unidentified female W-linked determinant may be required for ovary differentiation. Nevertheless, overexpression of the W-linked candidate sex determinant HINTW (also known as ASW or WPKCl) in male embryos does not induce ovary differentiation [2]. Gonad development in birds is in many aspects similar to that of mammals. As in mammals, the avian primordial gonads develop from the intermediate mesoderm, on the ventromedial surface of the embryonic kidney (mesonephros). Germ cells colonize the genital ridge by day 3.5 and the formation of primitive sex cords occurs by day 5 [3]. The gonads of ZZ and ZW embryos remain indistinguishable until day 6.5–7 (stage 30 of the Hamburger and Hamilton classification [4]), when the first histological signs of sex differentiation become visible. This process includes the thinning of the cortex and development of testicular cords enclosing germ cells in ZZ gonads. In the ZW left gonad, somatic and germ cells proliferate in the cortex, which thickens considerably, while the cords of the medulla become vacuolated, forming lacunae. The germ cells of the left ZW gonad enter in meiosis asynchronously from day 15 [5,6,7]. The meiotic entry of female germ cells is then completed before hatching. Comparative studies have demonstrated that genes involved in the sex differentiation pathway are conserved in vertebrates; nevertheless their roles, their regulation and their expression time-windows during this process can differ. For instance, similar to mammals, AMH and SOX9 are upregulated in the chicken testis during gonad development. However, in contrast to
mammals, *AMH* is also expressed in embryonic female gonads in chicken but at a lower level, and the onset of its expression precedes that of SOX9 [8]. Since both proteins are found in Sertoli cells, SOX9 may be involved in maintaining *AMH* expression [9]. In addition, the chicken model presents two particular features. The first, in contrast to mammals, gonad differentiation is sensitive to exogenous hormonal manipulation. Indeed, inhibition of CYP19A1 causes female to male sex reversal [10], while estrogen treatment of ZZ embryos leads to feminization of left gonads and regression of right gonads [11,12]. These experiments revealed a crucial role of estrogen production during avian ovary development and the fundamental role of regulation of CYP19A1 expression. There is a lack of detailed knowledge concerning molecular mechanisms leading to the activation of SOX9 and *AMH* expression in male gonads and to *FOXL2* and *CYP19A1* expression in female gonads in birds. Furthermore, the target and downstream pathways of these genes are poorly understood. Another particular feature of chicken gonadal differentiation is its left-right (L-R) asymmetry in the female. Gonadal development in male birds results in the formation of two functional testes. In female birds, only the left gonad becomes a functional ovary whereas the right reproductive system regresses. Recent studies revealed that left sided expression of *PITX2*, *RALDH2* and *SF1* leads to only the left sided expression of estrogen receptor alpha (*ESR1*) [11,13,14]. Estrogen production by embryonic ZZ gonads thus promotes left cortical development via *ESR1* and regression of the right gonad, where the level of expression of *ESR1* is dramatically decreased. In ZZ gonads which do not produce estrogens, the asymmetry remains silent. However, the other actors of complex molecular mechanisms of gonad asymmetry and the factors involved in gonad regression remain to be established. In the light of this current knowledge our study aimed to identify new candidate genes involved in male and female gonad differentiation and in L-R gonad asymmetry in birds. We therefore developed a large-scale real-time reverse transcription polymerase chain reaction (RT-PCR) approach on 110 selected candidate genes for investigation of their expression profiles during gonad differentiation and development in the chicken embryo. Selection of the set of candidate genes was mainly based on the literature. It included transcription factors, receptors, signalling molecules and enzymes known to be involved in gonad development in other vertebrate species, and more generally in reproduction and embryogenesis. A linear model was fitted and contrasts of interest were tested. The expression profiles were also explored using a hierarchical clustering method. Our study revealed many genes that behave differently between sexes and/or stage-specific groups including potential new actors of sex gonad differentiation and establishment of L-R gonad asymmetry.

**Results**

**Overall analysis**

The raw data set is presented in an online supplement (Table S1). For each date of sampling, the relative expression of each gene was obtained in two independent pools of gonads (biological replicates). To determine the consistency of our finding, we calculated correlation coefficients (r) of biological replicates. The correlation values of biological replicates ranged from 0.87 to 0.99 (Figure S1).

Based on the efficiency and specificity of Real-time RT-PCR, 110 genes were finally selected to identify differentially expressed genes and to cluster genes into groups with similar expression profiles. To assess the biological relevance of the genes assayed, we first performed hierarchical clustering of the samples (Figure 1) that generated two large groups corresponding to male and female gonads. In each group, cluster analysis discriminated two subgroups of samples, one that contained the early samples (day 5.5 to day 7.5 for female gonads and day 5.5 to day 8.5 for male gonads), and another that contained the later samples (day 8.5 to day 19.5 for female gonads and day 9.5 to day 19.5 for male gonads). Based on these findings, for the statistical analysis we chose to discriminate two periods during development: before day 7.5 and from day 8.5 referred to as the earlier and later periods respectively. Within these groups, almost all the biological duplicates were clustered together, showing the efficiency of the method and confirming the relevance of the samples and genes assayed. Furthermore, within these classes the samples were grouped according to whether they came from a right or left gonad, suggesting that there is differential expression of several genes between the two sides.

**Figure 1. Hierarchical Clustering of the biological samples.** Hierarchical clustering was performed using complete linkage algorithm applied on the similarity matrix based on the Pearson correlation coefficient. LO = left ovary; RO = right ovary; LT = left testis; RT = right testis. doi:10.1371/journal.pone.0023959.g001
Differential analysis and gene cluster analysis

Differential analysis was performed using a linear model. Lists of differentially expressed genes for each comparison were obtained after adjustment for multiple testing [15]. This analysis showed that 80.9% of genes displayed a statistically significant difference in their expression profiles in at least one of the comparisons. In order to group genes according to the similarity of their expression patterns, we also performed hierarchical clustering of genes with samples ordered on the basis of the developmental stage (from day 5.5 to day 19.5) (Figure 2).

As shown in Figure 2, analysis of the dendrogram revealed that genes were clustered in four main groups. The first group corresponded to genes with the highest relative expression (RE) in the testis (node 1). The second group corresponded to genes with a highest RE in later samples associated with preferential RE in the ovary (node 2). The third group contained genes with the highest RE in the ovary (node 3) while the last group corresponded to genes with the highest RE in early samples (node 4). Clusters were strongly supported by the existence of significant differences in gene expression between samples in particular comparisons, indicated by colored asterisks on the right of Figure 2.

Cluster C1 (Figure 2) contained forty-one genes, the majority of them displaying a preferential RE in the testis compared to the ovary. This cluster could be divided into three subgroups: C1.1, C1.2 and C1.3. The genes from subgroup C1.1 were not characterized by any similarity in their expression profiles. However, several genes showed sexual dimorphism (BMPR1A, NOTCH1 and SLIT1), among which SLIT1 was upregulated in the testis compared to the ovary in samples from day 8.5 (“later” period) while the RE of NOTCH1 was higher in the testis samples from the earlier period. In the second subgroup, (C1.2), ten genes were significantly upregulated in the testis compared to the ovary in both time periods (PTGR, DMR53, CFC1, DMR1, FANC, BMP2, CLDN11, INHA, SOX9 and TSKU) whereas IGF2 was upregulated in the testis only in the later period. In addition, the RE of three of these genes increased during testis development (CLDN11, INHA and SOX9). Interestingly, among genes with preferential higher RE in the testis, one gene (CFC1) was also downregulated during ovarian development. Representative expression profiles of this subcluster are shown in Figure 3 (See INHA and CLDN11). The third subcluster (C1.3) includes genes whose RE was also globally higher in the testis, and in addition, was increased during gonad development. Representative expression profiles of this subcluster are shown in Figure 3 (See ADAMTS12 and LOC427192). Similar to the AMH pattern, five genes demonstrated preferential testicular expression in both time periods (NR5A2, GHR, ADAMTS12, LOC427192 (corresponding to NIM1) and PAX5). Six genes were significantly more highly expressed in the testis samples than in the ovary samples in the later period (INHBA, INHBB, GDNF, GDF2, PDGFRA, and IGFBP4) and one gene (SMAD7) was preferentially expressed in the testis samples only in the early period. The RE of three genes (NR5A2, LOC427192 (corresponding to NIM1) and FGF18) was higher in the later period compared to the earlier period in both sexes. Furthermore, the RE of six genes increased only during testis development (INHBA, INHBB, GDNF, GDF2, PDGFRA and IGFBP4), while the RE of GHR, AMH, SMAD1 and DDX4 increased only during ovary development.

Cluster C2 (Figure 2) was characterized by genes whose RE was globally increased during ovary development. Indeed, seventeen genes were upregulated during ovary development between the earlier and later periods (AQP1, NROB1, FGF10, BMPR1B, IGF1, BMP2, AR, FSHR, CYP17A1, LHCGR, CYP11A1, HSD3B1, SMAD7, LOC416998 (corresponding to NALP1 protein) EGR1, PGR and TIMP3). Representative expression profiles of this cluster are shown in Figure 3 (see BMP2 and AR). Similarly, seven genes (NROB1, IGF1, FSHR, CYP11A1, HSD3B11, EGR1 and PGR) presented higher RE in later period compared to earlier period in the testis samples, and NOG expression increased during development but only in testis samples. In addition, several of the genes forming cluster 2 were preferentially expressed in the ovary compared to the testis. FSHR was significantly upregulated in the ovary in both periods. The RE of eight genes was significantly higher in the ovary compared to the testis in the later period (FGF10, BMPR1B, BMP2, AR, CYP17A1, LHCGR, SMAD7, and LOC416998 (corresponding to NALP1 protein)).

Cluster C3 (Figure 2) contained 21 genes preferentially expressed in the ovary compared to the testis. Of these, MHHM, FOXL2, CYP19A1 and HSD17B4 exhibited a stronger expression in the ovary in the earlier as well as in the later period. Moreover, eleven genes were preferentially expressed in ovarian samples compared to the testis in later period (FSH, ADAMTS1L1, GATA6, EMB, FGF9, BMP4, BMP7, FST, BMP2, GDF9 and SMAD2). Eight genes (GNRH1, ESR2, ADAMTS1L1, GATA6, EMB, HSD17B1, GDF9, JAK1 and TGFB2) exhibited upregulation during ovary development. Of these, GNRH1, EMB, JAK1 and TGFB2 were also more highly expressed in earlier testis samples compared to earlier ovarian samples. Interestingly, among the genes with a preferential RE in the ovary, five genes were downregulated in the testis during the later period (ADAMTS1L1, BMP4, BMP7, HSD17B4 and BMP3). The RE of one gene (MHHM) increased during testis development. The representative expression profiles of this cluster are shown in Figure 3 (See BMP2 and HSD17B4).

Cluster C4 (Figure 2) included twenty-six genes. These genes presented significantly preferential RE in the earlier samples (between day 5.5 and 7.5) compared to the later samples. Of these, eight genes exhibited higher RE in both sexes (ACVR2B, FRZB, ESR1, GATA3, GREM1, PAX2, PTX2 and GATA2) (see GREM1 and PAX2 in Figure 3). Six other genes presented preferential earlier RE only in testis samples (GAL, SST, GJA1, HOX7, WNT4, and GJB1). Furthermore, the RE of FOXO3 increased during ovarian development, whereas that of KIT increased during testis development. It was also observed that some genes in this cluster presented a sexual dimorphism in their expression patterns. Of these, NGF, ESR1, GREM1, GAL, GJA1, HOX7 and FOXO1A had sexually dimorphic expression profiles in the later period with significantly higher RE levels in the ovary. The RE of WNT4 was higher in the testis at the earlier stages whereas it was higher in the ovary at the later stages. Furthermore, KIT, BMP5, FSTL1, and ACVR2B were more highly expressed in the testis at the later stages.

As described above we also compared gene expression in left and right ovary and testis samples. We could not determine a specific cluster of asymmetrically expressed genes. This was probably due to the fact that only 24 out of 110 genes displayed significantly asymmetric expression profiles in at least one left-right side comparison and that the patterns of these genes were not similar enough to be clustered together. BARX1, SOX9, GDNF, NOG, CNGHRH, BMP3, ESR1, GAL and PTX2 were more highly expressed in the left ovary (See BMP3 profile in Figure 4) than in the right and the expression of ten genes (NR5A2, PAX3, DDx4, BMPR1B, IGF1, GNRH1, MHHM, BMP3, PTX2 and GJB1) was greater in the left testis than in the right (See GNRH1 profiles in Figure 4). At all stages, PTX2 was preferentially expressed on the left of male and female gonads. Interestingly, BMP3 was more highly expressed in the left ovary than in the right in the earlier and later periods. Moreover at the early ovary stages the
Figure 2. Hierarchical clustering of 110 gene expression patterns in right and left male and female chicken gonads. Each row represents a gene, and each column represents a sample. The samples at the top of the heatmap are set out according to sex (male and female), according to side (left or right) and according to development stage. Each cell in the matrix corresponds to an expression level, with blue for underexpression, yellow for overexpression, black for gene expression close to the median (see color scale) and grey for missing values. Genes are...
expression of *CGNRH-R* was greater on the left, whereas the expression of *NR5A2* and *GREM1* profiles in Figure 4). At the later ovary stages, three genes were upregulated on the left (*SOX9*, *IGF1*, and *GAL*) whereas three other genes were more highly expressed on the right (*CYP11A1*, *FST*, *PAX2*, *BMP4*, *PTX2* and *GJB1*) (see *GJB1* profile in Figure 4), and at the later testis stages *NR5A2*, *PAX5*, *IGF1*, *GNRH1* and *PTX2* were more highly expressed on the left.

**Whole-mount in situ hybridization**

We performed more detailed study of 3 genes (*CLDN11*, *ADAMTS12* and *BMP4*) by whole mount in situ hybridization (Figure 5). This analysis confirmed dimorphic expression of *CLDN11*, *ADAMTS12* and *BMP4* in male and female gonads, detected by real-time RT-PCR. *CLDN11* was highly preferentially expressed in the male gonads compared to the female gonads at day 8 and specifically expressed in the male gonads at day 10 (Figure 5). In the male gonads *CLDN11* expression was located in the testicular cords in the Sertoli cells. At day 8 faint *CLDN11* expression was observed in the cortex in the female gonads and it disappeared at day 10. In accordance with RT-PCR findings in situ hybridization analysis detected preferential expression of *ADAMTS12* in the testis at day 7, the time of histological differentiation of the gonads. The expression of *ADAMTS12* was located in the forming testicular cords. Based on RT-PCR findings the expression of *BMP4* in embryonic gonads becomes sexually dimorphic at day 10. *BMP4* was also more highly expressed in female than in male gonads at day 10 as detected by in situ hybridization. *BMP4* was expressed in the cortex and in the medulla but its expression was much greater in the cortex. In the male gonads *BMP4* was weakly expressed in testicular cords.

**Discussion**

The chicken is a very appealing comparative model to study molecular mechanisms leading to testis or ovary formation. Indeed, among other model species in which the female is heterogametic, only the chicken is characterized by a high degree of differentiation of sex chromosomes Z and W, which may be considered as functional equivalents of the mammalian highly differentiated XY chromosome system where the specific small sex chromosome Y is shared by the male. Furthermore, gonad development in chicken embryos presents other features such as asymmetry of the female reproductive system and the sensitivity of gonad differentiation to estrogens. These particular features may have consequences on the regulation of genes that trigger sex determination and gonad differentiation in this species. Our results provide valuable information on the genes involved in chicken gonad differentiation. Using real-time RT-PCR we compared the expression pattern of 110 candidate genes and as already applied for the study of the genetic program of trout gonad development, these expression patterns were analyzed by a clustering method and displayed in a color coded matrix [13]. It was first of note, that the hierarchical clustering of the samples was consistent with the genetic sex and with the time of sampling and clearly discriminated two periods in male and female gonad differentiation. The distinction between early and late male samples revealed a delay compared to the onset of the histological sex differentiation which occurs between day 6.5 and 7 [14]. This can be explained by the fact that the fold change in the RE of several genes increases or decreases in male gonads by days 8.5–9.5. For instance, this is the case for *PAX2*, *GATA2*, *AMH*, *GREM1*, *PITX2*, *SST*, *SOX9*, *DMRTR3*, *INHA*, *INHB4*, *INIB*, and *PDGFRA*. Second, hierarchical clustering of the genes discriminated four main gene clusters, three of which (C1, C2 and C3) contained genes exhibiting sexual dimorphism, and one cluster, C4, which mainly contained genes early regulated in both sexes, included new potential players (*GATA5*, *GATA2* and *PAX2*) of the development of the bipotential gonad. Similarly, some genes were upregulated in the later period in both sexes. This was the case for several genes from cluster 2 (e.g., *CYP11A1*, *HSD3B1*, *EGRI* and *PGR*) from cluster 1.3. These genes may be involved in gonad growth and steroid production.

Estrogens are known to have a critical role in avian sex determination and particularly in ovarian cortex development [12,16]. Therefore, as expected, in cluster C3 with a specific ovarian signature, we found *CYP19A1*, an enzyme involved in estrogen production [11,16,17,18] and its potential regulator *FOXL2* [19,20]. We also found in this experiment that the genes encoding other steroidogenic enzymes were preferentially expressed in the ovary, i.e. *CYP19A1* (C2) and *HSD17B4* (C3), consistent with the fact that the embryonic ovary produces more steroids than the testis [21,22,23]. One of the aims of this study was to find new potential regulators of enzymes involved in estrogen synthesis (in particular of *CYP19A1*) as well as estrogen targets. Of the genes upregulated in the developing ovary, *NGFB* (cluster C4) seems to be a good candidate because this neurotrophin is involved in the reproductive function in mammals [24,25,26,27,28,29,30]. Indeed, *NGF* mRNA expression in rodents and humans begins before primordial follicle formation [25,27,28], and it is closely involved in ovary development [24,26,29,30]. Furthermore, *NGF* seems to be a modulator of steroidogenesis in mammalian granulosa cells, decreasing progesterone production and increasing estradiol production by regulation of *FSHR* mRNA expression [31,32,33]. Thus *NGFB* could be involved in regulation of estrogen levels in chicken embryonic gonads. On the other hand, the upregulation of NGFB in the developing chicken ovary may be a consequence of *CYP19A1* upregulation since exposure to estradiol increases intraovarian *NGFB* expression in the postnatal rat ovary [34].

In the C1 cluster, we found genes that were specifically upregulated during testis differentiation. Some of them have been shown to be involved in vertebrate testis differentiation, including...
Figure 3. Expression profiles of some representative genes from the four main clusters (C1 to C4) identified during male and female sex differentiation. Cluster C1.2: INHA (inhibin alpha) and CLDN11 (claudin 11 (oligodendrocyte transmembrane protein)); cluster C1.3: ADAMTS12 (ADAM metallopeptidase with thrombospondin type 1 motif, 12) and LOC427192 (similar to hypothetical protein MGC42105); cluster C2: BMP2 (bone morphogenetic protein 2) and AR (Androgen receptor); cluster C3: BMP3 (bone morphogenetic protein 3) and HSD17B4 (Hydroxysteroid (17-beta) dehydrogenase 4); Cluster C4: PAX2 (paired box 2) and GREM1 (gremlin 1). For each histogram, female samples are represented by black squares and black lines and male samples by empty squares and dotted line. Results are represented with an arbitrary scale as the ratio between the expression of the specific gene and the mean expression of EEF1A1 and RPL15. Each square represents the mean of two different measurements from different biological samples for the same male or female population and the same sampling date.

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the chicken, (SOX9, DMRT1 and AMH) \[1,8,9,13,35,36,37,38\], and we used these genes to form the testicular cluster. Several genes joined the same cluster and were preferentially expressed in male gonads during the first stages of gonad differentiation (TSKU, NOTCH1, FSR, FANCG, CFC1, DMRT3, GHR, ADAMTS12, INHA, CLD11, PDGFR\(A\), NR3A2, BMP2 and LOC427192 (corresponding to NIM1)) suggesting that these genes would be good candidate early markers of male gonad differentiation. Six of these genes (FANCG, CFC1, ADAMTS12, DMRT3, GHR and LOC427192) are Z linked, and thus their preferential testicular expression may be the consequence of double chromosome dosage. Based on the fold change in their expression levels between the embryonic testis and ovary this could be the case for FANCG. However for 5 other genes the significant fold change in their expression levels between the embryonic testis and ovary indicates rather their specific regulation than the effect of the double Z chromosome dosage. Among these testicular genes, CLDN11 exhibited a highly conserved developmental expression profile. As demonstrated by \textit{in situ} hybridization in this study CLDN11 was specifically expressed in Sertoli cells during testis.
differentiation in the chicken similar to mouse [39]. CLDN11 is essential for tight junction formation and blood-testis barrier integrity. Absence of CLDN11 results in male sterility [40]. FANCG (Fanconi anemia complex G) is interesting because it is a member of a complex formed by FANCA, FANCC and FANCG, known to regulate germ cell proliferation in the mouse [41]. Another gene, ADAMTS12 (a desintegrin and metalloproteinase with thrombospondin type 1 motifs), is particularly interesting because, in addition to its role in the remodeling of the extracellular matrix and angiogenesis, this protein has been demonstrated to be an inhibitor of SOX9 expression during in vitro chondrocyte differentiation [42]. Similarly ADAMTS12 may be a modulator of SOX9 expression in developing chicken gonads since in this study ADAMTS12 expression was detected in forming testicular cords at the time of chicken sex differentiation. LOC427192 encodes a Serine/Threonine Kinase named NIM-1, which is a member of the AMPK-related kinase family that also includes testis specific genes SNRK and TSSKs [43,44,45]. This gene is conserved in the chimpanzee, dog, cow, mouse, rat, chicken, zebrafish and C.Elegans, but little is known about its functions; NIM-1 expression has been detected in many rat tissues but significant levels of NIM1 activity were observed only in the brain and in the testis [44]. This gene may thus have a specific role in chicken testis differentiation and function.

One particularly interesting finding of this study was that several members of TGFβ family exhibited sexually dimorphic expression.
profiles. Indeed, several Bone Morphogenetic Proteins (BMPs) were more highly expressed during chicken ovary development and clustered with CYP19A1. This was the case for transcripts of several BMP signaling molecules (BMP2, BMP4 and BMP7). Similarly, in cluster 2, that also contained genes whose RE was higher in the ovary (e.g. CYP17A1), we also found transcripts of BMP signaling molecule (BMP2), of one BMP receptor (BMPRIB) and of one inhibitory SMAD (SMAD7). It has already been shown that BMP7 is early expressed (day 4.5/5) in the chicken gonadal mesenchyme, becoming female specific from day 8 in the left and right medullae [46], consistent with our results. Similarly, BMP2 is overexpressed in the mouse embryonic ovary and BMP7 in the trout embryonic ovary. Nonetheless, in contrast to findings in the chicken, BMP7 is more highly expressed in the mouse testis from day 10.5 [47], and BMP4 expression is not sexually dimorphic in trout embryonic gonads [13]. All these results obtained in different vertebrates thus show that the sexual dimorphism of BMP expression is not totally conserved. These BMPs may be involved in germ cell proliferation, as demonstrated in murine testis cell culture where BMP2 and BMP4 exhibited a mitogenic effect on germ cells [48,49]. In addition to this potential role, BMPs may be involved in the regulation of steroid production, as shown in mammalian and hen granulosa cells [50,51]. Moreover, GDF9 (another TGFβ family member) also belonging to this ovarian cluster, is an oocyte-specific factor in mammalian early-stage follicles and seems to be essential for further follicle progression [50]. Furthermore this factor also regulates steroidogenesis by inhibiting FSHR expression [52]. Similarly to our findings, FST has been found to be overexpressed in the mouse ovary at embryonic day E12.5, which is the time of gonad differentiation [53,54]. Furthermore, in mammals, FST acts downstream from WNT4, in order to inhibit the coelomic vessel formation that is male-specific. In the chicken, in contrast to the mouse, there is no male-specific vessel, suggesting another role for FST in ovary development. However, in the mouse, FST is also involved in maintaining germ cells in the cortical region [55]. FST is known to neutralize BMP activity by non-competitive receptor binding [56,57] and thus FST may regulate BMP activity in chicken ovary development.

In contrast to the preferential ovarian expression of several BMPs during gonad development found in this study, genes encoding other members of the TGFβ family including subunits α, βA and βB of inhibin were upregulated in the developing testis. These subunits are involved in the formation of inhibins (αβA or αβB) or activins (βAβA, βAβB or βBβB). This is the first report of early sexually dimorphic expression of the βB and α inhibin subunits during chicken testis differentiation. Sexually dimorphic expression of inhibin subunit α in chicken embryonic gonads has been reported previously at later stages [58]. In the chicken, inhibin secretion by the testis has been shown to be higher than that by the ovary [59,60]. Inhibin A and activin A have been found to modulate androgen synthesis in testicular cells from 18- day-old chicken embryos in vitro [60]. In mammals, sexual dimorphism in inhibin production and secretion has been found in the rat [61], sheep [62], and bovine [63] as well as in humans and nonhuman primates [64]. In the mouse, activin B has been shown to contribute to the formation of the coelomic vessel, a male-specific artery critical for testis development [65]. However, as mentioned above, this particular vasculature structure is absent in the chicken embryonic testis. Moore et al. hypothesized that inhibins and activins could also regulate interstitial cell functions and increase spermatogonia proliferation [66]. CFCl1, another Z-linked gene upregulated in the testis throughout the developmental period, encodes the co-receptor of another member of the TGFβ family (nodal) which is known to be largely involved in vertebrate embryonic patterning, including germ layers and left-right axis specification [67]. In addition to its role as nodal co-receptor, CFCl1 has also been shown to antagonize activin signaling by binding to the activin-ACTRIB/1B complex and preventing its interaction with the ACTRIB receptor [68]; it might thus regulate activin activity in the developing testis.

This study also highlights many genes expressed asymmetrically between left and right developing gonads. The absence of a specific cluster for these genes could be explained by the fact that they are asymmetrically expressed at different periods of gonad development and in different sexes, and thus only a small number of genes exhibited similar asymmetric expression profiles. Moreover, the clustering is mainly constrained by the differences between gene expression levels between ovarian and testis samples and between early and later samples. As expected, we found that PITX2 was more highly expressed in left gonads than in right gonads in both sexes. PITX2 has been shown to be involved in the asymmetric development of the ovary [69]. Furthermore, several other genes were specifically upregulated in the left ovary (IGF1, CGNRH-B, BMP3, PITX2 and G4L) or in the left testis (NR5A2, PAX5, DDX4, IGF1, GNRH1, BMP7 and PITX2). The presence of genes differentially expressed between the right and the left male gonads, which both become functional testes, could reflect the primary asymmetric program present in the gonads of both sexes. Indeed, PITX2, which is essential for the establishing the asymmetry, is overexpressed on the left side in both sexes [69,70,71]. Moreover, the right testis retains the potential for regression, which could be induced by estrogens, while the left testis develops in an ovary or ovotestis under the same conditions [16,18,72]. The genes preferentially expressed in the left female gonad which will become a functional ovary, may have a role in the development of the cortex, which occurs only in the left side. Indeed, genes whose expression is restricted to the medulla, as for example those encoding steroidogenic enzymes or FOXL2, do not exhibit significant asymmetry in their expression patterns. However, this potential role in the development of the cortex of female left side upregulated genes should be confirmed by their cellular localization. Moreover, some genes are specifically increased in the right ovary which regresses from day 8; this is the case for NR5A2, NROB1, CYP11A1, EMB, FST, GREM1 and PAX2. These genes could be markers of ovary digenesis.

To conclude, this study reports a picture of gene expression during chicken gonad development from undifferentiated gonads up to latest embryonic testicular and ovarian stages before hatching and reveals strong similarities between chicken gonad sex differentiation and that of other vertebrate species, as well as several specific features of this process in the chicken. This study also identified new candidate genes which could be potential actors of female and male pathways whose roles should be further investigated. Our findings also suggest that gonad differentiation in the chicken involves a complex interplay between different members of the TGFβ protein family whose precise function remains to be elucidated.

Materials and Methods

Ethics statement

All experiments were carried out with respect for the principles of laboratory animal care. Approval of the ethics committee is not necessary because in France experiments involving the chicken embryo are not considered as animal experiments.
Animals
Fertilized eggs from White Leghorn chickens were incubated at 37.8°C and 40% humidity. Eggs were then removed every half day between day 5.5 and day 7.5, every day between day 7.5 and 10.5 and every two days between 10.5 and 19.5. Chicken embryos were quickly decapitated immediately after removal from the eggs and before any dissection.

Tissue collection
At each stage, the left and the right gonads were dissected separately, put individually in RNAlater® (Ambion) and stored at −20°C. A small piece of extragonadal tissue from each embryo was dissected, frozen immediately in liquid nitrogen and stored at −80°C prior to sex determination. After sexing of embryos the gonads were pooled (10–25 gonads per pool depending on the stage) according to sex, stage and side. Two pools of gonads were combined for each time point analysis. Samples were stored in RNAlater® at −20°C until RNA extraction.

Genetic sexing of chicken embryos
Genomic DNA was extracted according to Estoup et al. [73]. Small pieces of tissue were incubated for 2 hours at 55°C in 0.2 ml of 10% Chelex100 resin (Bio-Rad) containing 200 μg/ml proteinase K. After inactivation of proteinase K by heating at 95°C for 10 min, samples were centrifuged at 10,000 g for 1 min. PCR sexing was performed on 2 μl of genomic DNA as described by Clinton [74].

Total RNA extraction and Reverse Transcription
Total RNA was extracted using TRI Reagent (Euromedex) according to the manufacturer’s instructions. RNA quality and concentration were determined using an Agilent 2100 Bioanalyser according to the manufacturer’s instructions. For cDNA synthesis, 2 μg of total RNA were treated with 1 unit of RQ1 RNase-free DNase (Promega) according to the manufacturer’s instructions and then denatured in the presence of a mix of oligoDT (250 ng) and random hexamers (62.5 ng) for 5 min at 70°C. Reverse transcription (RT) was performed first at 25°C for 10 min and then at 42°C for 50 min using Moloney murine leukemia virus reverse transcriptase in the presence of dNTP (0.5 mM) and RNAsine (RNase inhibitor, 20 U). (All products were from Promega).

Primer design
Chicken candidate genes were chosen according to a literature survey on the basis of their involvement in the sex differentiation cascade in vertebrates or more generally in embryogenesis and reproduction (Table S2). The chicken sequences resulted from searches in international public databases. New chicken genes were verified to be true orthologs of mammalian genes using a reciprocal blast hit strategy [75]. The physical location of genes identified on chicken chromosomes was retrieved from both mapview [76] and from blat search [77]. The primers were purchased from Eurogentec or from Sigma Genosis.

Real time PCR
Real-time RT-PCR (qPCR) was carried out on an ABIPrism 7000 (Applied Biosystems). Reactions were performed in 20 μl with 200 nM of each primer and 5 μl of a 30× dilution of the RT reaction and the qPCR Mastermix plus for SybrGreenI (Eurogentec). EEF1A and RPL13 were used as reference genes to normalize expression levels of genes assayed. Two independent runs were performed for each reference gene. For each set of primers, the efficiency of the PCR reaction (linear equation: $y = \text{slope} \times x + \text{intercept}$) was measured in triplicate on serial dilutions of the same cDNA sample (pool of reverse-transcribed RNA samples). Real-time PCR efficiency (E) was calculated using the following equation: $E = \left(10^{1\text{slope}}\right)^{-1}$. Melting-curve analysis was also performed for each gene to check the specificity of RT-PCR products. The relative amount of the target RNA (R) was then determined using the following equation:

$$R = \frac{E^{-C_{\text{gene}}}}{\text{mean}(R_{C1,6} - E_{\text{EEF1A}, 2 - E_{\text{EEF1A}, LFG-14.5}, 2 - R_{\text{RPL13}}})}$$

in which Ct is the cycle threshold and Rc1 and 2 are corrected relative reference gene expressions for runs 1 and 2 calculated as explained below. In order to take into account only fold changes in the expression levels of reference genes between samples but not the differences in the expression levels of reference genes (ref) in the sample, the expression levels of reference genes in each sample (samplei) were adjusted against the relative amount of EEF1A of run1 (EEF1A-run1) in one of two day 14.5 left female gonad samples (LFG-14.5) according to the equation:

$$R_{\text{ref}} = \left(E_{\text{EEF1A}, \text{RUN1}} \times C_{\text{EEF1A - RUN1}} - LFG-14.5 \times C_{\text{EEF1A - LFG-14.5}} \right) \times \left(C_{\text{ref}} - C_{\text{EEF1A - RUN1}} \right)$$

where $\Delta C_{\text{I}} = C_{\text{ref in LFG-14.5}} - C_{\text{ref in samplei}}$

Whole-mount in situ hybridization analysis
Whole-mount in situ hybridization was performed using digoxigenin-labeled riboprobes. For the probe synthesis the 434, 407 and 824 bp PCR fragments corresponding to CLDN11, ADAMTS12 and BMP4 respectively, were amplified from the embryonic gonad cDNA using the following pairs of primers: 5’-TCCTCTTAGAAAAACAGGCGCAAG-3’, 5’-AGTGAGAAACCAACGGAATTCAGG-3’ for CLDN11 and 5’-CAGCTGTTACGTTGCCGCTCT-3’ and 5’-AGGGAGGCTACTTTCCGTGACA-3’ for ADAMTS12, and 5’-CCACAGATCCATCGTTCGAA-3’ for BMP4. The PCR fragments were cloned in the pCRII-TOPO vector (Invitrogen) and the plasmids were sequenced to confirm the presence of the correct insert. The riboprobes for CLDN11, ADAMTS12 and BMP4 were synthesized using an in vitro transcription kit (Roche Diagnostics) according to the manufacturer’s instructions on the phenol-chloroform purified PCR products obtained by amplification of the inserts of interest from the plasmids using M13 reverse and M13 forward primers. The embryonic gonads at different developmental stages were dissected on ice with underlying mesonephros and fixed in 4% paraformaldehyde overnight. The hybridization procedure was performed as described previously [78]. For sectioning the gonads were embedded in phosphate buffered saline pH 7.5 containing 0.5% gelatin, 15% BSA, 2.2% glutaraldehyde, and 50 μM sections of the gonads were cut using MICROCUT H1200 (Biorade).

Data Analyses
Real time PCR data were used to cluster genes and biological samples using a hierarchical clustering method [79] implemented in the T-mev4 software (MultiExperiment Viewer). The expression levels were first log-transformed and then the rows (genes) were median-centered and normalized, and the columns (samples) were normalized. A complete linkage method was used on the similarity matrix based on the Pearson correlation coefficient in order to organize biological samples and to identify genes with a
similar expression pattern. In order to distinguish between missing values due to PCR anomalies (grey colour on Fig. 5) and undetermined values due to a too weak gene expression level in the samples, we attributed a very low value (1 × 10^−15) to these later samples before proceeding with the clustering. This was done only in the case of data obtained for CYP19A1 transcript measurement in male gonad samples, in which this gene is normally very weakly expressed, and therefore is often not determined by real time RT-PCR. As only the left ovary becomes completely functional, the comparisons of expression levels between the testis and the ovary, and between the earlier and later samples, were made using left gonads. We defined the comparisons of interest as: A) all left male and female samples; B) left “early” female and left “early” male samples; C) left “late” female and left “late” male samples; D) left “early” and left “late” female samples E) left “early” and left “late” male samples; F) left and right female samples; G) left and right “early” female samples; H) left and right “late” female samples; I) left and right male samples; J) left and right “early” male samples; K) left and right “late” male samples. Differential analysis was performed on the dataset of the expression levels log-transformed using a linear model with one factor defined by the combination of the levels of the factors sex (male/female), side of the embryonic gonad (left/right) and developmental stages (early/late) with the Limma R package [80]. Contrasts of interest were those of expression levels between the testis and the ovary, PCR. As only the left ovary becomes completely functional, the comparisons of expression levels between the testis and the ovary, were made using left gonads. We defined the comparisons of interest as: A) all left male and female samples; B) left “early” female and left “early” male samples; C) left “late” female and left “late” male samples; D) left “early” and left “late” female samples E) left “early” and left “late” male samples; F) left and right female samples; G) left and right “early” female samples; H) left and right “late” female samples; I) left and right male samples; J) left and right “early” male samples; K) left and right “late” male samples. Differential analysis was performed on the dataset of the expression levels log-transformed using a linear model with one factor defined by the combination of the levels of the factors sex (male/female), side of the embryonic gonad (left/right) and developmental stages (early/late) with the Limma R package [80]. Contrasts of interest were tested and p values were adjusted for multiple testing using Benjamini and Hochberg’s method to control the false discovery rate at the threshold fixed at 0.05 [15].

**Supporting Information**

**Figure S1** Scatter plots of mRNA levels of 110 genes obtained from biological sample duplicates (Left ovary 1 vs Left ovary2; day 12.5). Each black spot denotes a data point. The diagonal black line denotes X = Y identity (TIF).

**Table S1** Relative expression values of 110 genes in embryonic chicken gonads detected by real time RT-PCR. Each value in the table represents the expression level of the assayed gene detected by real time RT-PCR and normalized against the expression level of two reference genes EEF1A and RPL13 as described in the section “Materials and Methods”. Each row corresponds to a gene; each column corresponds to a sample. The sex and the side of embryonic gonads in the samples are indicated. F = female, M = male, L = left, R = right. “#VALEUR!” indicates that the sequence was undetermined.

**Table S2** Names, symbols, accession numbers and chromosomal location of sequences of chicken genes and sequences of corresponding forward and reverse primers used in real time RT-PCR.

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**Author Contributions**

Conceived and designed the experiments: MSG. Performed the experiments: GAC IC MSG. Analyzed the data: GAC MSG. Contributed reagents/materials/analysis tools: GAC MSG. Wrote the paper: GAC MSG. Statistical analysis of the data: CHA.

**References**

1. Smith CA, Roeseler KN, Ohnesorg T, Cummins DM, Farlie PG, et al. (2009) The avian Z-linked gene DMRT1 is required for male sex determination in the chicken. Nature 461: 267–271.
2. Smith CA, Roeseler KN, Sinclair AH (2009) Genomic evidence against a role for W-linked histidine triad nucleotide binding protein (HINTW) in avian sex determination. Int J Dev Biol 53: 59–67.
3. Tsunekawa N, Naito M, Sakai Y, Nishida T, Nocé T (2000) Isolation of chicken vasa homolog gene and tracing the origin of primordial germ cells. Development 127: 2741–2750.
4. Hamburger V, Hamilton HL (1992) A series of normal stages in the development of the chick embryo. 1951. Dev Dyn 195: 231–272.
5. Hughes GC (1963) The Population of Germ Cells in the Developing Female Chick. J Embryol Exp Morphol 11: 513–536.
6. Ueshima A, Fujimoto T (1991) A fine morphological study of germ cells in asymptomatically developing right and left ovaries of the chick. Anat Rec 230: 378–386.
7. Civimini A, Mastrolia L (1986) Ultrastructural localization of acid phosphatase in germ cells of chicken embryo left ovary. Exp Cell Biol 54: 94–105.
8. Orel E, Peceu C, Mattei MG, Josso N, Picard JY, et al. (1998) Early expression of AMH in chicken embryonic gonads proceeds testicular SOX9 expression. Dev Dyn 212: 522–532.
9. Orel E, Mazaui S, Picard JY, Magre S, Carre-Eusebe D (2002) Different patterns of anti-Mullerian hormone expression, as related to DMRT1, SF-1, WT1, GATA-4, Wnt-4, and Lhx9 expression, in the chick differentiating gonads. Dev Dyn 225: 221–232.
10. Elbrecht A, Smith RG (1992) Aromatase enzyme activity and sex determination in chickens. Science 255: 467–470.
11. Nakabayashi O, Kikuchi H, Kikuchi T, Mizuno S (1998) Differential expression of genes for aromatase and estrogen receptor during the gonadal development in chicken embryos. J Mol Endocrinol 20: 193–202.
12. Vaillant S, Guemene D, Dorizzi M, Peceu C, Richard-Mercier N, et al. (2003) Degree of sex reversal as related to plasma steroid levels in genetic female chickens (Gallus domesticus) treated with Fadrozole. Mol Reprod Dev 65: 420–428.
13. Baron D, Houlgate R, Foster A, Guiguen Y (2005) Large-scale temporal gene expression profiling during gonadal differentiation and early gametogenesis in rainbow trout. Biol Reprod 73: 959–966.
14. Smith CA, Sinclair AH (2004) Sex determination: insights from the chicken. Bioessays 26: 120–132.
15. Benjamini YH (1995) Controlling the false discovery rate - a practical and powerful approach to multiple testing. Journal of the royal statistical society seriesB 57: 289–300.
16. Scheib D (1983) Effects and role of estrogens in avian gonadal differentiation. Differentiation 23 Suppl: 867–92.
17. Abinavatno, Shimada K, Yoshioka K, Saito N (1996) Effects of aromatase inhibitor on sex differentiation and levels of P450 (17 alpha) and P450 arom messenger ribonucleic acid of gonads in chicken embryos. Gen Comp Endocrinol 102: 241–246.
18. Abinavatno, Shimada KS, Sugishima N, Shikawa T, E (1997) Sex-reversal effects of non-steroidal aromatase inhibitor on aromatase(P450arom) mRNA expression in adult chicken gonads. Jpn Poult Sci 34: 158–168.
19. Govoroun MS, Pannetier M, Pailhoux E, Cocquet J, Brillard JP, et al. (2004) Isolation of chicken homolog of the FOXL2 gene and comparison of its expression patterns with those of aromatase during ovarian development. Dev Dyn 231: 839–870.
20. Hudson QJ, Smith GA, Sinclair AH (2005) Aromatase inhibition reduces expression of FOXL2 in the embryonic chicken ovary. Dev Dyn 233: 1052–1055.
21. Gasc JM (1980) Estrogen target cells in gonads of the chicken embryo during sexual differentiation. J Embryol Exp Morphol 53: 331–342.
22. Guichard A, Cedard I, Haffen K (1973) “[Comparative aspect of the synthesis of sex steroids by chick embryonal gonads at different stages of development [study in organ culture in the presence of radioactive precursors]].” Gen Comp Endocrinol 20: 16–28.
23. Guichard A, Cedard I, Mignot TM, Scheib D, Haffen K (1978) “Radioimmunoassay of steroids produced by chick embryo gonads cultured in the presence of some exogenous steroid precursors.” Gen Comp Endocrinol 39: 9–19.
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24. Disen GA, Garcia-Ruiz C, Ojeda SR (2009) Role of neurotrophic factors in early ovarian development. Semin Reprod Med 27: 24–31.

25. Disen GA, Hirshfeld AN, Malamed S, Ojeda SR (1995) Expression of neurotrophins and their receptors in the mammalian ovary is developmentally regulated: changes at the time of folliculogenesis. Endocrinology 136: 4681–4692.

26. Disen GA, Romero C, Hirshfeld AN, Ojeda SR (2001) Nerve growth factor is required for early follicular development in the mammalian ovary. Endocrinology 142: 2070–2086.

27. Anderson RA, Robinson LL, Brooks J, Spears N (2002) Neurotrophins and their receptors are expressed in the human fetal ovary. J Clin Endocrinol Metab 87: 580–587.

28. Abir R, Fisch B, Jin S, Barnert M, Ben-Haroush A, et al. (2005) Presence of its NGF and its receptors in ovaries from human fetuses and adults. Mol Hum Reprod 11: 229–236.

29. Spears N, Molinski MD, Robinson LL, Fulton N, Cameron H, et al. (2003) The role of neurotrophin receptors in female germ-cell survival in mouse and human. Development 130: 5481–5491.

30. Paredes A, Romero C, Disen GA, DeChiara TM, Reichard L, et al. (2004) TrkA receptors are required for follicular growth and oocyte survival in the mammalian ovary. Dev Biol 267: 430–440.

31. Romero C, Paredes A, Disen GA, Ojeda SR (2002) Nerve growth factor induces the expression of functional FSH receptors in newly formed follicles of that ovary. Endocrinology 143: 1485–1494.

32. Mattoli M, Barbier B, Gui L,Lucidi P (1999) Nerve growth factor production in sheep antral follicles. Domest Anim Endocrinol 17: 361–371.

33. Salas C, Julio-Pepe R, Valladates M, Pommer R, Vega M, et al. (2006) Nerve growth factor-dependent activation of trkA receptors in the human ovary reveals in synthesis of follicle-stimulating hormone receptors and estrogen secretion. J Clin Endocrinol Metab 91: 2396–2405.

34. Sotomayor-Zarate R, Dorfman M, Paredes A, Lara HE (2008) Neonatal exposure to estradiol valerate programs ovarian sympathetic innervation and follicular development in the adult rat. Biol Reprod 78: 673–680.

35. Cederroth CR, Pitetti JL, Papaioannou MD, Nef S (2007) Genetic programs that regulate testicular and ovarian development. Mol Cell Endocrinol 265–266: 3–9.

36. Raymond CS, Kettlewell JR, Hirsch B, Bardwell VJ, Zarkower D (1999) Expression of Dmtr1 in the genital ridge of mouse and chicken embryos suggests a role in vertebrate sexual development. Dev Biol 215: 208–220.

37. Raymond CS, Murphy MW, O’Sullivan MG, Bardwell VJ, Zarkower D (2000) Dmtr1, a gene related to worm and fly sexual regulators, is required for mammalian testis differentiation. Genes Dev 14: 2587–2595.

38. Jakob S, Lovell-Badge R (2001) Sex determination and the control of Sox9 expression in mammals. Feb J 278: 1002–1009.

39. Hellai A, Ji J, Mauduit C, Deschdile C, Tabone E, et al. (2000) Developmental and hormonal regulation of the expression of oligodenndrocyte-specific protein/claudin 11 in mouse testis. Endocrinology 141: 3012–3019.

40. Mazaud-Guittot S, Meugnier E, Pessini S, Wu X, Vital H, et al. (2001) Claudin deficiency in mice results in loss of the Sertoli cell epithelial phenotype in the testis. Biol Reprod 82: 202–213.

41. Nadler JJ, Braun KE (2000) Fanconi anemia complementation group C is required for proliferation of murine primordial germ cells. Genesis 27: 117-123.

42. Bai XH, Wang DW, Luan Y, Yu XP, Liu CJ (2009) Regulation of chondrocyte hypertrophy by BMP4/ALK3/SMAD5 signaling pathway in the mouse testis: a potential role of BMP4 in spermatogonia differentiation. J Cell Sci 116: 485–497.

43. Moore RK, Otsuka F, Shimasaki S (2003) Molecular basis of home morphogenetic protein-15 signaling in granulosa cells. J Biol Chem 278: 304–310.

44. Menke DB, Page DC (2002) Sexually dimorphic gene expression in the developing mouse gonad. Gene Exp Paters 2: 359–367.

45. Feijen A, Goumans MJ, van den Eijnde-van Raai AJ (1994) Expression of activin subunits, activin receptors and follistatin in postimplantation mouse embryos suggests specific developmental functions for different activins. Development 120: 3621–3637.

46. Yao HH, Matzuk MM, Jorgez CJ, Menke DB, Page DC, et al. (2004) Follistatin operates downstream of Wnt in mammalian ovary organogenesis. Dev Dyn 230: 210–215.

47. Bailemans W, Van Hul W (2002) Extracellular regulation of BMP signaling in vertebrates: a cocktail of modulators. Dev Biol 250: 231–250.

48. Jerumaa S, Yamamoto TS, Takagi C, Uchiyama H, Natsunoe T, et al. (1998) Direct binding of follistatin to a complex of bone-morphogenetic protein and its receptor inhibits ventral and epididymal cell fates in early Xenopus embryos. Proc Natl Acad Sci U S A 95: 9337–9342.

49. Safi M, Onagbesan OM, Volmkaert G, Vanmontfort D, Bruggeman V, et al. (2001) Developmental expression of activin/Inhibin-alpha- and beta-A-subunits in the gonads of male and female chick embryos. Gen Comp Endocrinol 122: 304–311.

50. Rombaums L, Vanmontfort D, Berglham LR, Decupere E, Verhoeguen G (1994) Contribution of the fetal adrenal to circulating immunomodulatory inhibin in the chicken embryo. Biol Reprod 51: 926–933.

51. Rombaums L, Vanmontfort D, Decupere E, Verhoeguen G (1996) Inhibin and activin have antagonistic paracrine effects on gonadal steroidogenesis during the development of the chicken embryo. Biol Reprod 54: 1229–1237.

52. Ashland JE, Schwarz NB (1991) Changes in serum immunoreactive inhibin and follicle-stimulating hormone during gonadal development in male and female rats. Biol Reprod 45: 295–300.

53. Albers N, Hart CS, Kaplan SL, Grumbach MM (1989) Hormone ontogeny in the ovine fetus. XXIV: Porcine follicular fluid “inhibin” selectively suppress plasma follicle-stimulating hormone in the ovine fetus. Endocrinology 125: 675–678.

54. Torney AH, Robertson DM, Hodgson YM, de Kretser DM (1990) In vitro bioactive and immunoinhibitory inhibin concentrations in bovine fetal ovaries and testes throughout gestation. Endocrinology 127: 2939–2946.

55. Rabinovici J, Goldsmith PC, Roberts VJ, Vaughan J, Vale W, et al. (1991) Localization and secretion of inhibin/activin subunits in the human and subhuman primate fetal gonads. J Clin Endocrinol Metab 73: 1141–1149.

56. Yao HH, Aardema J, Helhlkem K (2000) Sexually dimorphic regulation of inhibin beta A in establishing gonadal vasculature in mice. Biol Reprod 74: 978–983.

57. Moore A, Krummen LA, Mather JP (1994) Inhibins, activins, their binding proteins and receptors: interactions underlying paracrine activity in the testis. Mol Cell Endocrinol 100: 81–86.

58. Tian T, Mung AM (2006) Nodal signals pattern vertebrate embryos. Cell Mol Life Sci 63: 672–683.

59. Gray RC, Harrison CA, Vale W (2003) Cripto forms a complex with activin and type II activin receptors and can block activin signaling. Proc Natl Acad Sci U S A 100: 5193–5198.

60. Ishimaru Y, Komatsu T, Kasahara M, Kato-Fukui Y, Ogawa H, et al. (2008) Mechanism of asymmetric ovarian development in chick embryos. Development 135: 677–685.

61. Nakamura Y, Yamamoto Y, Usui F, Mushika T, Ono T, et al. (2007) Migration and proliferation of primordial germ cells in the early chicken embryo. Poult Sci 86: 2102–2113.

62. Guioli S, Lovell-Badge R (2007) PTFX2 controls asymmetric gonadal development in both sexes of the chick and can rescue the degeneration of the right ovary. Development 134: 4199–4208.

63. Eiches RJKH (1997) Genotypic and phenotypic sex reversal. Perspectives in avian endocrinology. Journal of endocrinologyLtd, Bristol. pp 57–67.

64. Ensoue A, Pauquet, D (1996) Rapid one-tube DNA extraction for reliable detection of fish polymorphic markers and transgenes. Mol Mar Biol Biotechnol 5: 295–298.

65. Clinton M, Haines L, Belloir B, McBride D (2001) Sexing chick embryos: a one-tube reaction for reliable detection of fish polymorphic markers and transgenes. Mol Mar Biol Biotechnol 10: 169–185.

66. Smyth GK (2005) LIMMA: linear models for microarray data. In: Springer, ed. Bioinformatics and Computational Biology Solutions using R and Bioconductor. New York: R. Gentleman, V. Carey, S. Dudoit, R. Irizarry, W. Huber pages. pp 370–420.