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Different Patterns of Anti-Müllerian Hormone Expression, as Related to DMRT1, SF-1, WT1, GATA-4, Wnt-4, and Lhx9 Expression, in the Chick Differentiating Gonads

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ABSTRACT **In mammals, anti-Müllerian hormone (AMH) is produced by Sertoli cells from the onset of testicular differentiation and by granulosa cells after birth. In birds, *AMH* starts to be expressed in indifferent gonads of both sexes at a similar level and is later up-regulated in males. We previously demonstrated that, unlike in mammals, the onset of *AMH* expression occurs in chick embryo in the absence of SOX9. We looked for potential factors that might be involved in regulating AMH expression at different stages of chick gonad differentiation by comparing its expression pattern in embryos and young chicken with that of *DMRT1*, *SF-1*, *WT1*, *GATA-4*, *Wnt-4*, and *Lhx9*, by in situ hybridization. The results allowed us to distinguish different phases. (1) In indifferent gonads of both sexes, *AMH* is expressed in dispersed medullar cells. *SF-1*, *WT1*, *GATA-4*, *Wnt-4*, and *DMRT1* are transcribed in the same region of the gonads, but none of these factors has an expression strictly coincident with that of *AMH*. *Lhx9* is present only in the cortical area. (2) After this period, *AMH* is up-regulated in male gonads. The up-regulation is concomitant with the beginning of *SOX9* expression and a sex dimorphic level of *DMRT1* transcripts. It is followed by the aggregation of the *AMH*-positive cells (Sertoli cells) into testicular cords in which AMH is coexpressed with *DMRT1*, *SF-1*, *WT1*, *GATA-4*, and *SOX9*. (3) In the females, the low level of dispersed medullar *AMH* expression is conserved. With development of the cortex in the left ovary, cells expressing *AMH* accumulate in the juxtacortical part of the medulla, whereas they remain dispersed in the right ovary. At this stage, *AMH* expression is not strictly correlated with any of the studied factors. (4) After hatching, the organization of left ovarian cortex is characterized by the formation of follicles. Follicular cells express *AMH* in conjunction with *SF-1*, *WT1*, and *GATA-4* and in the absence of *SOX9*, as in mammals. In addition, they express *Lhx9* and *Wnt-4*, the latter being also found in the oocytes. (5) Moreover, unlike in mammals, the chicken ovary retains a dispersed *AMH* expression in cortical interstitial cells between the follicles, with no obvious correlation with any of the factors studied. Thus, the dispersed type of *AMH* expression in indifferent and female gonads appears to be bird-specific and not controlled by the same factors as testicular or follicular *AMH* transcription.** **© 2002 Wiley-Liss, Inc.**

Key words: AMH; MIS; DMRT1; SOX9; SF-1; WT1; GATA-4; Wnt-4; Lhx9; Cvh; aromatase; chick embryo; testis; ovary; sex differentiation

INTRODUCTION

The anti-Müllerian hormone (AMH), or Müllerian inhibiting substance (MIS), a glycoprotein of the transforming growth factor-β (TGF-β) superfamily secreted by embryonic testes, is responsible for the regression in males of Müllerian ducts, the anlagen of the female reproductive ducts. This hormone has only been found in amniote vertebrates. In mammals, *AMH* expression starts in males at the onset of Sertoli cell differentiation and persists in adults late after regression of the Müllerian ducts (Vigier et al., 1983). In females, ovarian *AMH* expression is delayed, starting around birth in granulosa cells of primary follicles (Bézard et al., 1987). *AMH* has been cloned in chick (Carré-Eusèbe et al., 1996) and is also expressed in embryonic ovaries (Hutson et al., 1981). The function of AMH in the ovaries is unclear, although it may play a role in ovarian follicle maturation (Durlinger et al., 2001; McGee et al., 2001).

AMH is at the crossroads of sex determination and sex differentiation. After the transcription factors *SRY* and *SOX9*, it is the first identified product character-

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izing Sertoli cells in mammals. Sertoli cell differentiation will determine the fate of gonad germ and somatic cells and ultimately the sex of the individual. AMH itself controls the evolution of the genital tract and may also influence Leydig cell function (Racine et al., 1998). In birds, synthesis of AMH in embryos of both sexes makes the picture more complicated.

In chick embryo, gonadal organogenesis begins around the 4th day of incubation (Merchant-Larios et al., 1984) with the identification of the genital ridge, a thickening of the coelomic epithelium on the medial aspect of the mesonephros in which primordial germ cells, migrating from the germinal crescent, are going to settle. The origin of the gonad somatic components is still under debate. It has been suggested that these cells derive from the coelomic epithelium, from subjacent mesenchyme, or from the mesonephros (reviewed in Merchant-Larios et al., 1984). Histologically, ovaries and testes can be distinguished after 6.5 days of incubation by the differentiation, in male gonads, of testicular cords formed by Sertoli and germ cells delimited by a basement membrane. In contrast, the ovarian organization is only completed after hatching with formation, in the cortex, of follicles harboring the oocytes.

Left-right gonadal asymmetry, in birds, adds a degree of complexity to the process of differentiation. Asymmetry is manifested, from 3 days of incubation in both sexes, by a greater number of primordial germ cells colonizing the left gonad anlage. The difference is higher in females than in males, constituting an early evidence of some sex differentiation of the gonads (Van Limborgh, 1968). Additionally, volume and surface epithelium thickness are greater in the left gonad in both sexes. These differences fade away in males, whereas in females, only the left ovary and left Müllerian duct fully develop and become functional.

The mechanism underlying the determinism of gonadal sex has been extensively studied in mammals and involves numerous factors (for reviews, see Swain and Lovell-Badge, 1999; Capel, 2000). These factors may be implicated directly in the control of *AMH* expression or indirectly by triggering the sertolian pathway. Downstream of *SRY*, the first gene of the male sex determining cascade, located on the male-specific Y chromosome, *SOX9* is necessary (Foster et al., 1994; Wagner et al., 1994) and sufficient (Vidal et al., 2001) for male differentiation. It activates the *AMH* promoter in vitro (de Santa Barbara et al., 1998) and in vivo (Arango et al., 1999). In addition to these two key factors, SF-1 (steroidogenic factor-1), an orphan nuclear receptor, stimulates transcription of the *AMH* promoter in vitro (Shen et al., 1994) and in vivo (Arango et al., 1999). An interaction with WT1 (Nachtigal et al., 1998) and the zinc finger GATA-4 factor (Tremblay et al., 2001) enhances this effect. It may be antagonized by Dax-1 (Tremblay and Viger, 2001). SF-1 is also an activator of the expression of enzymes involved in steroidogenesis, in particular aromatase (P450-arom), which catalyzes the conversion of androgens to estrogens (Lynch et al., 1993). Another factor implicated in gonadal morphogenesis is the LIM domain-containing homeobox factor Lhx9, of which deficiency leads to an arrest in gonad development before sex differentiation (Birk et al., 2000). DMRT1, another element of the testicular pathway, is a DM domain transcription factor whose inactivation leads to testis degeneracy, germ cell meiosis failure, and arrest in Sertoli cell differentiation in mice (Raymond et al., 2000) and to sex reversal in hemizygote XY humans (Raymond et al., 1999a). Besides these transcription factors, Wnt-4, a member of the Wnt family of secreted locally acting cell signals, is required for proper female differentiation. Inactivation of this gene leads to a masculinization of female gonads with a Leydig cell-type early expression of steroidogenic enzymes (Vainio et al., 1999). Wnt-4 has been shown to stimulate *Dax-1* expression (Jordan et al., 2001).

In birds, the determinism of sex is chromosomal as in mammals but differs in its implementation and is still poorly understood. The male is homogametic (ZZ) and the female heterogametic (ZW). The absence of Z-chromosome gene dosage compensation is the current dogma, presently under controversy (McQueen et al., 2001, Kuroda et al., 2001). Sex determination might result either from the double dosage of a Z-chromosome gene escaping dosage compensation in males or from the presence of feminizing gene(s) on chromosome W, or from a combination of both processes (for a review, see Clinton, 1998).

In birds, genes for testicular or ovarian differentiation can be activated in the opposite sex under abnormal conditions interfering with estrogen production or action (reviewed by Clinton, 1998). These inversions of the genetic pathways show that, in birds, unlike in eutherian mammals, the determination or maintenance of female gonad differentiation is very dependent on an early ovarian estrogen production.

No sex chromosome-specific *SOX* gene homologous to the mammalian sex determining gene *SRY* has been found in birds (Griffiths, 1991). The other factors listed above for their involvement in sex differentiation in mammals have orthologs expressed in bird gonads, but their roles remain to be determined. *SOX9* mRNA appears in the embryonic male chick gonad 1 day after the onset of *AMH* expression. This finding rules out the possibility that this factor is required for the initiation of *AMH* transcription at this early stage (Oréal et al., 1998). In female gonads, *AMH* expression occurs in the absence of SOX9. The avian *DMRT1* gene is located on Z chromosomes (Nanda et al., 1999) and is expressed more strongly in male than in female embryonic gonads (Raymond et al., 1999b; Smith et al., 1999a, Shan et al., 2000). *DMRT1* appears as a candidate testis-determining gene in the hypothesis of a Z gene double-dosage mechanism of sex determination. On the other hand, a gene expressed from chromosome W in birds, *Wpkci*, also called *ASW*, has been suggested as a candidate feminizing gene (reviewed by Ellegren, 2001).

In the present study, we have performed a comparison of the expression patterns of *DMRT1*, *SF-1*, *WT1*, *GATA-4*, *Wnt-4*, and *Lhx9* with that of *AMH*, by parallel in situ hybridization, in embryonic and postnatal male and female chick gonads. Determining the localization and timing of expression of these factors will help to elucidate the precise roles they may play in gonad sex differentiation and especially in the control of *AMH* gene transcription in birds. We accorded special attention to *AMH* expression during follicle formation in postnatal ovaries, which has not been studied extensively, and we report the expression of *Lhx9* and *Wnt-4* genes in relation to folliculogenesis. This study allowed us to distinguish different types of AMH expression, with presumably different modes of regulation, because the same regulation factors are not systematically coexpressed with the hormone.

RESULTS AND DISCUSSION

Analysis of *AMH*, *DMRT1*, *SOX9*, *SF-1*, *WT1*, *GATA-4*, *Wnt-4*, and *Lhx9* expression was performed by in situ hybridization of digoxigenin-labeled antisense RNA probes on cryostat sections of male and female differentiating chick gonads. Adjacent sections were used to compare expression profiles. *Lhx9* is the avian ortholog of the mammalian gene (Failli et al., 2000), previously named *LH-2B* and studied in chick limb development (Nohno et al., 1997). Expression of *Cvh*, the chick homologue of the Drosophila *Vasa* gene, was used as a marker of germ cells (Tsunekawa et al., 2000). *Aromatase* transcripts were used to identify cells involved in the last step of estrogen biosynthesis. Immunolocalization of fibronectin on the same or adjacent sections was used to monitor the structure of gonads. The main stages of the differentiation process will be presented successively: (1) gonadal territory before the onset of *AMH* expression, (2) indifferent gonads expressing AMH, (3) male gonads at the time of AMH up-regulation and formation of testicular cords, (4) female gonads before the formation of the follicles, (5) postnatal left ovary containing young follicles. When informative, left and right gonads are compared. Time of incubation for the embryos (E) and age post-hatching for the chicken (P) are given in days. Embryonic stages of development (HH) have been identified according to Hamburger and Hamilton (1951).

Gonadal Territory Before the Onset of AMH Expression

Expression of the different genes in transverse embryo sections was examined at HH24 (E4.0), at the beginning of the genital ridge formation before the onset of *AMH* expression, which occurs at HH25 (Oréal et al., 1998). The genetic sex of the animals was determined by polymerase chain reaction (PCR). The series of hybridization results presented in Figure 1A, although corresponding to a female, is representative of both sexes because patterns are identical.

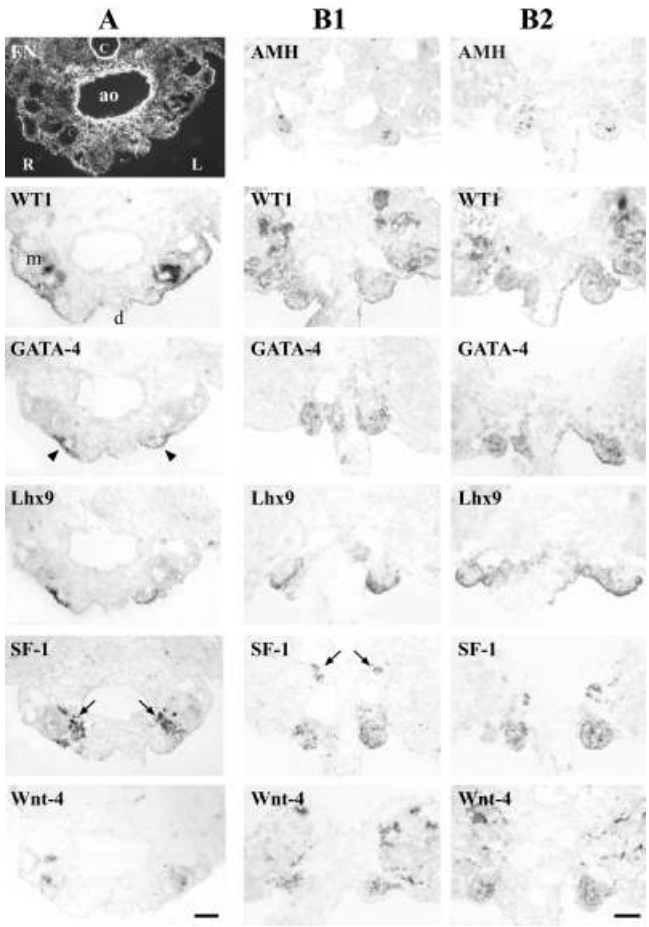

Fig. 1. Chick embryo "indifferent" gonads. Fibronectin (FN) is detected by histoimmunology, expression of *AMH*, *WT1*, *GATA-4*, *Lhx9*, *SF-1*, and *Wnt-4* by in situ hybridization of digoxigenin-labeled RNA probes on cryostat sections. **A:** Serial transverse sections of a female chick embryo at Hamburger and Hamilton stage (HH) 24 (embryonic day [E] 4.0). Female (**B1**) and male (**B2**) chick embryo gonads at HH27 (E5.0). m, mesonephros; d, dorsal mesentery; ao, aorta; c, chorda. Orientation is the same for all the sections: L, left; R, right. Arrowheads indicate the position of the genital ridges; arrows point to adrenals. At E5.0, *AMH* is expressed in a few cells of the medullar part of the gonads in both sexes. None of the factors displays a sexually dimorphic pattern of expression. Scale bars = 100 μm in A, B2 (applies to B1,B2).

As seen from fibronectin immunostaining, the genital ridge is not yet identifiable at the surface of the mesonephros. *WT1* is widely expressed in the coelomic epithelium, the subjacent mesenchyme and in mesonephric structures, as shown previously (Kent et al., 1995), in accordance with a role in gonadal and renal morphogenesis (Kreidberg et al., 1993).

At this stage, two factors, *GATA-4* and *Lhx9*, display a restricted expression. Both are expressed in the same small area of the coelomic epithelium on the ventromedial aspect of the mesonephros near the root of the dorsal mesentery, as mouse or porcine *GATA-4* homologues (Viger et al., 1998; McCoard et al., 2001). No expression is seen in the depth of the mesonephros. The

limited expression of these two factors, which will later be expressed specifically in the gonads, indicates the position of the presumptive genital ridge.

Transcripts for the steroidogenic factor SF-1 are detected in the urogenital ridge from stage HH21 by whole-mount in situ hybridization (Smith et al., 1999b). At HH24 (Fig. 1A), they are essentially located in groups of cells in the depth of the mesonephros that correspond to the adrenal anlage (Hatano et al., 1994; Luo et al., 1994). *SF-1* expression is very limited in the presumptive genital ridge. Only a small number of positive cells are present in the region expressing *GATA-4* and *Lhx9*. Such a delay in *SF-1* expression in the genital ridge, compared with *Lhx9*, is in agreement with the conclusions drawn from Lhx9-deficient mice (Birk et al., 2000). Lhx9 is presumably required for proliferation of the gonad cell population able to express *SF-1*.

Wnt-4, in both sexes, is expressed essentially in the mesonephros. *DMRT1*, in accordance with its detection from stage HH19 by reverse transcriptase (RT)-PCR (Raymond et al., 1999b), displays very faint expression in the coelomic epithelium overlying the mesonephros and no obvious sex difference is noticed (not shown). At this stage, the *AMH* gene is not transcribed and *SOX9* is not detected in the genital ridge (Oréal et al., 1998).

In conclusion, gonadal commitment occurs in a territory at the crossing of expression domains for several differentiation factors not necessarily restricted to the genital ridge or strictly overlapping. *GATA-4* and *Lhx9* transcripts present the most specific location.

Dispersed Medullar *AMH* Expression in the Indifferent Gonads

We have shown previously (Oréal et al., 1998) that the first cells positive for *AMH* mRNA appear at the same time, stage HH25, in male and female gonads morphologically indistinguishable and thus called "indifferent." This phase is illustrated at HH27 (E5.0) in Figure 1B (B1 for the female and B2 for the male). The gonads are already well identified as protrusions at the surface of the mesonephroi composed of an outer epithelial zone or "cortex," wider in left gonads, and an inner fibronectin-positive region containing irregular clusters of cells, the so-called "medulla." *AMH* transcripts are found in a few cells dispersed in the inner fibronectin-positive part of the gonads. No expression is seen in the cortical area.

WT1, *GATA-4*, *Lhx9*, *SF-1*, and *Wnt-4* (Fig. 1B) are expressed in the gonads without any obvious sexual dimorphism. *WT1*, *GATA-4*, and *SF-1* expression is generalized to the entire gonads. *WT-1* is also expressed in mesonephric structures and *GATA-4* in the root of the dorsal mesentery. Two distinct groups of cells expressing *SF-1* correspond to gonads and adrenals (Hatano et al., 1994). *Lhx9* and *Wnt-4* have expression patterns limited to specific regions of the gonads: *Lhx9* in the outer gonad epithelial layer and subjacent cells, and *Wnt-4* essentially in the inner part

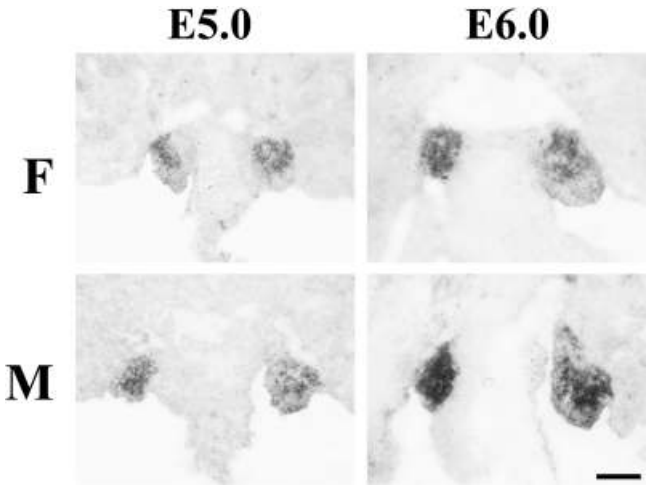

Fig. 2. *DMRT1* expression in chick embryo gonads at Hamburger and Hamilton stage (HH) 27 (embryonic day [E] 5.0) and HH28 (E6.0), in female (F) and male (M) embryos. A significant *DMRT1* sex dimorphism is apparent at E6.0. Scale bar = 100 μm.

of the gonads and in mesonephric structures. At HH27 (E5.0), as seen in Figure 2, *DMRT1* is expressed throughout the gonads and, because of the limitations of the in situ hybridization technique, we are not able to reveal a clear sexual dimorphism at this stage, unlike other authors (Raymond et al., 1999b; Smith et al., 1999a). In these gonads, as shown previously (Oréal et al., 1998), *SOX9* transcripts are not detected, whereas they are abundant in surrounding tissues (not shown).

Thus at this stage, *AMH* expression is gonad-specific but not sex-specific. This early transcription occurs in both sexes in the absence of SOX9 and has no equivalent in mammals. *DMRT1*, *SF-1*, *WT1*, *GATA-4*, and *Wnt-4* are expressed in the inner part of the gonads, where the *AMH* expressing cells arise. However none of these factors has an expression pattern limited enough to be the unique inducer of *AMH* transcription, which may be triggered by a specific dosage of several factors coexpressed in a limited number of medullar cells or by another yet unidentified factor. *Lhx9* does not appear to be concerned with *AMH*, because its expression domain is different.

Establishment of the Sexual Dimorphism

As previously reported (Oréal et al., 1998), *AMH* expression increases dramatically in male gonads from HH28 and a low level of *SOX9* transcripts is detected, which is conserved at HH29 and HH30, that is, during more than 1 day, before a real increase concomitant with testicular cord formation. Cells expressing *AMH* organize into clusters before forming well-delineated testicular cords. We have chosen to present, in Figure 3, the expression pattern of the different factors at HH30+ (E7.0), an intermediate stage characterized by *AMH* up-regulation in male gonads before the appearance of well-defined sex cords. Comparison is made

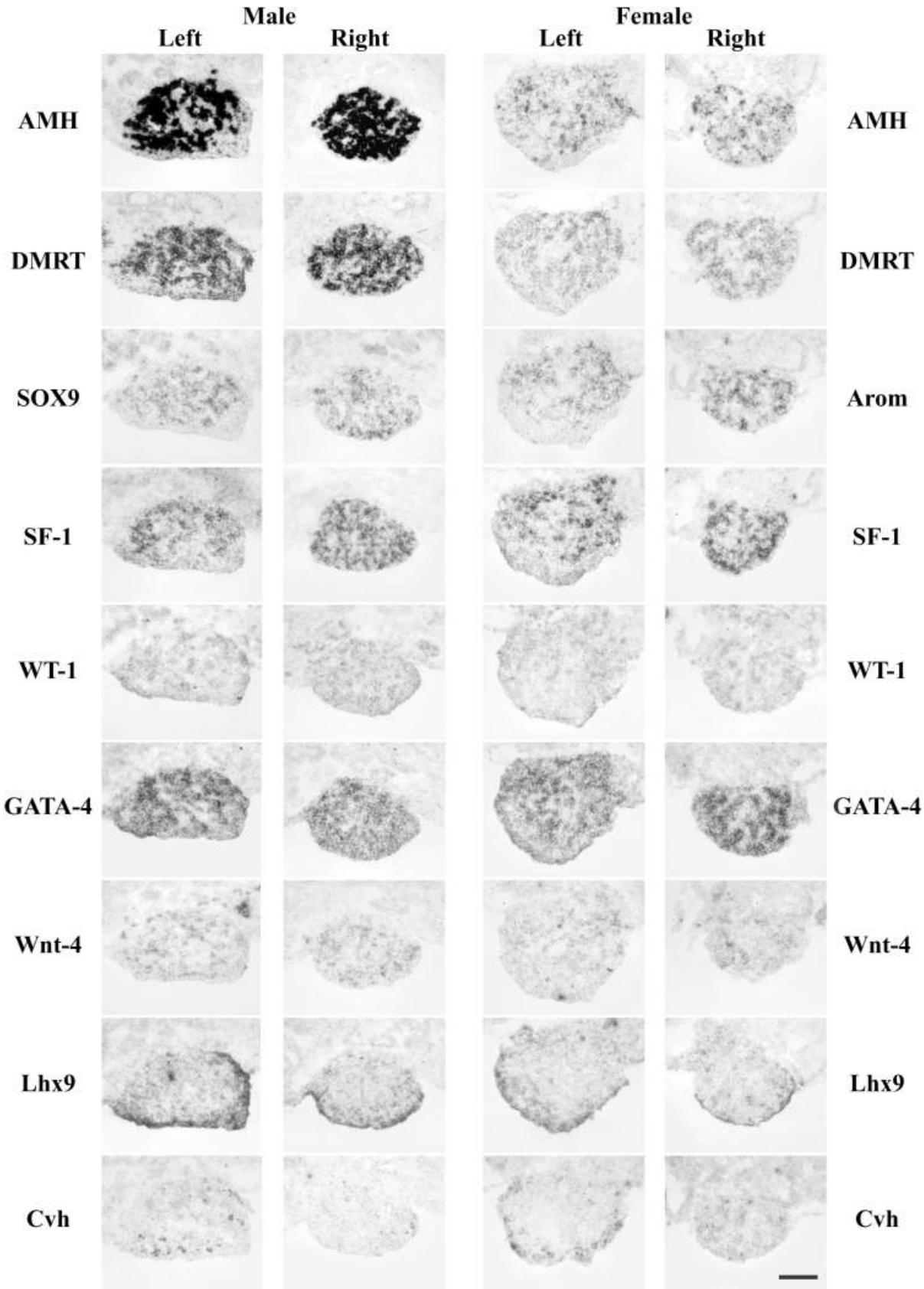

Fig. 3. Male and female embryonic gonads at Hamburger and Hamilton stage (HH) 30+ (embryonic day [E] 7.0). Expression of *AMH*, *DMRT1*, *SOX9* (male only), *aromatase* (Arom) (female only), *SF-1*, *WT1*, *GATA-4*, *Wnt-4*, *Lhx9*, and *Cvh*. Male gonads are characterized by a high level of expression of *AMH* and *DMRT1*, the expression of *SOX9*, and the formation of the testicular cords. Female gonads express *aromatase* and retain a dispersed medullar pattern of *AMH* expression. Other factors do not present a significant sexual dimorphism. In both sexes, left–right asymmetry is reflected in the germ cell number and the wider left cortical area characterized by *Lhx9* expression contrasting with the general or medullar-specific pattern of the other factors. Scale bar = 100 μm.

with female gonads at the same age. The left–right asymmetry is visible in both sexes. Left gonads possess a distinct cortical area, whereas right gonads are restricted to the medullar part surrounded by a thin cortical layer.

Sexual dimorphism is manifested in the expression of *AMH*, *DMRT1*, *SOX9*, and *aromatase*. In testes, *AMH* is highly expressed in aggregates of medullar cells, whereas the cortical area is almost devoid of expression. In the right testis, because of the reduced cortical thickness, *AMH* expression gets closer to the surface. In some places, these clusters are getting organized into more compact cords. In contrast, in the female, the "dispersed medullar" pattern of *AMH* expression described for the indifferent gonads is retained. Expression in left and right female gonads is similar and much lower than in testes.

DMRT1 expression appears clearly dimorphic at HH28 (E6.0), as shown in Figure 2, and furthermore, at HH30+ (E7.0) (Fig. 3), being more than twice higher in male than in female gonads as previously determined (Smith et al., 1999a). The transcripts are colocalized with those of *AMH* in the forming testicular cords. Some expression is also detected in the cortical region. In female gonads, the expression is low, more homogeneous than that of *AMH*, and preferentially located in the dense medullar cords.

We have previously shown that the formation of testicular cords is correlated with a strong increase in *SOX9* expression at HH31 (Oréal et al., 1998). At HH30+ (E7.0), an intermediate stage, *SOX9* transcripts are absent in female gonads (not shown) and moderately expressed in clusters of cells in testes with a distribution similar to that of *AMH* (Fig. 3).

In contrast with these factors, which have a male-specific expression or up-regulation, *aromatase* is expressed exclusively in female gonads (the negative male gonads are not shown). Transcripts, which are detected from E6.0 in accordance with published data (Yoshida et al., 1996), are present at similar levels in left and right gonads, in groups of cells dispersed within the medulla (Fig. 3). Estrogens may play an immediate role in ovarian differentiation, in particular on cortex development, because the estrogen receptor is already expressed in the cortex from E4.5 in both sexes (Smith et al., 1997). Indeed, estrogens are necessary for female gonad differentiation because inhibition of aromatase activity results in masculinization of the female gonads with induction of *SOX9* expression, high levels of *AMH*, and formation of testicular-like cords (Nishikimi et al., 2000; Vaillant et al., 2001).

The other factors, *SF-1*, *WT1*, *GATA-4*, *Wnt-4*, and *Lhx9*, do not display any significant sexual dimorphism. *SF-1* transcripts are located in the medullar part of the gonads and appear colocalized with those of *AMH* in testes. Expression levels per cell are heterogeneous; however, the global level appears similar in both sexes as found at this stage by RNAse protection (Smith et al., 1999b). Thus, *SF-1* expression is not down-regulated in female chick embryos as it is in mice (Shen et al., 1994). *WT-1* and *GATA-4* gene transcription occurs preferentially, in both sexes, in dense regions of the medullar zone corresponding mainly but presumably not exclusively to the forming testicular cords in testis.

Wnt-4 displays a low expression. In both sexes, its expression decreases after E6.0 to reach background in the second half of the incubation (not shown). In contrast, in mice, *Wnt-4* is down-regulated in male gonads while continuously expressed in embryonic ovaries. This sexual dimorphism has been related to the delayed start of steroidogenesis in females. *Wnt-4* is supposed to prevent synthesis of androgens in mouse ovaries because enzymes involved in this synthesis and not present in normal embryonic ovaries are expressed in *Wnt-4* knockout mice (Vainio et al., 1999). Wnt-4 does not play the same role in chick gonads, where steroidogenic enzymes are expressed early in both sexes (Yoshida et al., 1996; Nomura et al., 1999).

Lhx9 expression pattern, in both sexes, reflects the left–right asymmetry of cortical areas. As stated previously, the transcripts are preferentially in the gonad surface epithelium and subjacent cells, a region devoid of *AMH* expression. The inner limit of the *Lhx9* transcription domain is not well defined, and dispersed positive cells are seen within the medulla.

Location of the gonocytes was determined by using the germ cell lineage marker *Cvh*. The previously described asymmetry of colonization of gonad anlagen by primordial germ cells (Van Limborgh, 1968) is obvious: the number of *Cvh*-positive cells is higher in the left than in the right gonad and in female than in male. At HH30+ (E7.0), in the male left gonad, germ cells are mostly located at the periphery of the gonad and not systematically associated with the *AMH*-expressing cells in the forming testicular cords. In the left ovary, they are essentially cortical. In both sexes, only a few dispersed germ cells are visible in the right gonad.

Thus, at this stage, we observe in the testes a new type of AMH pattern, that we would call "cordonal," with a high level of expression. This change is associated with the sexually dimorphic expression of *DMRT1* and *SOX9*. Conversely, female gonads, which do not have SOX9 but express *aromatase*, retain the low-level, "dispersed medullar" *AMH* expression pattern.

Cordonal AMH Expression in Male Gonads

With progression of the differentiation, testicular cords containing Sertoli and germ cells become separated from a mesenchymal interstitial tissue by a basement membrane. At P1, the day of hatching (Fig. 4), the male cordonal type of gonad structure and pattern of *AMH* expression are more defined than in early stages. *SOX9*, *DMRT1*, *WT1*, and *GATA-4* are colocalized with *AMH* in somatic cells of the testicular cords. *SF-1* is expressed both inside the cords like *AMH*, and at a significant level in interstitial islets corresponding

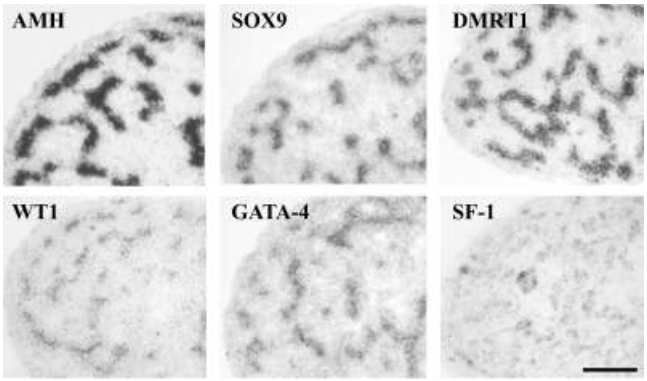

Fig. 4. Neonate testis. Expression of *AMH*, *SOX9*, *DMRT1*, *WT1*, *GATA-4*, and *SF-1* at posthatching day 1. *AMH* colocalizes with the other factors in testicular cords. Scale bar = 100 μm.

to the steroidogenic Leydig cells. *Lhx9* is not present in the testicular cords (not shown).

Testicular cords provide a clear situation in which *DMRT1*, *SOX9*, *SF-1*, *WT-1*, and *GATA-4* are coexpressed with *AMH* in the same cells, the Sertoli cells. Therefore, any one of these factors may potentially have an effect on its regulation. By contrast, *Lhx9*, which has a different pattern of expression, is not involved in *AMH* control in the embryo. Only *SOX9* and *DMRT1* are up-regulated in male gonads as is *AMH*. These two factors might be responsible for the increase in *AMH* transcription; however, such a time correlation is not enough to establish proof. Conversely, a repressive action of estrogens in female gonads cannot be ruled out.

Juxtacortical Medullar AMH Expression in Female Gonads

In female gonads, the second half of the embryonic life is characterized by the development of the left ovarian cortex. This stage is illustrated in Figure 5, in embryos at E14 and in newly hatched chicken at P1. The left ovary is made of a fibronectin-positive medulla containing lacunae and a well developed, essentially fibronectin-negative, cortex. Occasional strands of fibronectin-positive material often in continuity with the medulla split up the cortex, sometimes up to the surface epithelium, infiltrating between fibronectin-negative cell clusters. The right gonad, which stops growing, is essentially composed of a medulla covered by a thin epithelium.

In the left ovary, *AMH*-expressing cells, previously dispersed throughout the medulla, accumulate in the juxtacortical part of the medulla, a dense fibronectin-positive tissue. Furthermore, some *AMH*-positive cells are included in the strands of fibronectin-positive material splitting up the cortex. The cortex plays presumably an active role in this phenomenon. Indeed, the right gonad in which cortex does not develop retains the "dispersed medullar" pattern of *AMH* expression, as shown at E14 (Fig. 5).

In the left ovary, the cortex is characterized by the presence of the germ cells, labeled by the *Cvh* probe, as seen at E14. They are associated in groups where they divide by synchronous mitosis (Erickson, 1974). Premeiotic DNA synthesis starts between E15 and E16, and by E17 leptotene and zygotene figures are detected (Ukeshima and Fujimoto, 1991). A few germ cells remain dispersed in the medulla in the left as in the right (not shown) gonads.

At E14, *Lhx9* expression is strictly restricted to the gonad surface epithelium and to somatic cells of the fibronectin-negative parts of the cortex. The inner limit of its expression is now well defined as the limit of the cortex. At P1, a double-labeling for fibronectin and *Lhx9* at high magnification shows that the cortical fibronectin-negative nests are composed of clusters of *Lhx9* mRNA-negative cells, presumably germ cells, surrounded by one layer of *Lhx9*-positive cells. In some places, the *Lhx9*-positive surface epithelial layer is in continuity with the nests. *Lhx9* and *AMH* are clearly expressed in different cells at this step.

During this period, *aromatase* transcripts are found in the external half of the medulla, sometimes in cells lining the smaller lacunae. In contrast with *AMH*, *aromatase*-expressing cells do not accumulate at the border of the cortex and do not invade the cortical area, as seen at E14.

SF-1 expression, at E14, is still heterogeneous in intensity and distributed throughout the medulla in both gonads. It may correspond to more than *aromatase*- and *AMH*-expressing cells. Notably, *SF-1* is expressed in the depth of the medulla where these two genes display little expression. *SF-1* transcripts are not particularly abundant in the juxtacortical medulla and in the fibronectin-positive strands that invade the cortex where *AMH* is expressed. Thus, its involvement in *AMH* expression in these cells is questionable.

Expression of *Wnt-4* (Fig. 5), *WT1*, and *GATA-4* (not shown) is very low in E14 ovaries. Down-regulation of *GATA-4* in the fetal ovaries was also observed in mouse (Viger et al., 1998).

In conclusion, in embryonic and neonate female gonads before the formation of follicles, *AMH* is expressed in more or less dispersed cells in the medulla and the fibronectin-positive cortical strands. This "juxtacortical medullar" localization is an evolution of the previous "dispersed medullar" expression without any major change. This expression pattern is completely separate from that of *Lhx9*. It does not correlate strictly with those of *WT1*, *GATA-4*, *SF-1*, and *Wnt-4* nor with that of *aromatase*.

Cortical *AMH* Expression in Female Gonads

After hatching, development of the left ovarian cortex is characterized by the arrest of the oocyte meiotic prophase at the diplotene stage and the formation of follicles, completed by P22 (Ch'in Suang et al., 1979). We present in Figure 6 three steps of this differentiation: at P7, P21, and P28. We observe the invasion of

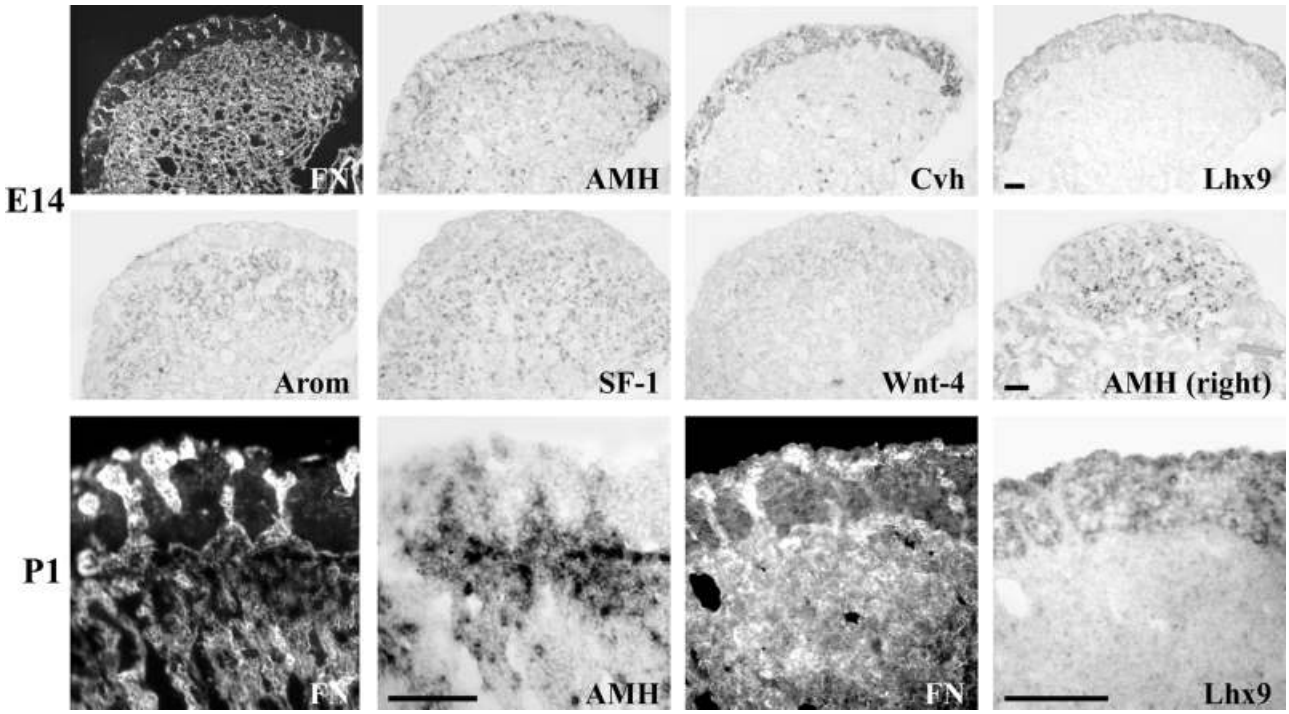

Fig. 5. Female gonads at embryonic day (E) 14 and posthatching day (P) 1. At E14: immunostaining of fibronectin (FN), expression of *AMH*, *Cvh*, *Lhx9*, *aromatase* (Arom), *SF-1* and *Wnt-4* in the left ovary, and *AMH* expression in the right gonad (last picture). Fibronectin and adjacent *AMH* are double labeling of the same section. At P1: expression of *AMH* and *Lhx9* in the left ovary. In both cases, the image of the same section labeled for fibronectin is given. *AMH* is expressed in cells located in juxtacortical medullar and cortical fibronectin-positive regions, whereas *Lhx9* is present in the surface epithelium and in cells at the periphery of the cortical fibronectin-negative nests. Groups of germ cells occupy the center of the nests. Scale bars = 100 μm.

the cortical region by fibronectin-positive material, which makes it less distinct from the medulla. The cortical germ cell clusters dissociate and form fibronectin-negative follicles composed of a single oocyte surrounded by a layer of cuboid follicular cells (granulosa cells). Expression of *Cvh* mRNA is observed in oocytes, as shown at P7.

At P7, *AMH* transcripts are present in the cortical area in two different locations. As in mammals, they are expressed in the follicular cell layer. But, unlike in mammals, they are also found in abundance in unorganized cortical interstitial cells located in the fibronectin-positive tissue that separates the follicles. Little expression remains in the medulla. During the following weeks, the cortex undergoes an important development and becomes lobulated. At P21, the ovary contains small follicles at the periphery and larger ones underneath, both expressing *AMH*. *AMH* remains also highly expressed in interstitial cells. At P28, the situation evolves toward a more structured pattern. *AMH* is expressed in the follicles and to a lesser extent in cortical interstitial cell clusters getting organized into discontinuous circles around the follicles. Few *AMH*-positive cells remain dispersed. To ensure the specificity of the labeling, we have tested a second *AMH* probe corresponding to the 3′-untranslated part of the *AMH* mRNA and got identical results, ruling out the possibility of cross-reactions with other members of the TGF-β superfamily.

During this period, *Lhx9* is expressed in the epithelium covering the gonad that is sometimes irregular and even exfoliating, as seen at P7. *Lhx9* is also expressed in the follicular cells of young follicles. At P21, the expression is high in small follicles. It decreases with their growth. Thus, in the ovarian cortex, the follicular cells of young follicles express at the same time *AMH* and *Lhx9*, a situation that was not observed at earlier stages in gonad development.

Another striking event concerns the expression of *Wnt-4*, which had declined to nearly background level in ovaries before hatching. In the week after hatching, this gene starts to be highly expressed in the follicular cells, as seen at P7. Wnt-4, which is an autoregulator of the mesenchymal to epithelial transformation in kidneys (Kispert et al., 1998), is expressed here in cells that organize to form the epithelial follicular layer. No expression is observed outside the follicles. At P14, *Wnt-4* mRNA is revealed both in the follicular cell layer and in the oocyte of some of the follicles (not shown). During the following weeks, follicular cells remain positive and oocytes accumulate a high amount of *Wnt-4* mRNA as seen at P21. RNA synthesis starts in oocytes from the early diplotene (Ch'in Suang, 1979) and various mRNAs accumulate within the oocyte cytoplasm

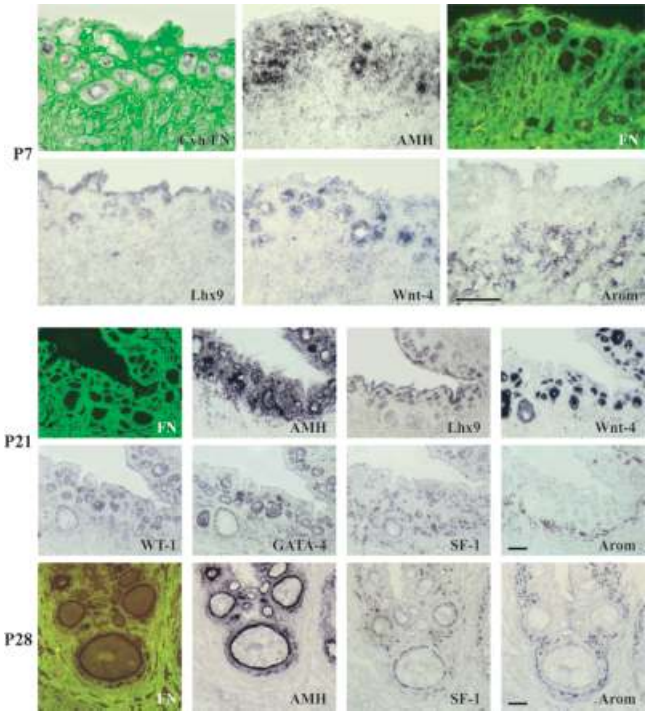

Fig. 6. Postnatal left ovary at P7, P21 and P28, formation of the follicles. Immunostaining of fibronectin (FN) and expression of *Cvh*, *AMH*, *Lhx9*, *Wnt-4*, *WT1*, *GATA-4*, *SF-1*, and *aromatase* (Arom). At P7, the double labeling of a single section for *Cvh* and fibronectin was superimposed by digital image processing; AMH and fibronectin on its right is a double-labeled section. *AMH* is expressed in follicular cells and in cortical interstitial cells. Scale bars = 100 μm.

to be translated after fertilization. *Wnt-4* mRNA may be either stored for use in the first steps of embryogenesis or immediately translated, and the protein secreted by the growing oocyte may play a role in oocyte maturation or follicle maintenance. Such a postnatal expression of *Wnt-4* in granulosa cells and in oocytes, not yet documented in mammals, would explain the degeneration of the oocytes and subsequent differentiation of cord-like structures observed in *Wnt-4* mutant female mice after birth (Vainio et al., 1999).

Follicular cells of small and large follicles also express *WT1* and *GATA-4* at a level higher than the cortical interstitial tissue, as seen at P21. *WT1* expression has also been reported in laying hen immature follicles (Chun et al., 1999). *SF-1* is present in the follicular cells and heterogeneously in interstitial cortical cells. However, low if any expression of *SF-1* is observed outside the follicles in the cortical region that expresses *AMH* at high level as seen at P21 and P28.

During this period, an evolution in the distribution of *aromatase*-expressing cells occurs. They were located in the ovarian medulla from E6. At E14 (Fig. 5), they were mostly found in the subcortical zone, where they still are at P7 (Fig. 6), unlike *AMH*-expressing cells that have invaded the cortex. *Aromatase* cells do not enter the cortex but remain underneath and organize progressively in scale-like clusters around the larger follicles, as seen at P21. In the following week, the limit between cortex and medulla becomes imprecise and the *aromatase*-expressing cells form a layer underlying and progressively surrounding the most developed follicles, as seen at P28. This structure is likely the anlage of the theca cell layer. In adult hens, *aromatase* is expressed in the theca externa of preovulatory follicles (Nitta et al., 1991).

In summary, in the chick immature left ovary, *AMH* transcripts are present in two different locations. First, they are expressed in the follicles, in the follicular cell layer surrounding the oocyte. This expression is similar to what is observed in mammals where *AMH* is expressed in granulosa cells of primary and small antral follicles (Bézard et al., 1987) and colocalizes with that of *WT1*, *GATA-4*, and *SF-1*. In addition, chick follicular cells express *Wnt-4*, and *Lhx9* is present in the youngest follicles. *SOX9* is not expressed. Second, *AMH* is also transcribed in cortical interstitial cells located between the follicles, which, at later stages, become oriented around them. Contrary to the follicular cells, which are epithelial, these interstitial cells are dispersed within a mesenchymal fibronectin-positive tissue. They do not express *Wnt-4* or *Lhx9*. Expression in these cells of *WT1*, *GATA-4*, and *SF-1* is low, if any. By its localization and the factors it expresses, this cell population appears to be the same as the "dispersed medullar" cells observed in the ovary at earlier stages. It has no equivalent in mammalian ovaries, in which *AMH* is neither expressed in the embryo nor outside the follicles.

Do these two different sets of *AMH*-expressing cells observed in the chick postnatal left ovary correspond to two independent cellular populations? In this case, follicular cells would then derive from cortical *Lhx9*-positive cells that start to express *AMH* when forming the follicles. In the other case, follicular cells would derive from medullar cells already expressing *AMH* that acquire *Lhx9* expression as they organize into the follicular layer. Other studies will be necessary to answer this important question.

CONCLUSION

This study of *AMH* transcription, conducted in parallel with that of several differentiation factors possibly involved in its regulation and in gonad sex determination and/or differentiation, has allowed us to distinguish different types of *AMH* expression in chick gonads. Two types have equivalents in mammals: expression in testicular cords and in ovarian follicles.

In the male, *AMH* is highly expressed in the Sertoli cells, which acquire an epithelial structure as they associate and form the testicular cords. In these cells, *AMH* is coexpressed with *SOX9*, *DMRT1*, *SF-1*, *WT1*, and *GATA-4*, which all may potentially play a role in its regulation, *SOX9* and *DMRT1* having a sex-dimorphic pattern.

In the female left ovary after hatching, *AMH* is transcribed in the follicular or pregranulosa cells, as in mammals. In these epithelial cells, *AMH* is coexpressed with *SF-1*, *WT1*, *GATA-4*, and also, in chick, with *Wnt-4* and *Lhx9*. This expression occurs in the absence of SOX9. However, chick differs from mammals in that *AMH* is also expressed from E5.0 in dispersed cells of the medullar part of indifferent gonads in both sexes. This expression precedes, in the male, *SOX9* expression, the testis-specific *AMH* up-regulation, and the formation of testicular cords. This type of expression is conserved in the medulla of female gonads during embryonic life with some modifications in its distribution in the left gonad, and persists in the left ovary several weeks after hatching as a dispersed cortical expression in interstitial cells between the follicles. This bird-specific type of *AMH* expression occurs in fibronectin-positive regions, is independent of SOX9 and Lhx9, and does not seem to be strictly correlated with *SF-1*, *WT1*, *GATA-4*, or *Wnt-4* expression. The combination of factors controlling the *AMH* promoter activity in these various situations appears different and deserves further study.

EXPERIMENTAL PROCEDURES

Sex Determination

Commercial white Leghorn eggs were incubated at 38°C. In young embryos, the genetic sex was determined according to Griffiths et al. (1996). Fragments of the *CHD-W* and *CHD-Z* genes, located on W and Z chromosomes, respectively, are amplified from genomic DNA by using primers P2: 5′ TCTGCATCGCTAAATCCTTT 3′ and P3: 5′ AGATATTCCGGATCTGATA 3′. The 120-bp PCR products are digested with the restriction enzyme *Hae*III, which cleaves only the PCR fragment derived from *CHD-Z*. In males (ZZ), the PCR product is fully cleaved into two smaller bands, whereas in females (ZW), only half of the material is digested.

Tissue Preparation

Dissected gonads, associated or not with the mesonephros, depending on the age, were fixed 1 hr in 2% paraformaldehyde-phosphate buffered saline (PBS). After washing in PBS with increasing concentrations of sucrose (0, 12, 15, and 18%), specimens were embedded in Tissue-Tek O.C.T. compound (Miles, Inc., Kankakee, IL) and frozen at −20°C. Cryostat sections (5-mm thickness) were mounted on slides coated with 2% 3-aminopropyltriethoxysilane (Sigma, St. Louis, MO, A-3648) and stored at −20°C.

In Situ Hybridization

In situ hybridization was performed as previously described (Oréal et al., 1998) by using digoxigenin-labeled riboprobes. The *AMH* probe (Carré-Eusèbe et al., 1996) corresponds to an 821-bp fragment of chick cDNA (accession no. X89248, position 2121-3108) subcloned into pBluescript KSII(+) (Stratagene). After linearization of the plasmid, transcription took place with T7 or T3 RNA polymerases by using digoxigenin-labeled UTP generates the sense or antisense probes. A second AMH probe, corresponding exclusively to the 3′ untranslated sequence, was isolated by PCR from the cDNA clone. Other chick probes were obtained by RT-PCR. The PCR fragments were cloned into Bluescript or pGEM-T Easy (Promega) vectors, sequenced with the Sequenase sequencing kit (Amersham), and transcribed as above by using T3, T7, or Sp6 RNA polymerases. Genbank accession numbers and nucleotide positions are as follows: *AMH*, X89248, position 3475-4000; *Aromatase*, J04047, position 247-674; *Cvh*, AB004836, position 1-685; *DMRT1*, AF211349, position 11-709; *GATA-4*, U11887, position 1-608; *Lhx9* (also called LH-2B), L35566, position 4-624; *SF-1*, AB002404, position 731-1554; *SOX9*, U12533, position 751-1373; *Wnt-4*, D31900, position 866-1383; *WT1*, X85731, position 124-731. Hybridization with sense probes verified the specificity of the signal.

Immunofluorescence

To identify gonadal structures, immunofluorescence was performed as previously described (Oréal et al., 1998) by using as primary antibody a rabbit anti-human plasma fibronectin serum (Life Technologies, Grand Island, NY). The reaction was performed on sections previously treated by in situ hybridization (double labeling) or adjacent sections. Sections were washed in PBS, and processed for indirect immunofluorescence detection as described (Fridmacher et al., 1995).

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