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Mechanism of asymmetric ovarian development in chick embryos

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In most animals, the gonads develop symmetrically, but most birds develop only a left ovary. A possible role for estrogen in this asymmetric ovarian development has been proposed in the chick, but the mechanism underlying this process is largely unknown. Here, we identify the molecular mechanism responsible for this ovarian asymmetry. Asymmetric *PITX2* expression in the left presumptive gonad leads to the asymmetric expression of the retinoic-acid (RA)-synthesizing enzyme, *RALDH2*, in the right presumptive gonad. Subsequently, RA suppresses expression of the nuclear receptors *Ad4BP/SF-1* and estrogen receptor α in the right ovarian primordium. Ad4BP/SF-1 expressed in the left ovarian primordium asymmetrically upregulates cyclin D1 to stimulate cell proliferation. These data suggest that early asymmetric expression of *PITX2* leads to asymmetric ovarian development through up- or downregulation of *RALDH2*, *Ad4BP/SF-1*, estrogen receptor α and cyclin D1.

KEY WORDS: Pitx2, Asymmetry, Chick, Estrogen, Ovary

INTRODUCTION

The vertebrate body plan is organized by three orthogonal axes: the anterior-posterior, dorsal-ventral and left-right (L-R) axes. A complex series of genetic interactions controls proper L-R axis development, which leads to later L-R asymmetry of the visceral organs. This process is evolutionarily conserved in vertebrates and relies upon genes encoding transcription factors and growth factors (Levin, 2005; Raya and Belmonte, 2006). *PITX2*, a member of the conserved bicoid-type homeobox gene family (Gage et al., 1999), has been implicated in the establishment of L-R asymmetry through its expression in the left lateral plate mesoderm. Indeed, *Pitx2*-knockout mice exhibit abnormal visceral organ asymmetry (Lin et al., 1999; Lu et al., 1999).

In mammals, the gonads develop bilaterally through the orchestrated action of a number of genes (Ross and Capel, 2005; Swain and Lovell-Badge, 1999). Many of these genes have been identified through the study of knockout mice and patients suffering from gonadal developmental abnormalities. However, no gonad defects identified so far have exhibited clear laterality, and no genes involved in gonad development are expressed with L-R asymmetry. Unlike mammals, most female birds develop ovaries only on the left side, while males develop bilateral testes. During early developmental stages before sexual differentiation, chick embryonic gonads show no obvious morphological L-R asymmetry and consist of two components, the cortex and medulla (Smith and Sinclair, 2004). After sexual differentiation, testicular development occurs bilaterally in the male (genetically ZZ). The testicular cords appear in the medulla where Sertoli and Leydig cells differentiate, while the cortex regresses and eventually disappears. In female birds (genetically ZW), the left cortex proliferates and develops into the ovary, whereas the right cortex disappears (Smith and Sinclair, 2004). Studies of chick gonad development implicate estrogen in asymmetric ovarian development. Indeed, estrogen receptor α (*ERα*) is expressed in the left but not the right cortex (Nakabayashi et al., 1998) of both sexes (Andrews et al., 1997). However, it has not been known how asymmetric *ERα* expression is induced and whether or not asymmetric estrogen signaling is related to asymmetric ovarian development.

Retinoic acid (RA) plays a variety of roles throughout development (Niederreither et al., 1999; Sakai et al., 2001) and functions by binding to the nuclear receptors RAR and RXR to regulate gene expression. The synthesis and distribution of RA is controlled by the coordinated expression of genes encoding RA-synthesizing retinaldehyde dehydrogenases (*RALDH1*, *RALDH2* and *RALDH3*) (Zhao et al., 1996; Niederreither et al., 1997; Swindell et al., 1999; Mic et al., 2000; Niederreither et al., 2002) and RA-metabolizing cytochrome P450 proteins (*CYP26A1*, *CYP26B1* and *CYP26C1*) (Fujii et al., 1997; Swindell et al., 1999; MacLean et al., 2001; Tahayato et al., 2003; Reijntjes et al., 2004). Because RA is involved in cell growth and differentiation, cell-cycle accelerator and suppressor genes have been studied as potential RA targets (Chen and Ross, 2004; Dragnev et al., 2004; Guidoboni et al., 2005).

Here, we investigate the roles of *RALDH2*, *PITX2*, *ERα* and *Ad4BP/SF-1* (Ad4-binding protein/steroidogenic factor 1) in L-R asymmetric ovarian development in chicks. We demonstrate that a genetic cascade including these factors differentially regulates cyclin D1 gene expression in the left and right cortices and eventually causes asymmetric cell proliferation.

MATERIALS AND METHODS

Whole-mount in situ hybridization

Chick cDNA was supplied as follows: *Ad4BP/SF-1* from Dr Sutou (Kudo and Sutou, 1997); *RALDH2* and *CYP26A1* from Dr Eichele (Swindell et al., 1999); *RALDH1* and *RALDH3* from Dr Noda (Suzuki et al., 2000); *RARβ*

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from Dr Nohno (Nohno et al., 1991); *CYP26B1* (clone ID: ChEST239e5), *RXRα* (clone ID: ChEST142D11), *RXRβ* (clone ID: ChEST102B17), *RXRγ* (clone ID: ChEST248B14), *LHX9* (clone ID: ChEST664O12) and cyclin D1 (clone ID: ChEST223D23) from the Medical Research Centre Geneservice (Cambridge, UK); and *RARα* (clone ID: pgp1n.pk009.m12) from the Delaware Biotechnology Institute, University of Delaware (Newark, DE). cDNA encoding mouse Pitx2c was supplied by Dr Semina (Semina et al., 1996). Chick cDNA for *PITX2a* was amplified by 5′-RACE (Invitrogen, Carlsbad, CA) with the primer shown in Table 1. Chick cDNA for *PITX2c*, *RARγ* and *ERα* was amplified with primers listed in Table 1. As the probe for *PITX2a* contains a region common to *PITX2b*, the probe detects both *PITX2a* and *PITX2b*. These cDNAs were cloned into pCR II-TOPO vector (Invitrogen) and digoxigenin-labeled probes were prepared according to the manufacturer's instructions (Roche Diagnostics, Penzberg, Germany). Whole-mount in situ hybridization was performed as described (Yoshioka et al., 1998). After staining, the embryos were embedded in 2% gellan gum followed by sectioning with a microslicer (Dosaka EM, Kyoto, Japan).

Cell number counting and cell proliferation assay

Differentiation stages are defined according to Hamburger and Hamilton (Hamburger and Hamilton, 1951). Three embryos at stage 27 [embryonic day 5-5.5 (E5-5.5)] and 29 (E6-6.5) were fixed with 4% paraformaldehyde (PFA)/PBS and cross-sectioned. Five sections containing the gonad were randomly selected for each embryo and were labeled with 4′,6-diamidino-2-phenylindole (DAPI) and mouse anti-cytokeratin monoclonal antibody (1:100; Lab Vision Corp., part of Thermo Fisher Scientific, Fremont, CA) for 1 hour at 37°C to visualize the cortical region (Oreal et al., 1998). To detect cytokeratin, Alexa-Fluor-555-labeled goat anti-mouse antiserum (1:200; Molecular Probes, Willow Creek Road, Eugene, OR) was used for secondary labeling. DAPI-positive nuclei in the cortical (cytokeratin-positive) and medullary (cytokeratin-negative) regions were counted and summed across all five sections for each embryo. The mean and standard deviation were determined using data from all three embryos. The relative cell numbers are presented as described in the figure legends. Bromodeoxyuridine (BrdU)-labeling of chick embryos and detection of the BrdU-labeled cells were basically performed as described elsewhere (Yoshioka et al., 2005). In brief, 1 μl BrdU solution (10 mg/ml in H_2O) was injected into untreated or manipulated (as described below) embryos through the vitelline vein at stage 27 or 29. After 1 hour incubation, the embryos were fixed with 4% PFA/PBS and cross-sectioned. Three embryos were used for each experiment. Five sections containing the gonad were randomly selected for each embryo and were subjected to immunofluorescence. The sections were incubated with sheep anti-BrdU antiserum (1:300; Exalpha Biological, Watertown, MA) and mouse anti-cytokeratin monoclonal antibody for 1 hour at 37°C. Alexa-Fluor-488-labeled donkey anti-sheep antiserum (1:200; Molecular Probes) and Alexa-Fluor-555-labeled goat anti-mouse antiserum were used for secondary detection. The number of BrdU-positive and total DAPI-stained cells in the cortical and medullary regions was determined. Statistical analysis and calculation of relative cell numbers are described above.

Manipulation of chick embryos

Manipulation of chick embryos of both sexes was performed as described previously (Yoshioka et al., 2005). To construct *Pitx2-en*, the repressor domain of *Drosophila engrailed* (amino acids 1-296) (Jaynes and O'Farrell, 1991) was fused to the carboxy-terminus of *Pitx2c*. cDNAs for *Pitx2*, *Pitx2-en*, *Ad4BP/SF-1* and alkaline phosphatase (*AP*) were cloned into the avian viral vector RCASBP (A) and transfected into chick embryonic fibroblasts to generate virus-producing cells. *Pitx2*, *Ad4BP/SF-1* and *AP*-expressing cells were implanted into the right lateral plate mesoderm of stage 11–12 embryos at the level of the presumptive gonad, while the *Pitx2-en* and *AP*-expressing cells were implanted into the left presumptive gonad (see Fig. S1A in the supplementary material). After being incubated for 72 (stage 27) or 96 hours (stage 29), the embryos were fixed with 4% PFA-PBS and subjected to whole-mount in situ hybridization or the cell proliferation assay described above. AG1-X2 ion-exchange resin beads (150–200 μm diameter; BioRad Laboratories, Hercules, CA) were soaked in 10 μM all-*trans*-RA (Wako Pure Chemical Industries, Osaka, Japan) or 12.7 mM retinoid antagonist AGN 193109 (Allergan, Irvine, CA) (Mercader et al., 2000) dissolved in dimethylsulphoxide (DMSO). The beads were washed with PBS, and implanted into the coelomic epithelium of the left or right presumptive gonad region at stage 18-19 (see Fig. S1B in the supplementary material). After 48 (stage 27) or 72 hours' (stage 29) incubation, the embryos were subjected to whole-mount in situ hybridization or the cell proliferation assay.

Table 1. Oligonucleotide primers used for 5′-RACE and PCR to synthesize in situ probes

Primer	Sequence (5′ to 3′)
For 5′-RACE	
PITX2a	CACGCAGGCTGAGACAAGTTTA
For PCR	
PITX2c	ATGAGTTGCATGAAGGACCCG
	TGCGGCCTCGGGGCTGGAGGT
RARγ	CAGCAGGGCGCACCAGGAGAC
	GTCCTGCCGGTCCCCGCAGAT
ERα	GCCTCTGGAGTTACCCTGCTG
	GCTCCCTTTCTCATTGGTACT

Sexing of tissue samples

The sex of chick embryos was determined by the presence or absence of the W chromosome. To detect the W chromosome, the W-specific *Xho*I repeat was amplified by PCR with genomic DNA as a template. *β-actin* was amplified as a control (Kent et al., 1996).

RESULTS

Left-right asymmetric morphology and gene expression in chick embryonic gonads

To better understand the detailed morphological changes that occur in the chick gonad before sexual differentiation, we prepared cross sections at stage 27 and stage 29 (see Fig. S2A-D in the supplementary material). No morphological differences were observed between the right and left cortices at stage 27, with both cortices composed of one or two layers of columnar epithelia. By stage 29, the left cortex became thicker with several more layers than the right cortex. The number of left cortical cells increased by 2.1-fold during this time, but no change in the number of right cortical cells was evident (Fig. 1A). This asymmetric event was observed in both female (ZW) and male (ZZ). By contrast, the number of cells in the medulla increased approximately four-fold bilaterally (Fig. 1B).

As differences in cell number can arise from differential cell proliferation and/or cell death, we examined cell proliferation by BrdU incorporation in embryos at stage 27 (Fig. 1C) and 29 (Fig. 1D). BrdU-positive proliferating cells were visualized by immunofluorescence (Fig. 1C,D, green) and the cortical region of the developing gonad was visualized by anti-cytokeratin staining (Fig. 1C,D, red) (Oreal et al., 1998). The number of BrdU-positive cells in the left cortices of ZW and ZZ embryos were 1.8-fold and 1.6-fold higher, respectively, than in the right cortices at stage 27 (Fig. 1E), and this bias persisted through stage 29. By contrast, there was no significant asymmetry observed in the BrdU incorporation in the medullae during the same time period (Fig. 1F). Next, we examined whether apoptotic cell death occurs asymmetrically in the developing gonad. Only a few TUNEL-positive cells were detected on both sides of the cortex and medulla, and their numbers did not increase between stages 27 and 29 in either sex (see Fig. S2G,H in the supplementary material). Taken together, these data indicate that increased proliferation in the left cortex, rather than increased apoptosis in the right cortex, is primarily responsible for the observed asymmetric cortical development.

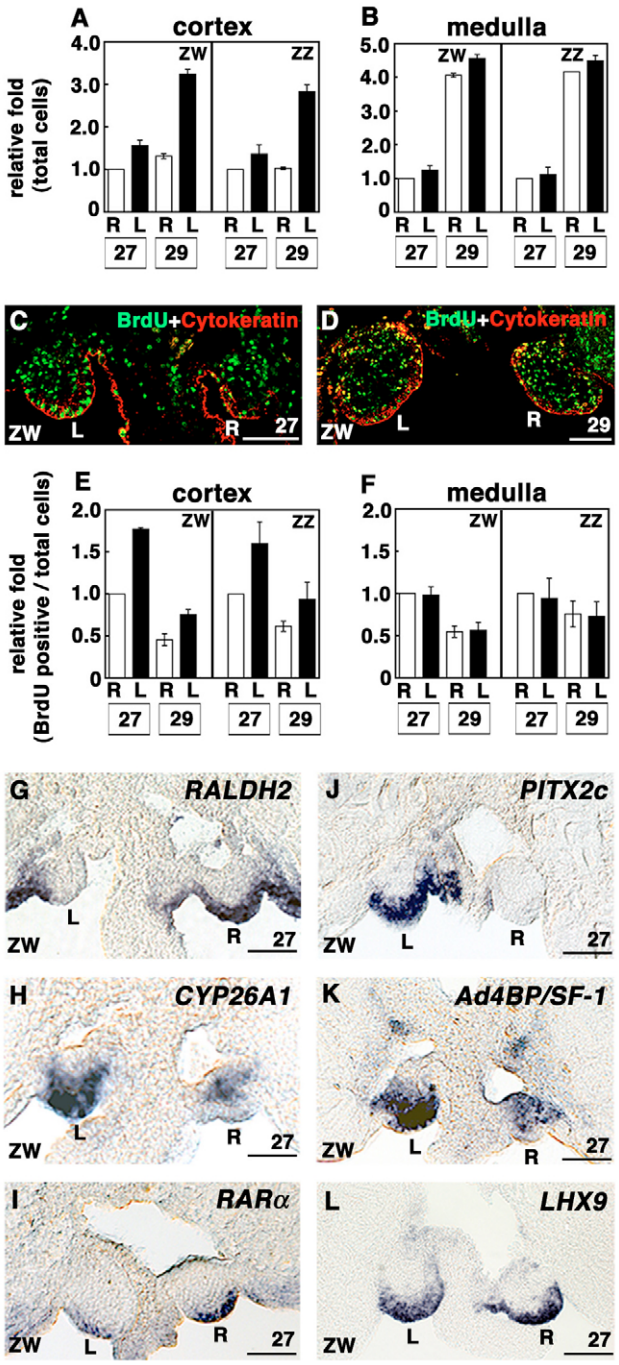

Fig. 1. L-R asymmetric development and gene expression in developing gonads of chick. (**A**,**B**) The number of cortical and medullary cells was determined as described (see Materials and methods and Fig. S2E,F in the supplementary material). The relative fold changes in the number of cortical (A) and medullary (B) cells are plotted, with the numbers in the right cortex and right medulla of the female gonad at stage 27 set at 1. (**C-F**) Cell proliferation was examined at stage 27 (C) and stage 29 (D). The developing gonads in BrdU-labeled embryos were sectioned and subjected to immunostaining for BrdU (green) and cytokeratin (red) (C,D). Relative fold changes in the number of BrdU-positive cells in the gonadal cortex and medulla are plotted, with the numbers in the right cortex (E) and right medulla (F) of the female gonad at stage 27 set at 1. (**G-L**) The expression of *RALDH2* (G), *CYP26A1* (H), *RARα* (I), *PITX2c* (J), *Ad4BP/SF-1* (K) and *LHX9* (L) in the embryonic gonad at stage 27 was examined in both sexes by whole-mount in situ hybridization. Sections of female (ZW) gonads are shown. R and L indicate right and left, respectively. ZW and ZZ indicate female and male, respectively. Scale bars: 100 μm.

Although the gonad is still structurally symmetric at stage 27, we hypothesized that some genes are expressed asymmetrically at this stage. As RA signaling has been implicated in cell proliferation, we examined the expression of genes involved in RA signaling (*RALDH1*, *RALDH2*, *RALDH3*, *CYP26A1*, *CYP26B1*, *RARα*, *RARβ*, *RARγ*, *RXRα*, *RXRβ* and *RXRγ*). In addition, we examined the expression of genes implicated in establishing the L-R asymmetric body plan (*PITX2a*, *PITX2b* and *PITX2c*). Interestingly, *RALDH2* and *CYP26A1* were expressed asymmetrically in a mutually exclusive fashion at stage 27. *RALDH2* was expressed in the right cortex (Fig. 1G), whereas *CYP26A1* was expressed in the left cortex and in both sides of the medullae (Fig. 1H). *RARα* (Fig. 1I) and *RXRα* (data not shown) expression was stronger in the right cortex than in the left, and was not detected in the medullae, suggesting that the right cortex is activated by RA signaling. *PITX2c* was expressed only in the left cortex, showing a good correlation with a previous report (Fig. 1J) (Logan et al., 1998). As there is genetic evidence that *Ad4BP/SF-1* and *LHX9* are essential for gonad development (Birk et al., 2000; Luo et al., 1994), we also examined their expression. The pattern of *Ad4BP/SF-1* expression was similar to that of *CYP26A1* (Fig. 1K), whereas *LHX9* was expressed symmetrically (Fig. 1L). Interestingly, asymmetric gene expression occurred only in the cortex in both sexes. The other genes examined were not expressed in the gonad at stage 27.

Effects of RA signaling on L-R asymmetry of embryonic gonads

We next wished to determine the functional relationship between the asymmetrically expressed genes. The complementary expression of *RALDH2* and *CYP26A1* suggested that a higher concentration of RA is present in the developing right cortex. As RA signaling is probably related to the absence of *PITX2* and *Ad4BP/SF-1*, we hypothesized that RA downregulates *PITX2* and *Ad4BP/SF-1* in the right cortex, while in the left cortex, inhibition of RA signaling leads to the upregulation of a variety of genes. To test this hypothesis, we implanted beads soaked in RA or an RA antagonist (RAA) in the region of the developing left or right gonad, respectively, at stage 19 and the treated embryos were then incubated to stage 27. In the left gonad implanted with the RA beads, *Ad4BP/SF-1* expression was substantially reduced in the cortex but persisted in the medulla (Fig. 2A, arrowhead; number of affected male embryos/total number of male embryos manipulated (male)=12/14; number of affected female embryos/total number of male embryos manipulated (female)=13/15). However, the expression of *PITX2c* in the left cortex was not downregulated by RA (Fig. 2B; male=0/11, female=0/12). Conversely, implantation of the RAA beads increased the expression of *Ad4BP/SF-1* in the right cortex (Fig. 2C, arrowhead; male=12/15, female=13/16), but the RAA beads did not affect the expression of *PITX2c* in the right cortex (Fig. 2D; male=0/10, female=0/11). Implantation of DMSO beads had no effect on expression of either *Ad4BP/SF-1* (Fig. 2E; male=0/12, female=0/14) or *PITX2c* (Fig. 2F; male=0/11, female=0/11).

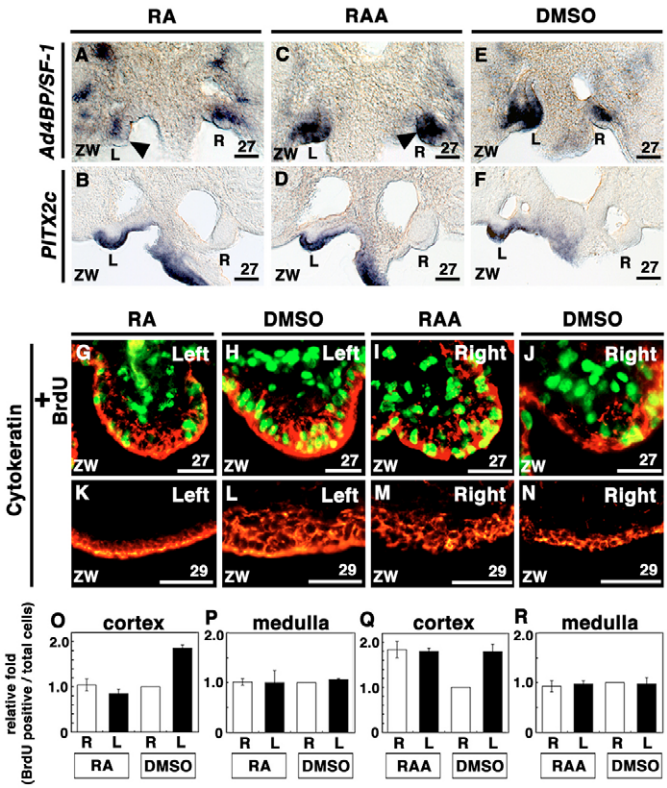

Fig. 2. Effects of RA signaling on the expression of *PITX2c*, *Ad4BP/SF-1* and cell proliferation in chick. (**A-F**) RA and RAA beads were implanted into the left (A,B) and right (C,D) presumptive gonad regions of both sexes, respectively, at stage 18-19. As controls, DMSO beads were implanted (E,F). 48 hours after implantation (stage 27), the embryos were subjected to whole-mount in situ hybridization with *Ad4BP/SF-1* (A,C,E) and *PITX2c* (B,D,F) probes, then the embryos were sectioned. Sections of female (ZW) samples are shown. Arrowheads in A and C indicate affected expression of *Ad4BP/SF-1*. (**G-N**) The effects of implantation of RA (G) and RAA (I) beads on cell proliferation were examined at stage 27 in female embryos. DMSO beads were used as a control (H,J). BrdU was injected into the operated embryos 1 hour before fixation, then gonads were stained with antibodies for BrdU (green) and cytokeratin (red). Effects of RA (K) and RAA (M) beads on cortical thickness were examined by staining with anti-cytokeratin antibody 72 hours after the implantation (stage 29). DMSO beads were used as controls (L,N). (**O-R**) Cell proliferation was analyzed quantitatively in gonads of embryos implanted with RA (O,P), RAA (Q,R) and DMSO beads at stage 27. Total DAPI-stained cells (data not shown) and BrdU-positive cells in the gonadal cortex (cytokeratin-positive) (O,Q) and medulla (cytokeratin-negative) (P,R) were counted. Relative fold changes in the number of BrdU-positive cells in the cortex and medulla are plotted, with the number of BrdU-positive cells in the right cortex of DMSO-bead-implanted embryos set at 1. Scale bars: 100 μm in A-J; 50 μm in K-N.

As the L-R cortical asymmetry seen at stages 27-29 is caused by increased cell proliferation rather than cell death, we examined whether RA signaling affected cortical cell proliferation. After BrdU was injected into bead-implanted embryos, we counted the number of BrdU-positive cells at stage 27. Interestingly, the number of BrdU-positive cells decreased in the left cortex implanted with RA beads compared with DMSO-treated embryos (Fig. 2G,H,O), but there was no corresponding effect in the medulla (Fig. 2P). Conversely, the number of BrdU-positive cells increased in the right cortex implanted with the RAA beads (Fig. 2I,J,Q), with no effect on the medullary cell number (Fig. 2R). We next examined the morphologic effects of bead implantation. By stage 29, the thickness of the left cortex, as visualized by cytokeratin immunostaining, was reduced in the RA-bead-implanted embryos (Fig. 2K,L), while the right cortex thickened following RAA bead implantation (Fig. 2M,N).

Effect of exogenous PITX2 and Ad4BP/SF-1 on the L-R asymmetry of embryonic gonads

Our data indicate that exogenous RA can suppress the expression of *Ad4BP/SF-1* but not *PITX2c*, suggesting that PITX2 inhibits the expression of *RALDH2* in the left cortex. To test this hypothesis, we prepared expression plasmids encoding either wild-type mouse Pitx2 or a dominant-negative form, Pitx2-En. The transcriptional activities of Pitx2 and Pitx2-En were examined using luciferase reporter gene assays. As expected, Pitx2 induced robust luciferase activity, whereas Pitx2-En suppressed transcription driven by Pitx2 (see Fig. S3 in the supplementary material).

We implanted cells producing either *Pitx2*- or *Pitx2-en*-encoding viruses at stage 11-12 into the presumptive right or left gonad, respectively. Because the viruses were replication competent, transgene expression expanded throughout the gonad region (Fig. 3A,D,G). Operated chick embryos were then incubated to stage 27, and the expression of *RALDH2* and *Ad4BP/SF-1* was examined. Exogenous expression of *Pitx2* led to reduced expression of *RALDH2* in the right cortex (Fig. 3B, arrowhead; male=11/14, female=12/16), and increased expression of *Ad4BP/SF-1* (Fig. 3C, arrowhead; male=10/14, female=12/15). By contrast, expression of *Pitx2-en* increased expression of *RALDH2* in the left cortex (Fig. 3E, arrowhead; male=8/11, female=10/13), and reduced expression of *Ad4BP/SF-1* (Fig. 3F, arrowhead; male=8/10, female=12/15). A control virus encoding *AP* did not affect expression of either *RALDH2* (Fig. 3H; male=0/13, female=0/15) or *Ad4BP/SF-1* (Fig. 3I; male=0/14, female=0/15).

Pitx2 is required for cell-type-specific proliferation during cardiac outflow tract (Kioussi et al., 2002) and pituitary gland (Kioussi et al., 2002; Zhu and Rosenfeld, 2004) development. Thus, we examined the effect of *Pitx2* and *Pitx2-en* on BrdU incorporation in the developing gonad. When cells producing *Pitx2*-encoding viruses were implanted into the right presumptive gonad region, the number of BrdU-positive cells increased by 2.0-fold in the right cortex at stage 27 (Fig. 3J,R), but proliferation in the medulla was not affected (Fig. 3S). By contrast, the number of BrdU-positive cells decreased in left cortices implanted with *Pitx2-en* virus-producing cells (Fig. 3L,T), whereas the medulla was not affected (Fig. 3U). Exogenous *AP* expression did not affect cell proliferation (Fig. 3K,M,R-U). Structurally, right cortices implanted with *Pitx2* virus-producing cells thickened by stage 29 (Fig. 3N,O), whereas *Pitx2-en*-implanted left cortices were thinner (Fig. 3P,Q).

Likewise, we examined the effect of exogenous *Ad4BP/SF-1* expression on the right gonad cortex (Fig. 4A). Expression of *PITX2c* nor *RALDH2* was unaffected following implantation of *Ad4BP/SF-1* virus-producing cells (data not shown). However, the number of BrdU-positive cells in the right cortex increased by 1.8-fold (Fig. 4B,C,F) and the cortical layer thickened (Fig. 4D,E). Proliferation of the right medulla was unaffected (Fig. 4G).

Regulation of cyclin D1 gene expression

Our data indicate that *PITX2*, RA and *Ad4BP/SF-1* are involved in asymmetric L-R cortical cell proliferation. In order to determine the mechanism by which these factors act, we examined their effect on the expression of the cell cycle regulators cyclin D1 and D2. At stage 27, cyclin D1 was expressed asymmetrically in the left cortex and

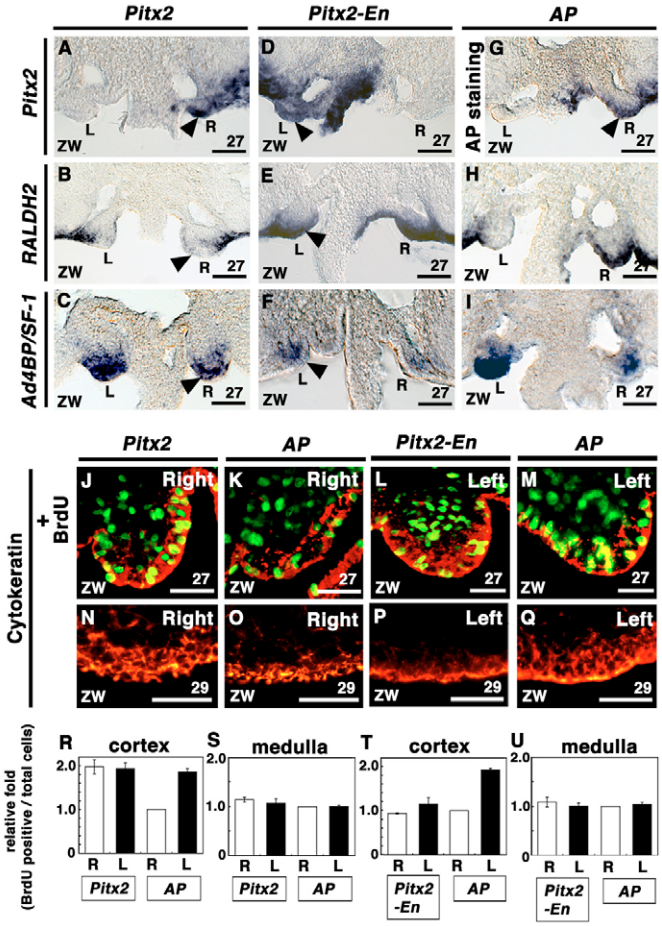

Fig. 3. Function of *PITX2* in the gonadal cortex before sexual differentiation. (**A-I**) Chick embryonic fibroblasts producing *Pitx2*- (A-C) and *Pitx2-en*- (D-F) encoding viruses were implanted into the right and left presumptive gonad regions of both sexes, respectively, at stage 11-12. As a control, cells producing *AP*-encoding virus were implanted into the right presumptive gonad regions (G-I). Distribution of the virus was examined by in situ hybridization for mouse *Pitx2* (A,D) and by AP staining (Morgan and Fekete, 1996) (G). Arrowheads in A,D,G indicate viral distribution in the developing gonads. 72 hours after implantation (stage 27), embryos were subjected to whole-mount in situ hybridization for *RALDH2* (B,E,H) and *Ad4BP/SF-1* (C,F,I). Arrowheads in B,C,E,F indicate altered expression of *RALDH2* and *Ad4BP/SF-1*. Sections of female (ZW) samples are shown. (**J-U**) The effect of exogenous *Pitx2* (J), *Pitx2-en* (L) and *AP* (K,M) on cell proliferation was examined in female embryos at stage 27. BrdU was injected into the operated embryos 1 hour before fixation, and their gonads were stained with antibodies for BrdU (green) and cytokeratin (red) (overlays in J-M). The effect of misexpressed *Pitx2* (N), *Pitx2-en* (P) and *AP* (O,Q) on cortical thickness was examined by staining with anti-cytokeratin antibody at stage 29. Cell proliferation was analyzed quantitatively in gonads misexpressing *Pitx2* (R,S) or *Pitx2-en* (T,U) at stage 27. AP was used as a control. The total number of DAPI-stained cells (data not shown) and BrdU-positive cells in the gonadal cortex (cytokeratin-positive) (R,T) and medulla (cytokeratin-negative) (S,U) was counted. Relative fold changes in BrdU-positive cell numbers in the cortex and medulla are plotted with the numbers in the right cortex and medulla misexpressing *AP* set at 1. Scale bars: 100 μm in A-M; 50 μm in N-Q.

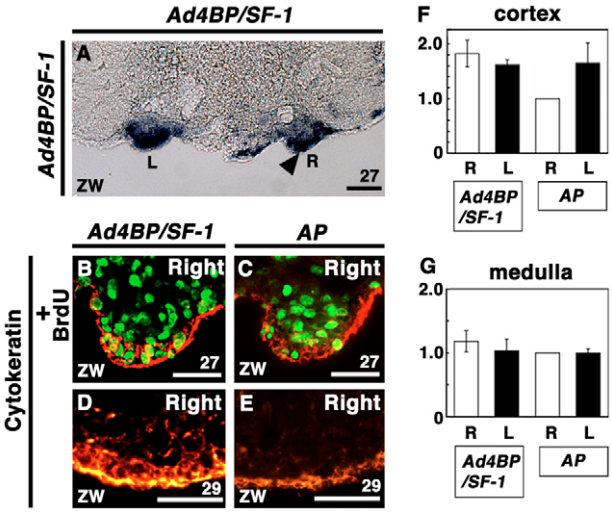

Fig. 4. Function of *Ad4BP/SF-1* in the gonadal cortex before sexual differentiation. (**A-E**) Chick embryonic fibroblasts producing *Ad4BP/SF-1* (A,B,D) and *AP* (C,E)-encoding viruses were implanted into the right presumptive gonad of both sexes at stage 11-12. The distribution of *Ad4BP/SF-1*-encoding viruses was examined by in situ hybridization for chick *Ad4BP/SF-1* (A). As chick *Ad4BP/SF-1* was used for the implantation study, in situ hybridization detected both endogenous and exogenous expression. The arrowhead in A indicates viral distribution in the developing right gonadal cortex, where *Ad4BP/SF-1* gene is not endogenously activated (see Fig. 1K). The effects on cell proliferation (B,C) and cortical thickness (D,E) were examined as described in Fig. 3. (**F**,**G**) Relative numbers of BrdU-positive cells in the cortex (F) and medulla (G). Scale bars: 100 μm in A-C; 50 μm in D,E.

bilaterally in the medulla (Fig. 5A), whereas cyclin D2 was transiently expressed on both sides of the cortex and disappeared by stage 29 (data not shown). As the expression of cyclin D1 overlapped with that of *CYP26A1* and *Ad4BP/SF-1*, we examined whether production of RA, PITX2 and Ad4BP/SF-1 leads to asymmetric cyclin D1 expression. RA and RAA beads were implanted as described. At stage 27, the expression of cyclin D1 was significantly downregulated in the RA-bead-implanted left cortices (Fig. 5B, arrowhead; male=5/8, female=6/10). Expression in the medulla was unaffected. By contrast, implantation of RAA beads in the right cortex stimulated expression of cyclin D1 (Fig. 5C, arrowhead; male=5/7, female=7/10). Embryos implanted with DMSO beads (Fig. 5D; male=0/9, female=0/9) resembled untreated embryos. When *Pitx2* virus-producing cells were implanted, cyclin D1 was upregulated in the right cortex (Fig. 5E, arrowhead; male=5/8, female=6/8), but forced expression of *Pitx2-en* reduced the expression of cyclin D1 in the left cortex (Fig. 5F, arrowhead; male=5/9, female=5/10). When cells producing *Ad4BP/SF-1*-encoding virus were implanted, the expression of cyclin D1 was upregulated in the right cortex (Fig. 5G, arrowhead; male=6/8, female=7/9). Finally, implantation of cells producing a control *AP*-virus did not affect the expression of cyclin D1 (Fig. 5H; male=0/7, female=0/9). These results indicate that cyclin D1 is negatively regulated by RA and positively regulated by PITX2 and Ad4BP/SF-1 in the developing gonad, although it remains to clarify whether the regulations are direct or indirect.

Regulation of asymmetric expression of ERα and CYP26A1

As estrogen signaling has been implicated in asymmetric ovarian development in chicks (Nakabayashi et al., 1998; Romanoff, 1960), we examined the expression of *ERα* at stage 27. As shown in Fig. 6A,

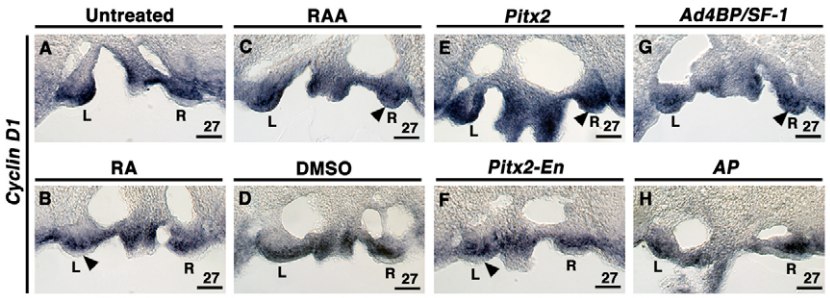

Fig. 5. Effect of RA, *PITX2* and *Ad4BP/SF-1* on cyclin D1 expression. (**A-H**) Beads soaked in RA (B), RAA (C) and DMSO (D), and chick embryonic fibroblasts expressing *Pitx2* (E), *Pitx2-en* (F), *Ad4BP/SF-1* (G) and *AP* (H) were implanted into embryos of both sexes as described in the legends to Figs 2 and 3. An untreated embryo is shown in A. Embryos were fixed at stage 27, and cyclin D1 expression was examined by whole-mount in situ hybridization. Sections of female (ZW) samples are shown. Arrowheads (B,C,E-G) indicate altered expression of cyclin D1. Scale bars: 100 μm.

ERα was expressed asymmetrically in the left but not right gonadal cortex, and symmetrically in both sides of the medulla (Andrews et al., 1997; Smith et al., 1997). This expression was identical in males and females (data not shown). When RA beads were implanted in the left developing gonad, the expression of *ERα* was significantly reduced in the left cortex (Fig. 6B, arrowhead; male=5/6, female=8/9). Conversely, RAA-bead-implantation in the right developing gonad increased the expression of *ERα* in the right cortex (Fig. 6C, arrowhead; male=5/7, female=7/9). Implantation of DMSO beads had no effect on the expression of *ERα* (Fig. 6D; male=0/6, female=0/7). Subsequently, we examined the effect of Ad4BP/SF-1 on *ERα* expression by implanting cells with *Ad4BP/SF-1*-encoding viruses in the right cortex. There was no change in cortical *ERα* expression in animals implanted with *Ad4BP/SF-1*-expressing cells (Fig. 6E; male=0/6, female=0/7) or control *AP*-expressing cells (Fig. 6F; male=0/5, female=0/5). These data suggest that asymmetric RA signaling in the developing chick gonad regulates asymmetric *ERα* expression, whereas Ad4BP/SF-1 is not involved in *ERα* expression. We further investigated whether or not estrogen regulates expression of *RALDH2*, *PITX2* and *Ad4BP/SF-1* by implanting estrogen-coated beads, but found no effect of estrogen on the expression of these genes (data not shown).

Likewise, we examined the effect of RA on the expression of *CYP26A1*. RA-bead-implantation in the left developing gonad (Fig. 6G, arrowhead; male=4/5, female=5/5) increased and expanded *CYP26A1* expression in the left medulla, while RAA-bead-implantation in the right developing gonad decreased *CYP26A1* expression in the left medulla (Fig. 6H, arrowhead; male=5/5, female=5/5). Implantation of DMSO beads into the left gonad had no effect (Fig. 6I; male=0/4, female=0/4). The effect of Ad4BP/SF-1 was also examined by implanting *Ad4BP/SF-1*-expressing cells into the right developing gonad. As was the case for control *AP*-expressing cells (Fig. 6K; male=0/5, female=0/4), *Ad4BP/SF-1*-expressing cells had no effect on the expression of *CYP26A1* (Fig. 6J; male=0/7, female=0/7). Taken together, these results show that asymmetric RA signaling in the developing gonad regulates asymmetric expression of *CYP26A1*, whereas Ad4BP/SF-1 does not.

RA signaling has been shown to activate *CYP26* gene expression during the development of several tissues (Swindell et al., 1999; Moreno and Kintner, 2004; Yashiro et al., 2004). Here, we show that *CYP26A1* expression is induced by RA signaling in the developing gonad. Interestingly, however, *CYP26A1* is not expressed in the right cortex. As the right cortex produces RA via RALDH2 and expresses *RARα* (Fig. 1I) and *RXRα* (data not shown), RA signaling is likely to be active there. Therefore, suppression of *CYP26A1* expression in the right cortex must be mediated by an unknown mechanism independent of RA signaling.

DISCUSSION

Differential gene cascades between L-R cortices

Although chick embryos form only a single ovary on the left-hand side, the gonad primordia initially develop symmetrically (Romanoff, 1960). Just before sex differentiation, the gonad cortex begins to develop morphological asymmetry, with the right cortex

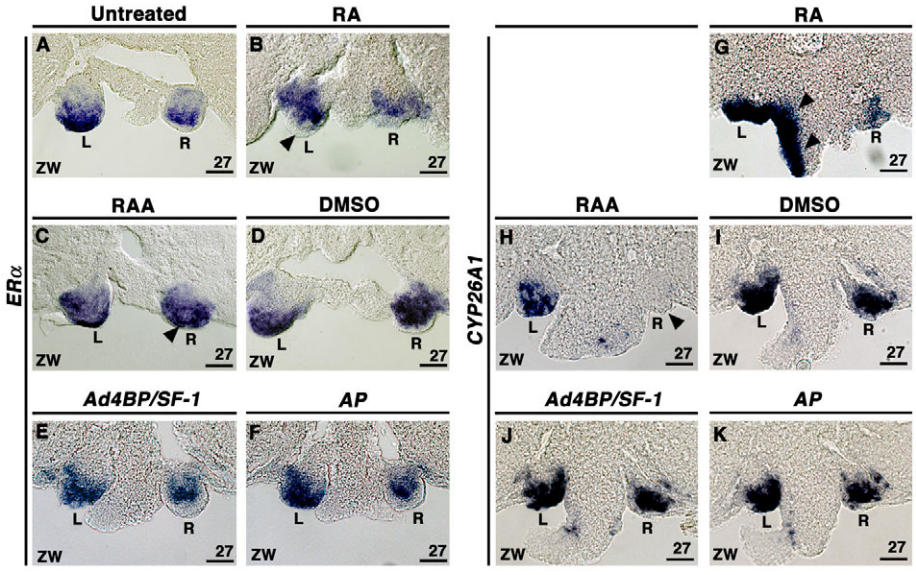

Fig. 6. Effects of RA signaling and *Ad4BP/SF-1* on the expression *ERα* and *CYP26A1*. (**A-K**) Beads soaked in RA (B,G), RAA (C,H) and DMSO (D,I), and chick embryonic fibroblasts expressing *Ad4BP/SF-1* (E,J) and *AP* (F,K) were implanted into embryos of both sexes as described in Figs 2 and 3. An untreated embryo is shown in A. The embryos were fixed at stage 27, and the expression of *ERα* (A-F) and *CYP26A1* (G-K) was examined by whole-mount in situ hybridization. Sections of female (ZW) samples are shown. Arrowheads in B and C, and in G and H, indicate affected expression of *ERα* and *CYP26A1*, respectively. Scale bars: 100 μm.

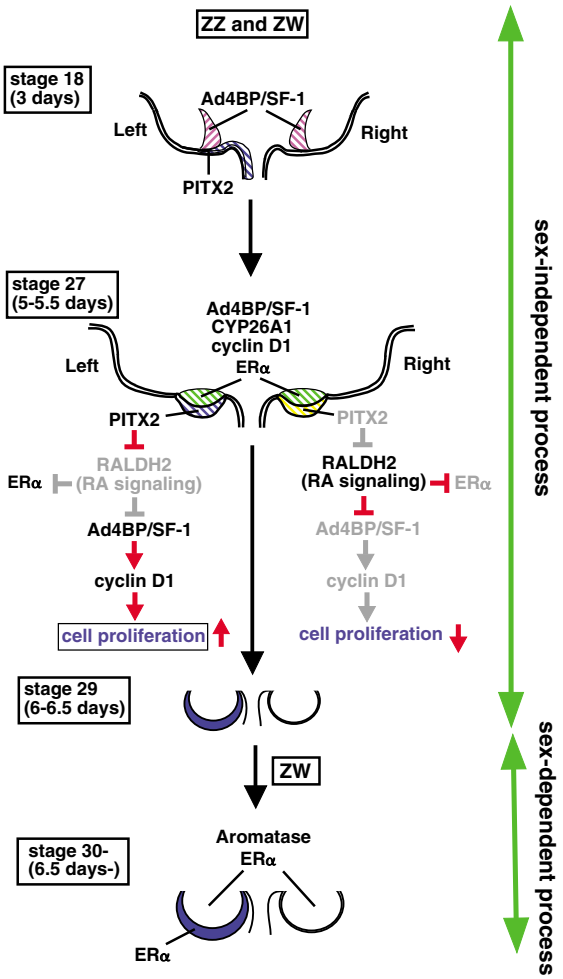

Fig. 7. Asymmetric gonad development in sexually differentiating chick embryos. Expression domains of *PITX2c*, *Ad4BP/SF-1*, *RALDH2*, *ERα* and cyclin D1 before sexual differentiation (stages 18 and 27) in the chick embryonic gonad, along with expression patterns of aromatase and *ERα* at sex-determined stages (stages 29 and 30) in the female chick embryonic gonad are illustrated. The events shown in the sex-independent process occur in both sexes. L-R differential genetic and functional cascades are indicated at stage 27, in accordance with our findings. The genes in black and gray are expressed and suppressed genes, respectively.

becoming thinner and the left cortex thickening. Given that the cortex contributes significantly to ovarian development, it seemed likely that the right gonad loses the ability to develop into an ovary at this stage. Thus, we focused on this step and investigated the molecular mechanism by which L-R asymmetry is induced in the gonadal cortices.

As summarized in Fig. 7, RA-synthesizing *RALDH2* is expressed asymmetrically in the right cortex, while RA-metabolizing *CYP26A1* is expressed in the left cortex. Likewise, *PITX2c*, *Ad4BP/SF-1* and *ERα* show asymmetric expression at the left cortex. Interestingly, the asymmetric expression of these genes occurs only in the cortex, suggesting that the initial step in L-R asymmetric ovarian development is dominated by differential expression of genes in the cortex. To investigate the functional relationship between these L-R asymmetric factors, we performed various experiments to test how forced gene expression or chemical treatment affects laterality of the developing gonads. Our data indicate that in the developing left cortex, PITX2 suppresses *RALDH2* expression, leading to reduced RA signaling and increased *Ad4BP/SF-1* and *ERα* expression. Subsequently, Ad4BP/SF-1 upregulates cyclin D1 expression and promotes cell proliferation. In the right cortex, lack of PITX2 results in upregulation of *RALDH2*, which in turn activates RA synthesis. RA then suppresses cell proliferation through downregulation of *Ad4BP/SF-1* and cyclin D1 (Fig. 7). Our current studies successfully demonstrate a functional relationship between the above components, although it still remains to be determined whether the regulation is direct or indirect.

It would be interesting to examine the later development of gonads implanted with *Pitx2*, *Pitx2-en* and *Ad4BP/SF-1*-expressing cells and RA and RAA beads. However, the operated embryos die at around stage 30, when L-R asymmetric ovarian development and gonadal sex differentiation begin, so we were not able to examine gonadal development in these animals. As operated embryos die even when control *AP*-expressing cells and DMSO beads are implanted, it appears that the embryos die from physical damage resulting from the operation itself. Novel techniques that cause less damage to the embryo are therefore necessary to address this question.

In the present study, we show that the genes involved in the initial L-R asymmetric development of the gonads are expressed similarly in both sexes, and initial morphogenetic events occur independent of sex (Fig. 7). Indeed, the medulla develops bilaterally, whereas the cortex develops only on the left in both sexes. This left-sided cortical development is thought to be important for left-sided ovarian development. Thereafter, the gonads display sexually dimorphic development, with bilateral testicular development in the male and left-sided ovarian development in the female. As estrogen activates development of the gonadal cortex into the ovary (Scheib, 1983), and estrogen is synthesized only in the female, left-sided ovarian development is achieved in the female but not the male. By contrast, as it is the medulla that contributes most to development of the testis, the testis develops bilaterally in males.

Mechanisms for L-R asymmetric cell proliferation

During the establishment of L-R asymmetry in ovarian development, cell proliferation appears to be accelerated in the left cortex and decelerated in the right cortex. Our data demonstrate that Ad4BP/SF-1 activates cyclin D1 transcription, which probably accelerates cell proliferation. Interestingly, LRH1 (NR5A2), which is highly homologous to Ad4BP/SF-1, activates cyclin D1 gene transcription through its direct interaction with β-catenin (Botrugno et al., 2004). Like LRH1, Ad4BP/SF-1 can interact directly with β-catenin to synergistically activate transcription (Mizusaki et al., 2003). Therefore, it is possible that Ad4BP/SF-1 functions in a similar manner to activate cyclin D1 gene transcription. Moreover, LRH1 activates cyclin E1 gene transcription by binding directly to its promoter. As the nucleotide sequence recognized by LRH1 is almost identical to that bound by Ad4BP/SF-1, Ad4BP/SF-1 may also regulate cyclin E1 gene transcription. In addition to the possible activation of cyclin D1 by Ad4BP/SF-1, Pitx2 also directly upregulates mouse cyclin D1 transcription (Kioussi et al., 2002; Zhu and Rosenfeld, 2004). The chick cyclin D1 promoter contains a putative PITX2-binding site, suggesting that PITX2 may also directly activate chick cyclin D1 gene expression. Moreover, the synergistic activation of LHβ transcription by Pitx2 and Ad4BP/SF-1 (Tremblay et al., 2000) suggests that cyclin D1 gene transcription might be synergistically regulated by these two factors. However, more work is necessary to determine the precise mechanism by which these factors interact.

The deceleration of cell proliferation in the right cortex appears to be mediated by inhibition of cyclin D1 expression by RA. Consistent with our present observations, other labs have shown that RA inhibits cyclin D1, cyclin E1 and cyclin-dependent kinase 6 in cultured cell lines (Balasubramanian et al., 2004; Chen and Ross, 2004; Sah et al., 2002). In addition to downregulating these cell cycle accelerators, RA stimulates the expression of several cell cycle inhibitors, including cyclin-dependent kinase inhibitor, *p27Kip1* and *p21Cip1* (Balasubramanian et al., 2004; Buzzard et al., 2003; Chen and Ross, 2004; Guidoboni et al., 2005). This is further supported by studies of *Raldh2* (also known as *Aldh1a2* – Mouse Genome Informatics) knockout mice (Lai et al., 2003), which exhibit reduced expression of *p27Kip1* (*Cdkn1b*) and *p21Cip1* (*Cdkn1a*), and increased proliferation of endothelial cells. These findings are consistent with our current observations in the developing gonad.

Interestingly, a novel role for RA was recently observed in the mouse embryonic gonad. Typically, germ cells enter meiosis in the embryonic ovary, whereas meiosis is retarded in the embryonic testis. Increased *Cyp26b1* expression in the male gonad leads to decreased RA relative to the female gonad, and the different levels of RA appear to regulate the sex-specific timing of meiotic initiation in germ cells (Bowles et al., 2006; Koubova et al., 2006). We did not observe sex-specific expression of *RALDH2* or *CYP26* in the chick gonad, but the meiotic regulation of these genes by RA could occur at a later stage, when sexual differentiation has already occurred.

Estrogen signaling and L-R asymmetric ovarian development

Previous work has shown that in ovo inhibition of estrogen synthesis by aromatase inhibitor induces bilateral female-to-male sex reversal (Elbrecht and Smith, 1992; Hudson et al., 2005; Smith et al., 2003; Vaillant et al., 2001a; Vaillant et al., 2001b). By contrast, although in ovo estrogen administration induces partial male-to-female sex reversal (ovotestis), it occurs only in the left gonad (Nakabayashi et al., 1998; Romanoff, 1960). This differential effect of estrogen has been thought to be due to the preformed differential potential of the left and right gonadal cortices (Mittwoch, 1998). Based on these observations, it was proposed that the differential potential of the cortices is established independently of estrogen signaling (Nakabayashi et al., 1998). Our present study demonstrates that RA signaling triggers two events in the right cortex: loss of responsiveness to estrogen through suppression of *ERα* expression, and inhibition of cell proliferation through suppression of *Ad4BP/SF-1* and thus cyclin D1 expression. As the left cortex retains both responsiveness to estrogen and the ability to undergo cell proliferation, it is not surprising that exogenous estrogen induces ovotestis only on the left side.

L-R asymmetric ovarian development is interesting from both an evolutionary and developmental standpoint. During L-R axis formation, L-R asymmetry at the node is transmitted to the lateral plate mesoderm by upregulation of *PITX2* expression on the left side, which is thought to result in asymmetric visceral organ development. Although the correlation between the L-R asymmetry of the body plan and ovarian development remains to be investigated, we have shown that *PITX2*, RA signaling, *Ad4BP/SF-1*, estrogen signaling and cyclin D1 are involved in the process of asymmetric ovarian development in the chick.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/4/677/DC1

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