Development 134, 4199-4208 (2007) doi:10.1242/dev.010249

PITX2 controls asymmetric gonadal development in both sexes of the chick and can rescue the degeneration of the right ovary

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The gonads arise on the ventromedial surface of each mesonephros. In most birds, female gonadal development is unusual in that only the left ovary becomes functional, whereas that on the right degenerates during embryogenesis. Males develop a pair of equally functional testes. We show that the chick gonads already have distinct morphological and molecular left-right (L-R) characteristics in both sexes at indifferent (genital ridge) stages and that these persist, becoming more elaborate during sex determination and differentiation, but have no consequences for testis differentiation. We find that these L-R differences depend on the L-R asymmetry pathway that controls the situs of organs such as the heart and gut. Moreover, a key determinant of this, *Pitx2*, is expressed asymmetrically, such that it is found only in the left gonad in both sexes from the start of their development. Misexpression of *Pitx2* on the right side before and during gonadogenesis is sufficient to transform the right gonad into a left-like gonad. In ZW embryos, this transformation rescues the degenerative fate of the right ovary, allowing for the differentiation of left-like cortex containing meiotic germ cells. There is therefore a mechanism in females that actively promotes the underlying L-R asymmetry initiated by *Pitx2* and the degeneration of the right gonad, and a mechanism in males that allows it to be ignored or overridden.

KEY WORDS: Sex determination, *Pitx2*, Left-right asymmetry, Chick embryo

INTRODUCTION

The establishment of left-right (L-R) axial patterning is crucial for the formation of several organs, including the heart and gut, for the correct asymmetric positioning of other organs, and for processes such as embryo turning (Levin, 2005; Raya and Belmonte, 2006; Shiratori and Hamada, 2006). However, early cues established from the midline that lead to stable differences in gene expression – for example, between left and right lateral plate mesoderm – can either be followed, leading to appropriate L-R patterning, or they can be overridden to permit symmetrical development. For example, in the absence of a protective signal provided by retinoic acid, the usually regular formation of somites occurs asymmetrically (Morales et al., 2007; Vermot and Pourquie, 2005). If both somitic mesoderm and lateral plate mesoderm (LPM) are subject to mechanisms promoting asymmetry, then this will also be true of intermediate mesoderm. Most intermediate mesoderm-derived structures usually develop with a considerable degree of symmetry. This is true of the urogenital system, which develops bilaterally. However, in all birds, with the exception of a few predatory species (Kinsky, 1971), female gonadal development is unusual in that only one functional ovary persists. This is normally on the left, while on the right side the ovary fails to differentiate and undergoes a degenerative fate along with its associated reproductive tract. Adult male birds have bilateral testes. There must, therefore, be a mechanism in females that actively promotes or permits L-R differences to occur, in contrast to one in males that overrides or ignores any underlying L-R asymmetry, if indeed the latter is present.

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Accepted 11 September 2007

The indifferent genital ridges arise on the ventromedial surface of the mesonephroi at around 3 days of embryonic development (E3) [equivalent to Hamburger and Hamilton (HH) stage 20 (Hamburger and Hamilton, 1992)], whereas sex-specific differentiation of the gonads becomes apparent from E6.5 (HH29-30). In ZZ embryos, both testes differentiate into functional organs with the inner medulla organised into seminiferous cords containing germ cells. In ZW embryos, an asymmetry between left and right gonads becomes very evident after E8 (HH34). While the left gonad develops a thick cortical layer with cords budding towards the medulla and enclosing most germ cells, the right fails to differentiate its epithelial layer into a cortex and all the germ cells remain in the medulla that, as in the left ovary, is made of degenerated cords. No further development occurs in the right gonad, which eventually loses germ cells and becomes vestigial (Carlon and Stahl, 1985).

Apart from the obvious functional asymmetry associated with ovarian development, one morphological difference between left and right gonads during embryonic development has been described: the left germinal epithelium is thicker than that on the right. This L-R difference is evident in both ZZ and ZW embryos within the indifferent genital ridges at HH24-25 and is maintained in both sexes through the initial steps of sex determination (HH28-30) (Carlon and Stahl, 1985). A few genes were also found to have L-R asymmetric expression patterns during ovarian development (Andrews et al., 1997; Gonzalez-Moran, 2005; Hoshino et al., 2005; Reed and Sinclair, 2002; Yoshioka et al., 2005), and one, *Bmp7*, has been shown to express asymmetrically in the indifferent ridges of both sexes (Hoshino et al., 2005). However, there have been no data linking any asymmetrically expressed factor to gonadal phenotype and to subsequent ovarian fate. Importantly, relatively little attention has been paid to the observation that asymmetries occur in both sexes from the indifferent stages of genital ridge development. These might indicate a convergence of the chick sex determination/differentiation pathway with the lateralisation signals that initiate L-R asymmetry within the early embryo.

In the last few years several studies have explored how these complex signals are interpreted at the level of single organs. One of the key genes of the L-R pathway found at the interphase between specification of the L-R biasing signal and its translation into asymmetric organ morphogenesis is *Pitx2*. This is expressed in the left LPM of mouse, chick, *Xenopus* and zebrafish, but its expression is also found and persists in organs undergoing asymmetric morphogenesis (Dagle et al., 2003; Gormley and Nascone-Yoder, 2003; Piedra et al., 1998; Schweickert et al., 2000; St Amand et al., 1998; Yoshioka et al., 1998). Misexpression of *Pitx2* in the right LPM is sufficient to alter the situs of heart, gut and embryonic rotation (Campione et al., 1999; Essner et al., 2000; Logan et al., 1998; Ryan et al., 1998). Loss-of-function experiments also support the idea that *Pitx2* plays important roles in the local generation of asymmetry within multiple organs (Ai et al., 2006; Dagle et al., 2003; Gormley and Nascone-Yoder, 2003; Kitamura et al., 1999; Liu et al., 2002; Shiratori et al., 2006). Moreover, the analysis of *Pitx2* conditional and hypomorphic mutations in mouse has shown that proper asymmetric morphogenesis of heart, lung and duodenum requires organ-specific thresholds of PITX2 activity, indicating the existence of organ-intrinsic mechanisms regulating asymmetric morphogenesis dependent upon dosage of PITX2 (Gage et al., 1999; Liu et al., 2001). Despite the enormous progress made towards the identification of the cascade of genetic interactions controlling the establishment and the propagation of L-R asymmetry signals in various vertebrates, there are no data addressing the importance of the lateralisation signal in chick gonad development.

We show here that perturbations of L-R signals also result in changes in the L-R asymmetry of the chick gonads. Furthermore, *Pitx2* is expressed in the left gonad and misexpression of *Pitx2* to the right is sufficient to induce symmetric development of the gonads as left isomers in both ZZ and ZW embryos. This transformation is sufficient to rescue the degenerative fate of the right ovary.

MATERIALS AND METHODS

Animals

Chick eggs were obtained from Winter Egg Farm (WEF), Royston, and from Henry Stewart (HS), Louth.

In-ovo drug treatment

At 0 hours of incubation, 5 ml albumen were removed from each egg and 2 ml were mixed with 400 μl Hank's Buffered Salt Solution (HBSS) with or without γ-lindane (Sigma) (40 μl from a 40 mg/ml stock in DMSO), and re-injected into the egg. Eggs were left to develop at 37.5°C until E7-8.5 (HH31-35).

RCAS(A) virus in-ovo infection

RCAS(A)Pitx2a virus (gift of Malcolm Logan, NIMR, London, UK) and control RCAS(A) virus expressing alkaline phosphatase (AP) were prepared by infection of the DF1 chick cell line as described (Logan et al., 1998). Eggs were infected at HH8-10 by injecting virus on the right side posterior to the last somite with a glass capillary needle and an Inject+matic pico-pump. Infected eggs were screened at E7 (HH31) and E12.5 (HH38-39). E7 (HH31) gonads were processed for *Pitx2* whole-mount in situ hybridisation. With some gonads, only the posterior part was analysed for *Pitx2* expression by whole-mount, and the anterior part was sectioned and analysed for other markers. Both WEF and HS eggs were used; HS eggs showed infection efficiencies that were consistently 3-4 times higher than those of WEF eggs.

Generation of RCAS(A)-Pitx2c construct and in-ovo electroporation

A chick *Pitx2c*-specific fragment was generated by PCR (Pfu Turbo, Stratagene) using the primers 5′-CCCAAGCTTGCGCTCCTTCTCCC-GTCAGCC-3′ and 5′-CTGGAGCTCCTGCGGCCTCGGGGCTGGAG-3′ on cDNA generated from E6.5 (HH29-30) gonadal RNA. This was cloned into pBluescript SKII$^-$ together with the 3′ common part of *Pitx2* fused to a triple HA-tag to obtain cPitx2c-HA. The full-length cDNA was cloned into the RCAS(A) retroviral vector. RCAS(A)Pitx2c DNA (1 μg/μl) was injected together with a plasmid ubiquitously expressing GFP into the right coelomic cavity of HH15-17 embryos (HS eggs), using a glass capillary needle and an Inject+matic pico-pump and then electroporated to the dorsal coelomic epithelium as described (Guioli et al., 2007).

Antibodies, immunohistochemistry and in situ hybridisation

The following antibodies were used: mouse monoclonals N-cadherin (1:200; Zymed) and fibronectin [1:1000; Developmental Studies Hybridoma Bank (DSHB)], rat monoclonal ERα (1:200) (Greene et al., 1984) and rabbit polyclonals LHX9 (Liem et al., 1997) and DMRT1 (1:1500). DMRT1 antibody was raised against the peptide PSIPSRGHLESTSDL from the chick protein and its specificity checked by comparing the protein expression pattern against the RNA expression pattern. Whole-mount and section in situ hybridisation protocols were as described (De Grandi et al., 2000; Dunwoodie et al., 1997). Probes specific for *Pitx2a* and *c* were generated by PCR of the specific N-terminal-encoding portion; the *Pitx2* generic probe includes sequences common to all isoforms. Urogenital ridges for histochemistry were fixed in 4% paraformaldehyde (PFA) at 4°C for 2 hours, rinsed in PBS at room temperature (RT), transferred to 30% sucrose/PBS overnight at 4°C, then embedded in OCT and stored at –80°C. Cryosections were rinsed 3× 5 minutes in PBS, blocked in PBS/0.1% Tween 20 (PBST), 0.5% BSA, 2% sheep serum for 2 hours at RT. Hybridisation with primary antibodies was at 4°C in blocking solution overnight, with the exception of ERα (overnight at 37°C). After 3× 10-minute washes in PBST at RT, sections were incubated with secondary antibodies (1:400) in PBST for 2 hours at RT, washed (3× 10 minutes in PBST) and mounted in Vectashield (Vector) containing DAPI.

RESULTS

The epithelium of left and right genital ridges differentiate asymmetrically in both sexes

Two regions are distinguishable within the indifferent genital ridge, the inner 'medulla', composed of primary cords, and the surrounding 'germinal epithelium'. The L-R gonadal differences reported so far at indifferent stages, including those to do with morphology (Carlon and Stahl, 1985) and asymmetric gene expression (Hoshino et al., 2005; Reed and Sinclair, 2002), all concern the epithelium. We therefore investigated this further, by analysing typical epithelial markers, and by revisiting the expression profile of gonad-specific markers, paying particular attention to their epithelial pattern. Fibronectin, one of the main components of basement membranes (Larsen et al., 2006), displayed a striking asymmetry. At E5 (HH26-27), the staining was similar within the two gonads, where it marked the basal laminae of the primitive medullary cords and germinal epithelium. By E6 (HH28), around the time of sex determination, fibronectin was still around the medullary cords on both sides, but a thick, although discontinuous, deposit was now present at the interface between epithelium and medulla only on the left. This difference was conspicuous in both differentiating testes and ovaries at E7 (HH31) and until E9 (HH35). By E12 (HH38), only the ovary maintained high levels of fibronectin within the expanding left cortical layer and at the interface with the medulla, whereas in the ZZ embryo the left-sided expression was downregulated to levels similar to those of the right (Fig. 1). These data reveal that the left and right epithelia have distinct properties with respect to laying down their basal lamina.

Next we found that the adhesion molecule N-cadherin (also known as cadherin 2) (Gumbiner, 2005) was highly expressed in the epithelial cells along the genital ridge and adjacent dorsal mesentery, but not along the mesonephros. As the protein localised to the cell membrane, it clearly marked the polarity of the cells and the difference in thickness between left and right. By E7 (HH31), in both

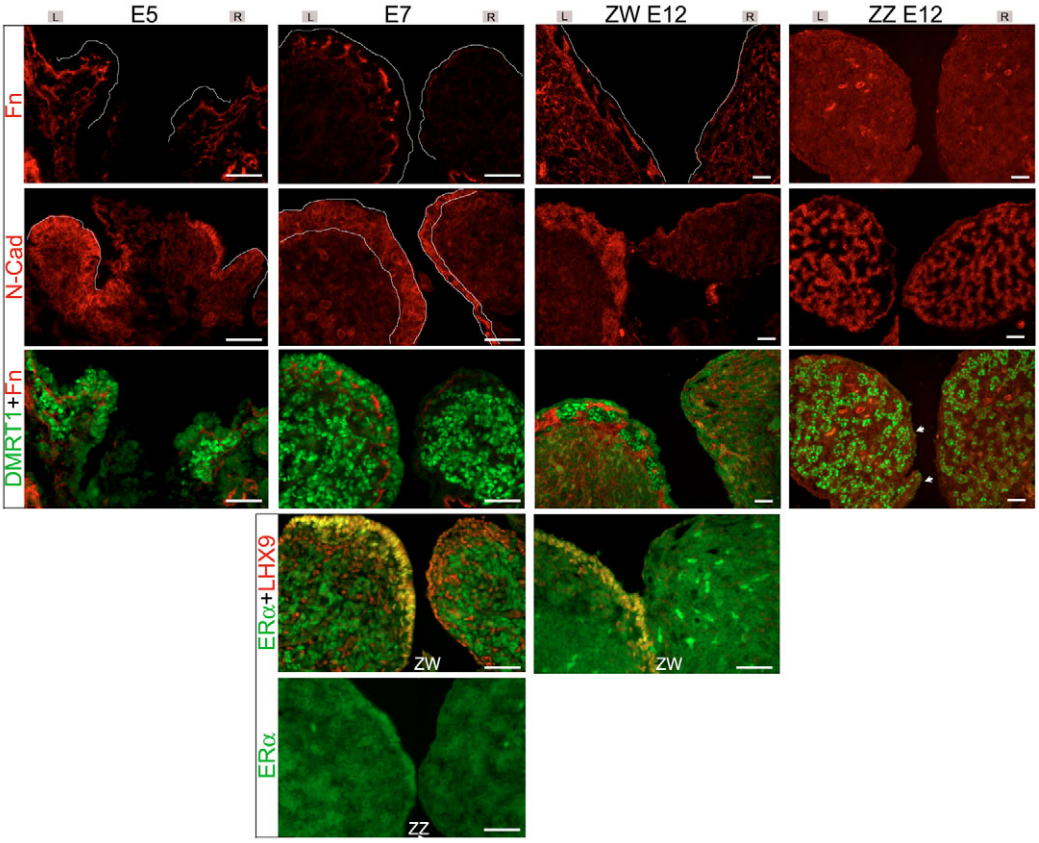

Fig. 1. Expression profile of L-R asymmetry markers within the chick genital ridges before and after sex determination. (Top three rows) Transverse sections stained for fibronectin (Fn, red), N-cadherin (N-Cad, red), and DMRT1 plus Fn (in green and red, respectively) at E5 (HH26-27), E7 (HH30-31) and E12 (HH38). At E5 and E7, ZZ and ZW embryos show similar L-R differences, and only sections from ZW embryos are shown. Arrows in the ZZ E12 panel highlight areas of the left epithelium that are still DMRT1-positive. (Bottom two rows) Expression of ERα (green) or ERα and LHX9 (red) at HH30-31 (left) and HH38 (right). L, left; R, right. Scale bars: 50 μm.

sexes the right epithelium had changed polarity, from columnar to flattened, while the left maintained its columnar shape, although having thickened to become stratified in the female. By E12 (HH38) in the male, the left epithelium was mostly flattened (Fig. 1).

DMRT1 (doublesex and mab3 related transcription factor 1) is known to be important for sex determination/differentiation in several vertebrate and invertebrate species (Koopman and Loffler, 2003). Its expression is very specific, being restricted to the gonads and, in chick, the Mullerian ducts (De Grandi et al., 2000; Raymond et al., 2000; Smith et al., 1999). We noticed that in both sexes DMRT1 was within the medulla of both gonads, and in the epithelial cells of the left (Fig. 1). This asymmetry was already evident at E5 (HH26-27) and maintained until E7 (HH31). After E7.5 (HH32), in the female the expression within the left epithelial cells was downregulated (the positive cells at E12 in Fig. 1 are germ cells), whereas in the male, epithelial cells were still weakly positive at E10 (HH36) and a few were detected even at E12 (HH38) at the edges of the gonad epithelium.

Several reports have described an asymmetric pattern of expression of estrogen receptor alpha (ERα; also known as ESR1), but with slightly different results (Andrews et al., 1997; Gonzalez-Moran, 2005; Nakabayashi et al., 1998). ERα is a nuclear receptor that controls gene transcription in response to estrogens. Its function in chick ovarian development has not yet been clarified, but much evidence suggests that it is essential for normal ovarian differentiation, as this is very much dependent on estrogens (Elbrecht and Smith, 1992; Kagami and Tomita, 1990). We found that *ERα* RNA is expressed from E6 (HH29) in both sexes, symmetrically within left and right medulla and asymmetrically in the epithelium, being only on the left (data not shown). The protein had a distribution similar to the RNA, but in the male it was very faint and mainly cytoplasmic. By contrast, in the female, ERα was nuclear and abundantly expressed by E7 (HH31) (Fig. 1). The L-R asymmetry in the female was even more evident by double staining with an antibody against LHX9, another transcription factor essential for gonadal development, at least in the mouse (Birk et al., 2000; Mazaud et al., 2002). In the chick, this marks the epithelium and its derivative cortical layer as well as a few medullary cells. We found that ERα and LHX9 co-localise only in cortical cells of the left gonad. After E7.5 (HH32), the female cortical expression of ERα increased relative to that in the medulla and, at E12 (HH38), although still seen in the epithelium and cortex, only very faint expression of ERα was detected in both the right and left medulla (Fig. 1).

These data provide clear evidence that the left and right gonads are asymmetric in both sexes from the indifferent stage. Furthermore, both sexes maintain gonadal asymmetric features well beyond the start of sex determination. It is therefore reasonable to propose that both sex determination pathways might be influenced by lateralisation signals. Our panel of markers provide readout of left and right identity; we have therefore used it as a tool to assess the 'situs' of the developing gonads in a series of functional experiments aimed to test the influence of the L-R pathway on gonadal development in ZZ and ZW embryos.

The L-R pathway controlling visceral organ situs interferes with the pathway regulating gonadal morphogenesis

Gap junction communication (GJC) in the chick blastoderm is required to establish early differences between left and right sides. Consequently, exposure to pharmacological compounds interfering with the junctions can cause heterotaxia or even complete situs inversus (Levin and Mercola, 1999). Lindane is a proven inhibitor

of GJC and relatively well tolerated by chick embryos in culture. We therefore added lindane in ovo prior to incubation and examined the embryos at E7-8 (HH31-35) for defects in the asymmetric development of the gut, as an indication of L-R pathway disturbances. Out of 76 embryos, six displayed clear inversion or malformation of gut looping. Five of these were analysed further for gonadal phenotype by assessing the expression profile of our panel of asymmetry markers. One embryo with complete situs inversus displayed gonads with complete L-R reversion of the cortical features (Fig. 2A). Of the four embryos with gut heterotaxia, one developed gonads with the usual L-R differences (data not shown), whereas the other three had symmetrical features as if they were left isomers (Fig. 2B,C). These data suggest that the signals controlling visceral organ asymmetries also control the 'situs' of the gonads.

Pitx2 is expressed in the left genital ridge and its expression correlates with 'leftness'

As PITX2 is the transcription factor at the interphase between the signals that initiate L-R differences and their translation into organ asymmetries, we analysed its expression pattern in the developing gonads of normal embryos. RNA in situ hybridisation was performed on E2-12 (HH21-38) embryos (Fig. 3A). At E2, *Pitx2* was expressed asymmetrically in the left somatoderm and splanchnic mesoderm, which contribute to the epithelium of the coelomic cavity. At E3, all the structures surrounding the left coelomic cavity were *Pitx2*-positive, including genital ridge and mesonephros. Whereas *Pitx2* was rapidly lost from the mesonephros just after E3, it was maintained in the gonad at all stages analysed. *Pitx2* localised to the gonad germinal epithelium and a few subjacent cells in both ZW and ZZ embryos, although in males the expression became almost undetectable by E12, marking very faintly the edges of the epithelium (data not shown).

Several *Pitx2* isoforms have been isolated that differ in the region 5′ to the DNA-binding domain (Cox et al., 2002; Essner et al., 2000; Schweickert et al., 2000; Yu et al., 2001). Two main isoforms, *Pitx2a* and *Pitx2c*, have been described in the chick (Yu et al., 2001); therefore, we also used in situ probes specific for the 'a' and 'c' regions. Only *Pitx2c* was detected in the developing left gonad (Fig. 3B). RT-PCR for *Pitx2a* and *Pitx2c* using RNA from E6-6.5 (HH29) left gonads confirmed the expression of *Pitx2c*, but also revealed a very faint band for *Pitx2a* (data not shown). These results suggest that the main isoform expressed within the developing gonad is *Pitx2c*, the isoform usually found correlated with L-R asymmetry (Schweickert et al., 2000; Yu et al., 2001).

We next examined *Pitx2* expression in the embryos treated with lindane. *Pitx2* was expressed in the right gonad of the embryo with complete situs inversus, but in both gonads of the three embryos with gut heterotaxia and gonadal left isomerism (Fig. 3C). So, in all samples the expression of *Pitx2* was found on the side(s) with 'left' characteristics, indicating that *Pitx2* might have a direct, instructive role in the situs-specific morphogenesis of the gonads.

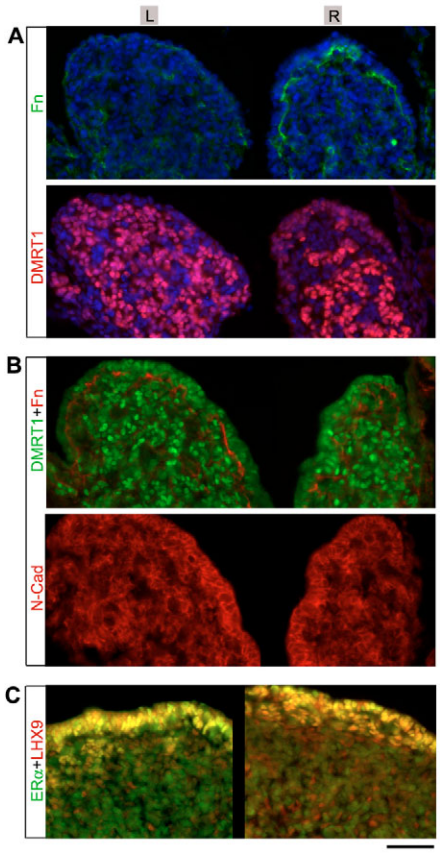

Fig. 2. Fluorescence images of transverse sections from gonads of chick embryos treated with lindane. (**A**) E8.5 (HH35) embryo with complete situs inversus. The gonads display inversion of the L-R differences within the epithelium as shown by fibronectin (Fn) and DMRT1; DAPI staining of DNA (blue). (**B**) E7 (HH31) ZZ embryo with gut heterotaxia double stained for DMRT1 and fibronectin, or for N-cadherin (N-Cad). (**C**) E7 (HH31) ZW embryo with gut heterotaxia double stained for ERα and LHX9. In B and C, both left and right gonads display a left pattern consistent with development as left isomers. L, left; R, right. Scale bar: 50 μm.

Misexpression of *Pitx2* to the right side at stage HH10 induces gonadal left isomerism

In order to misexpress *Pitx2* in the right gonad from as early as the gonad forms (HH20), we infected the right side of embryos at stage HH8-10 using RCAS virus expressing *Pitx2a* (RCAS-Pitx2a). Pilot experiments with a control virus expressing alkaline phosphatase (RCAS-AP) indeed showed that injection at HH8-10 posterior to the last somite results in the variable infection of tissues located between forelimb and hindlimb. These include body wall, mesonephros, gonad, dorsal mesentery and hindgut (data not shown).

Embryos from three independent experiments were examined at E7-7.5 (HH30-32). No embryo was identified showing any gross defect of heart or gut looping. This was expected, as no AP staining was observed within heart, foregut and midgut from the pilot or parallel control infections with RCAS-AP. In a proportion of RCAS-Pitx2a-infected embryos (16/98 using WEF eggs and 10/17 using HS eggs) the gonads showed bilateral expression of *Pitx2* (by in situ hybridisation), although the relative intensity varied from being much weaker to even slightly more intense on the right compared with the left side. In some instances, the expression pattern on the right was discontinuous along the anterior-posterior (A-P) axis (data not shown). A small number of control embryos (3/87 WEF eggs and 1/8 HS eggs) expressed bilateral *Pitx2*.

The anterior portion of twelve pairs of gonads infected with RCAS-Pitx2 and expressing bilateral *Pitx2* within the posterior half, were analysed with the battery of asymmetry markers. In eight samples, the right epithelium had acquired features of a left epithelium with fibronectin accumulation between epithelium and medulla and epithelial cells remaining columnar and DMRT1-positive. In the ZW samples, the right cortical layer expressed ERα

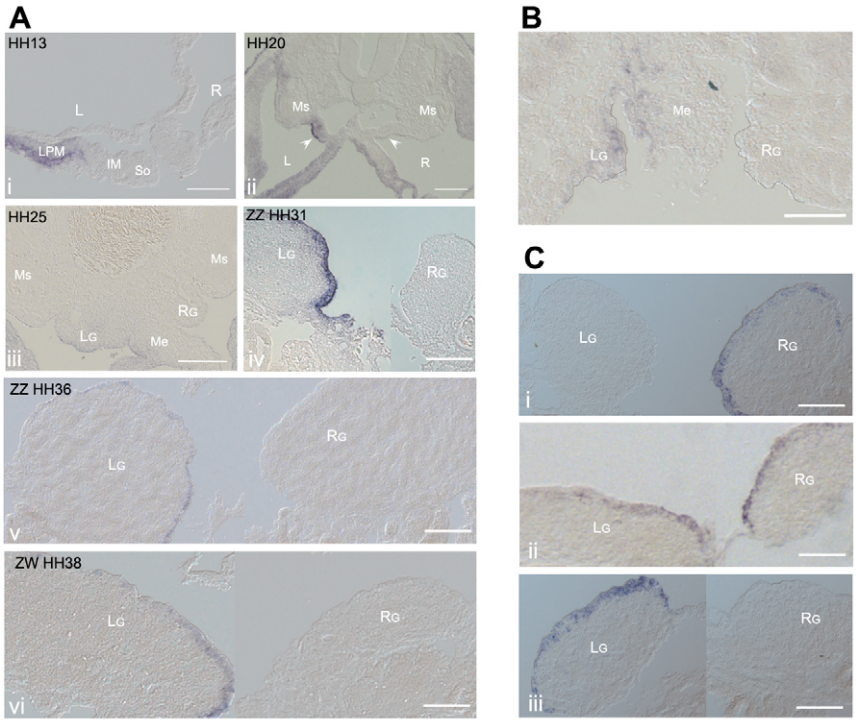

Fig. 3. *Pitx2* expression is asymmetric during gonadal development. (**A**) *Pitx2* expression between E2 and E12. (i) E2 (HH13) transverse section across the posterior part of the somitic region showing asymmetric expression of *Pitx2* in the left splanchnic and somatic lateral plate mesoderm. (ii) At E3 (HH20), *Pitx2* is asymmetrically expressed in the left gonadal region and weakly in the mesonephros. (iii) At E4.5 (HH25), *Pitx2* asymmetric expression is maintained in the left side of the dorsal mesentery and in the adjacent gonad, but is lost in the mesonephros. (iv-vi) *Pitx2* expression is maintained in the left gonad of both sexes at HH31(E7), HH36(E10), HH38(E12). (**B**) *Pitx2c* expression in left gonad and mesentery (E5; HH26). (**C**) *Pitx2* expression in E7 (HH31) gonads from samples injected with lindane. An embryo with complete situs inversus expresses *Pitx2* in the right gonad (i), whereas three out of four embryos with gut heterotaxia express *Pitx2* bilaterally (ii) and one only in the left gonad (iii). IM, intermediate mesoderm; LPM, lateral plate mesoderm; L, left; R, right; LG, Left gonad; RG, right gonad; Me, mesentery; Ms, mesonephros; So, somite. Scale bars: 100 μm.

in addition to that on the left (Fig. 4A,B). Indeed, it was no longer possible to distinguish the right from the left side, indicating that the gonads were undergoing symmetric development. In the other four samples – three males and one female – the right gonad showed an ambiguous phenotype with mixed features of right and left epithelium in terms of cell shape, DMRT1 and fibronectin expression (Fig. 4C); in the female, we did not detect ERα by immunostaining. In order to investigate this variability we reanalysed *Pitx2* expression on sections from the anterior part of all twelve pairs of gonads. The eight left isomers displayed clear bilateral *Pitx2* staining, whereas the four right gonads with an ambiguous phenotype displayed very patchy staining, mostly at the edge with the mesentery (Fig. 4A-C). These data show that the gonad situs is not randomised following the induction of *Pitx2* bilateral expression, but there is instead a strong correlation between the level of expression of *Pitx2* within the epithelium and leftness, indicating that PITX2 directs the morphogenesis of the gonad towards the left differentiation pathway. Distinct left characteristics might also respond with different sensitivities to PITX2.

As we performed our infection experiments with the *Pitx2a* isoform, we tested the gonads displaying left isomerism for expression of endogenous *Pitx2* by in situ hybridisation with the *Pitx2c*-specific probe. *Pitx2c* was found, as expected, on the left, but also on the right side within gonad and mesentery, indicating that the misexpression of *Pitx2a* to the right side at HH8-10 had induced the expression of *Pitx2c* (Fig. 4D).

In order to ascertain whether the left isomerism visualised at E7 (HH30-32) was translating into functional left isomerism, we screened some infected embryos at E12-13 (HH38-39). At these stages in ZW embryos, the degeneration of the right gonad is well underway and the left gonad has developed cortical cords embedding most germ cells. Out of 11 ZW embryos screened, six displayed a right ovary generally shorter than the left along the A-P axis, but resembling the left in the other two dimensions. These pairs were analysed for the expression of ERα to assess the status of the cortical somatic cells. In two cases, both left and right gonads showed similar expression of ERα and LHX9 within the epithelium at the cortical surface, and a weaker staining in some somatic cells of the cortical cords (Fig. 5). The expression of the meiotic marker γH2AX [phosphorylated histone H2AX (also known as H2AFX)] was also investigated to assess both the localisation of germ cells and their stage of maturation. Several studies of mouse meiosis have established that γH2AX marks condensing chromosomes in leptotene/zygotene stages of prophase (Mahadevaiah et al., 2001). We found that in chick, γH2AX is normally expressed in germ cells of the ovary at E12-13 (HH38-39) (Fig. 5A). Most of the positive cells were in the cortical cords of the left ovary, with a few additional cells scattered in the medulla on both sides. In our experiments, the two gonad pairs with symmetric expression of ERα also displayed a γH2AX pattern that was identical on left and right. This demonstrates the ability of the right gonad to form a niche for the germ cells that is equivalent to the one provided by the left (Fig. 5B). Three out of the four remaining pairs had an ambiguous phenotype. Meiotic germ cells were clustered in one or a few discrete cortical sites, sometimes extruding over the rest of the ovarian surface (Fig. 5C,D). These discrete areas were also double positive for ERα and LHX9 within the epithelium, as if they were pieces of left cortex. This indicates that specific cortical sites within these right gonads were undergoing differentiation (presumably responding to estrogens) and attempting to provide a niche for meiotic germ cell (i.e. oocyte) development. All six samples were tested for *Pitx2* expression on sections adjacent to those analysed for the cortical markers (Fig. 5). *Pitx2* was found along the right epithelium of the two left isomers. In the ambiguous samples containing discrete cortical clusters of germ cells, *Pitx2* was found in the portion of epithelium overlaying the sites of cortical differentiation, again strongly indicating that *Pitx2* interacts with the ovarian pathway to direct the differentiation of the ovarian cortex.

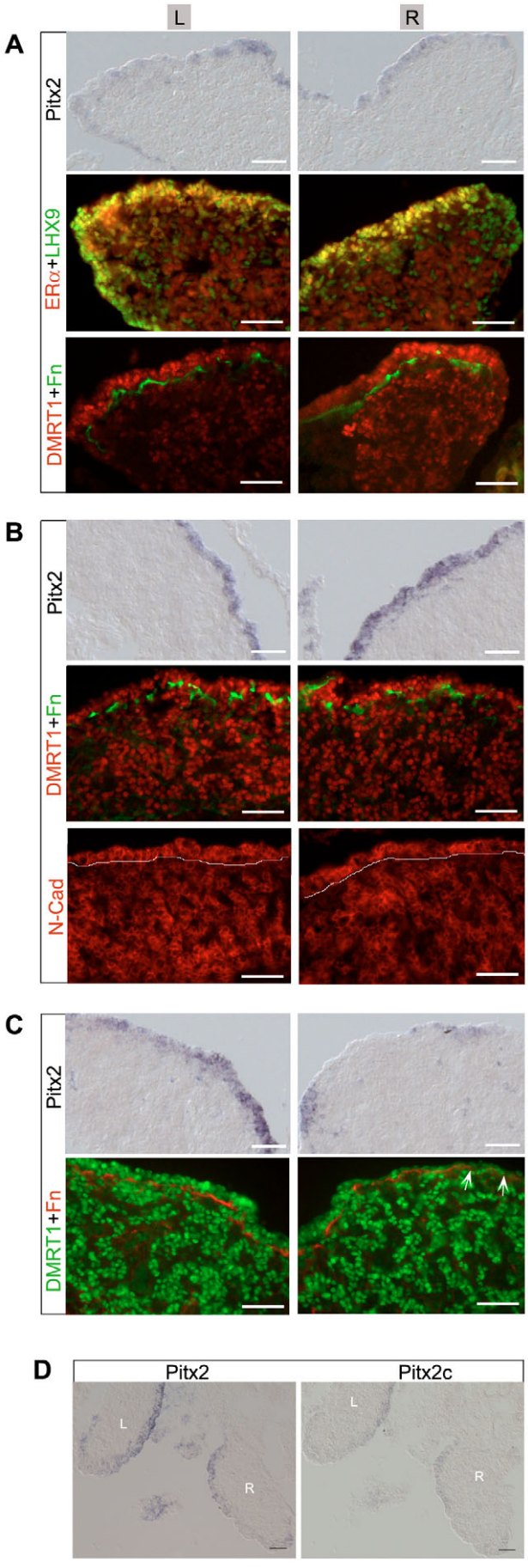

Fig. 4. Sections from three samples infected with RCAS-Pitx2a virus at HH8-10, screened at E7 (HH31) for expression of *Pitx2* in the right gonad and analysed with the battery of asymmetry markers. (**A**,**B**) ZW and ZZ chick embryos with bilateral expression of *Pitx2*. The gonads are left isomers as shown by staining with DMRT1, fibronectin (Fn), N-cadherin (N-Cad) (white line demarks the epithelium-medulla border), and ERα plus LHX9. (**C**) ZZ embryo with very patchy expression of *Pitx2* in the right gonad: its phenotype is ambiguous, with some epithelial DMRT1 and localised deposits of Fn, but some areas of the epithelium have flattened (arrows). (**D**) E7.5 (HH31-32) embryo with gonadal left isomerism following RCAS-Pitx2a infection. *Pitx2* expression is detected using either a probe for all *Pitx2* isoforms or one specific for *Pitx2c*. L, left gonad; R, right gonad. Scale bars: white, 50 μm; black, 100 μm.

Pitx2 misexpression in the right genital ridge is sufficient to induce cortical differentiation

Next we asked whether it was possible to revert the identity of the right gonad after this had already been set. We infected the right dorsal coelomic epithelium at HH15-17 by in-ovo electroporation of RCAS-Pitx2 DNA, mostly using the *Pitx2c* isoform. This procedure allows a few tissues to be targeted, including gonads, dorsal mesentery, mesonephros and Mullerian ducts (Guioli et al., 2007). In order to visualise the transfected area, the DNA was co-injected with a plasmid ubiquitously expressing GFP. The embryos were allowed to develop until E7-8 (HH31-34) and those with high levels of GFP expression within the gonads were analysed with the battery of asymmetry markers. In some gonads, epithelial expression of DMRT1 and deposits of fibronectin at the interface with the medulla were found on the right side, widespread along the A-P axis (Fig. 6A-C); in others, they were restricted to portions of the gonad in accordance with the distribution of GFP expression (data not shown). Epithelial expression of ERα was also found as discrete patches of cells (Fig. 6A,B) or more widespread (Fig. 6C) within the surface epithelium. In some infected gonads, the epithelium appeared rough, with 'humps' extruding in the coelomic cavity and/or lack of a proper border with the dorsal mesentery as if the gonad were fused to it. However, epithelial features of left identity were also present in these samples (Fig. 6D). We took advantage of the HA-tag fused to *Pitx2* in the construct to analyse the actual extent of infection in different samples. Ectopic *Pitx2* was found, at variable levels, in those tissues surrounded by dorsal coelomic epithelium, including gonads, mesonephros, dorsal mesentery and Mullerian duct. Areas along the A-P axis that were negative for HA within the gonad and mesentery had, as expected, right identity (data not shown). HA-positive samples or portions of it were associated with some degree of right-to-left transformation, as described above. These gonads were mosaic for HA-positive and HA-negative cells within the epithelium and medulla (Fig. 6B,D). Groups of adjacent epithelial cells expressing high levels of ectopic *Pitx2* were often found in the 'humps' (Fig. 6D). Similar results were obtained by misexpressing the *Pitx2a* isoform (data not shown). These data suggest that PITX2 expression in the right gonad after the start of gonadogenesis is sufficient to induce right-to-left changes within the gonad, but also that the location and levels of PITX2 within the gonadal cells might be important for proper differentiation. The process of extrusion could indeed result in portions of differentiating epithelium being lost. Moreover, those samples lacking a proper

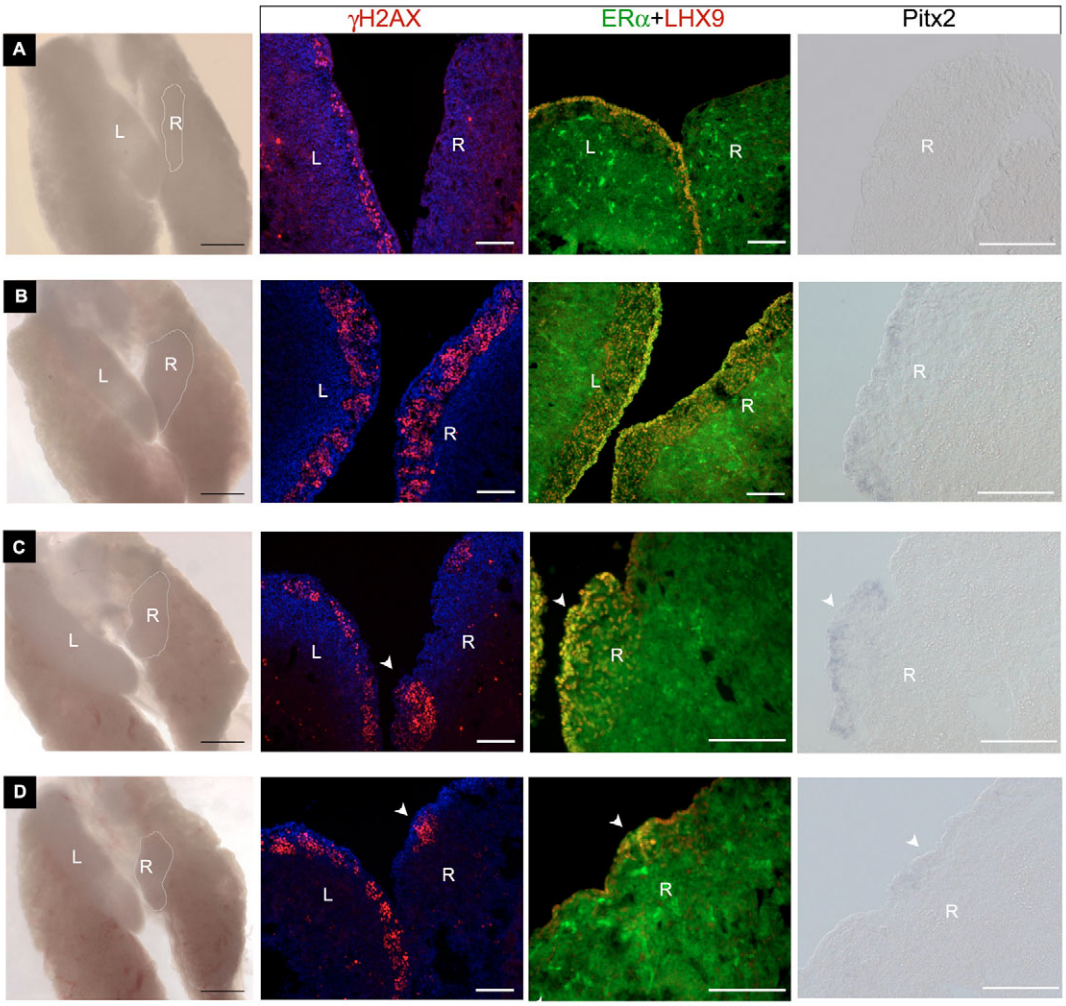

Fig. 5. E12-13 (HH38-39) gonads infected with RCAS-Pitx2a virus in ovo at HH8-10 analysed for the expression of γH2AX, ERα plus LHX9, and *Pitx2*. (**A**) E12 uninfected wild-type and (**B**,**C**,**D**) three infected samples showing some degree of right gonad 'puffiness'. Of the panels showing antibody staining for γH2AX and for ERα plus LHX9, in B the right gonad is indistinguishable from the left, whereas in C and D localised superficial areas of the right gonad contain clusters of meiotic germ cells and these areas are positive for epithelial ERα. In the panels showing in situ hybridisation for *Pitx2*, expression is in the entire epithelium (B) or in localised areas (C,D) of right gonads. Co-localisation of *Pitx2* and the left epithelial markers is evident. Arrowheads in C,D indicate left-like areas. L, left gonad; R, right gonad. Scale bars: white, 100 μm; black, 1 mm.

border between gonad and mesentery displayed high levels of ectopic PITX2 within the mesentery and the medial part of the gonad, suggesting that the correct dosage of PITX2 is required within both tissues for appropriate gonadal development according to the left pathway.

Some embryos from these electroporation experiments were allowed to develop to E12-13 (HH38-39). At this stage, the co-injected GFP was no longer visible. Gonad pairs from the female embryos were screened based on their morphology. In six out of 13 pairs, the right gonad showed some degree of 'puffiness' along the entire A-P axis, or part of it. Two were analysed further for germ cell localisation and cortical differentiation and found to have an ambiguous phenotype similar to that observed in some of the samples infected with the *Pitx2* virus at stage HH10. Meiotic germ cells were found in clusters in discrete areas in proximity to the epithelium and embedded in fibronectin (Fig. 7). These areas were enlarged relative to the rest of the ovarian surface, resembling pieces of cortex expressing ERα and LHX9. Expression analysis for the HA-tag revealed ectopic PITX2 widespread within both epithelium and medulla. The surface of these right gonads appeared rough, similar to the E7-8 electroporated samples. These data indicate that sex differentiation is sensitive to the dosage of PITX2 and that its expression is sufficient to both direct and redirect the morphogenesis of the gonad according to the left sex differentiation pathway and to produce a cortical layer containing meiotic germ cells.

DISCUSSION

According to Ramsdell (Ramsdell, 2005), three different endpoints of L-R patterning are possible: (1) directional looping of midline structures, notably the heart and gut, which generates an asymmetry within the organ as well as in its placement; (2) unilateral regression and/or persistence of structures that begin bilaterally, such as the spleen and the sixth pair of aortic arch arteries that persist only on the left; and (3) lateralisation of structures that begin symmetrically, such as the liver and lungs. Gonadal development in the chick would appear to fall into the second category, with the important difference that the endpoint is sex-specific.

Our data provide clear evidence that left and right gonads have distinct morphological and molecular characteristics in both sexes from the indifferent stage. Furthermore, these asymmetric features are maintained in both sexes well beyond the start of sex determination, making unlikely a model whereby the asymmetric fate of left and right ovaries is dependent on an asymmetry established within the ovarian pathway itself. Both sex differentiation pathways have to deal with the initial L-R differences and whereas the male pathway can overcome them, the female pathway cannot. We found no evidence for L-R differences within the medulla; instead, the epithelia display qualitatively distinct properties. Because testis cords differentiate within the medulla, and this is central to male development, it is possible that L-R differences within the epithelia have little impact on this process. Indeed, whereas in the mouse the gonad coelomic

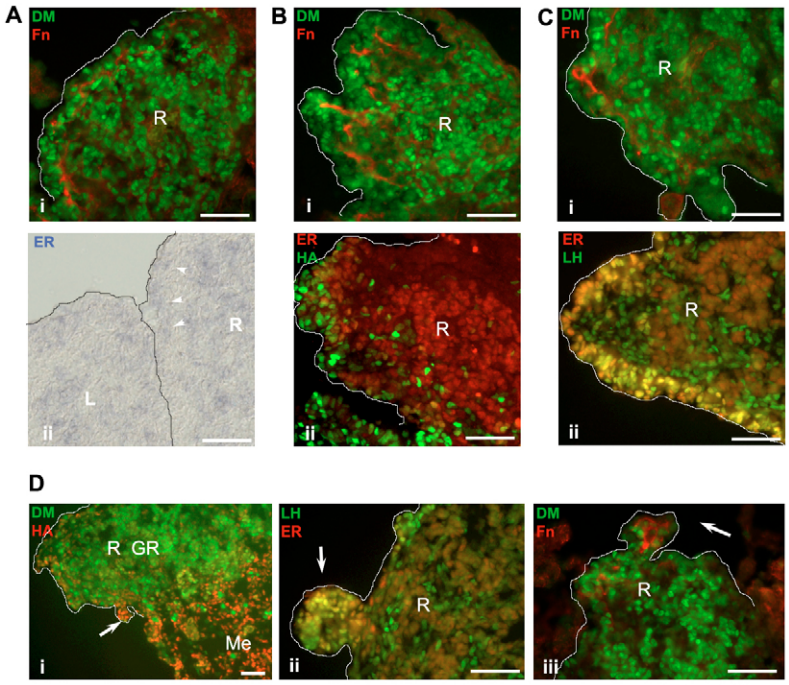

Fig. 6. Expression of L-R identity markers in gonads from chick embryos electroporated with RCAS-Pitx2c and GFP DNAs at HH15-16 and screened at E7-8 (HH31-34) for GFP expression. (**A-C**) Sections of the gonads from three infected embryos (A,B,C are ZZ, ZW and ZW, respectively) showing the epithelial expression of DMRT1 (DM) and accumulation of fibronectin (Fn) found along the A-P axis (Ai,Bi,Ci). These samples also express epithelial ERα (ER) to different degrees (Aii,Bii,Cii). The induction of epithelial ERα in the right gonad is independent of the sex as it is also present in the male embryo (Aii). HA-tag antibody staining shows a partial colocalisation between PITX2 and ERα (Bii). Double-staining for ERα and LHX9 (LH) shows colocalisation within the cortical layer of the infected right gonad (Cii). (**D**) High levels of ectopic PITX2 induce the formation of 'humps' at the surface epithelium (white arrows) and lack of a proper border between gonad and mesentery (i). The epithelial cells within the humps still express ERα plus LHX9 (ii), DMRT1 (i,iii) and accumulate high levels of fibronectin (iii). Scale bars: 50 μm.

epithelial cells are the source of Sertoli cells and therefore play an essential role in cord formation, this is not the case in chick, where the cords appear to form from the reorganisation of the primitive sex cords with no contribution from the coelomic epithelial cells (Carlon and Stahl, 1985; Sekido and Lovell-Badge, 2007). Moreover, at around E12 (HH38), testis expression of *Pitx2* is almost completely downregulated and the left epithelium has mostly flattened similar to the right side, suggesting that the male pathway has lost its epithelial L-R asymmetry in favour of right isomerism.

In the female, however, the central event is the differentiation of a cortex that provides the niche for the developing oocytes. At E6 (HH29), both left and right medulla start to express aromatase and produce estrogens, which are essential for chick ovarian development (Elbrecht and Smith, 1992; Kagami and Tomita, 1990). This is closely followed by the thickening of the left germinal epithelium, which later elaborates the cortex (Carlon and Stahl, 1985). The right epithelium, presumably owing to the absence of ERα, is unlikely to respond to estrogens. In fact, this is the only part of the gonads of either sex shown to lack target cells for estrogens (Gasc, 1980), providing a simple explanation for its inability to differentiate further.

We show that the gonad situs is dependent upon the signalling pathway controlling the establishment of the L-R body axis and, moreover, that *Pitx2* has a role in the lateralisation of the gonad and its morphogenesis. The gonads, at least in chick, add to the list of bilateral organs, such as the lungs, whose morphogenesis is directly linked to the lateralisation pathway (Shiratori and Hamada, 2006). In mammals, normal gonadal development leads to a pair of functional testes or ovaries. However, asymmetry is evident in disorders of sex differentiation leading to hermaphroditism, with ovaries being more common on the left side and testes and ovotestes on the right in humans, and, with opposite tendency, in mice (Mittwoch, 2001). *Pitx2* is expressed in mouse gonads, but this appears to be both male-specific and symmetrical (Coveney et al., 2007). If the lateralisation signals exert any influence, this effect is evidently buffered in normal development by the sex determination/differentiation pathways. In chick, however, the L-R

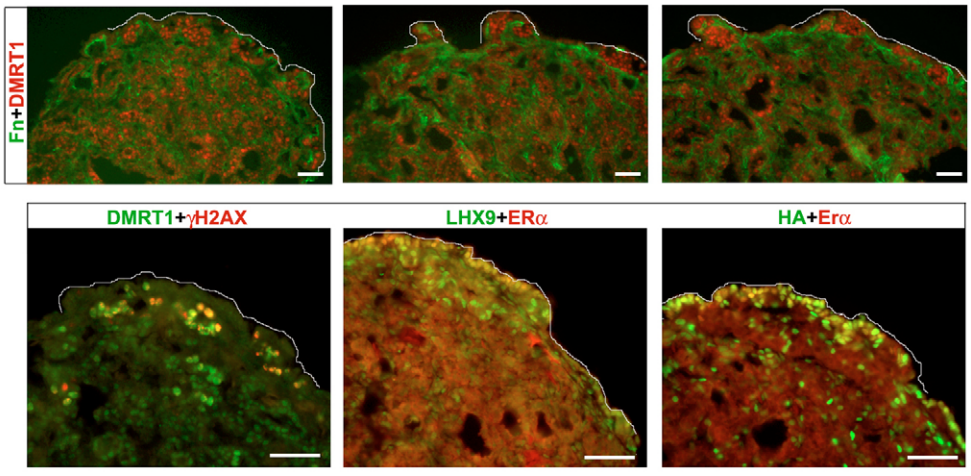

Fig. 7. Analysis of cortical differentiation in the right gonad of chick embryos electroporated with RCAS-Pitx2c at HH15-16 and screened at E12 (HH38). (Top row) Transverse sections along the A-P axis of one right gonad containing germ cells clusters (DMRT1-positive) surrounded by fibronectin (Fn). (Bottom row) Left-like cortex areas are positive for ERα plus LHX9 and contain germ cells positive for DMRT1 plus γH2AX. The HA antibody staining (right) shows widespread expression of ectopic PITX2 found in extensively infected right gonads. The line marks left-like cortex areas. Scale bars: 50 μm.

pathway via *Pitx2* has a direct, instructive role at organ level. *Pitx2* instructs the epithelium to differentiate according to a 'left' sex differentiation pathway. We show not only that there is always a correlation between the expression of *Pitx2* within the epithelium and a display of left identity, but also that the misexpression of *Pitx2* to the right side changes the fate of the gonad. First, *Pitx2* misexpression to the right at stage HH10 is sufficient to transform the right gonad into the mirror image of the left gonad. In males, this means the formation of testes with polarised left and right germinal epithelium and deposits of fibronectin well beyond the start of sex differentiation. In females, this allows the formation of a pair of ovaries with a stratified cortex containing most germ cells. As the RCAS virus infection leads to expression in the mesonephros, gonad and mesentery, we expected changes in gonadal fate to be the direct result of ectopic PITX2a in the gonad from the start of its formation. However, the finding that endogenous *Pitx2c* is expressed in the transformed gonads and adjacent mesentery in a precise pattern similar to that of the left side, suggests a more complex picture, whereby ectopic PITX2a induces PITX2c and then either the latter or both together induce a right-to-left transformation. A similar result was obtained in *Xenopus*, where ectopic expression of *Pitx2b* in the early whole embryo or animal cap explant induced *Pitx2c* expression in the right side of the heart (Schweickert et al., 2000). We also show that misexpression of *Pitx2* directed to the coelomic epithelium after the start of gonad development induces a right-to-left change. This means that *Pitx2* is sufficient to direct and redirect gonadal development towards a 'left' sex determination/differentiation pathway. PITX2 activity leads to the expression of all the genes in our asymmetry marker panel, including those likely to have a role in sex differentiation, notably ERα. PITX2 also regulates *Bmp7*, which is active in the left gonad from its formation (Hoshino et al., 2005), as expression of the gene is induced in the right gonad of embryos infected with the *Pitx2*-expressing virus either at HH8-10 or HH15 (see Fig. S1 in the supplementary material).

It has been reported that correct levels of PITX2 are essential for normal morphogenesis of organs such as heart, lung and duodenum, and that each organ has a different dosage requirement (Liu et al., 2001). This appears to be true also for chick gonads, as L-R epithelial asymmetry markers may well have different threshold sensitivities to PITX2 activity. For example, in some E7 infected gonads, all of the epithelium expresses DMRT1, whereas only patches of cells are ERα-positive. Moreover, it is evident that too much PITX2 is deleterious. In the infected gonads, the positive cells, both within medulla and cortical regions, express ectopic PITX2 at different levels. We observed that groups of highly expressing epithelial cells tend to forms humps extruding into the coelomic cavity where they may subsequently be lost. This phenotype is visible at E7, but also at E12, and might explain why we observe a discontinuous cortex in the E12 electroporated samples.

In conclusion, our results suggest a model whereby, in response to the signals that initiate L-R axis development, asymmetric expression of *Pitx2* in the gonadal coelomic epithelium, which is derived from LPM, confers 'leftness' to the resulting gonad, which also includes intermediate mesoderm. This permits development to continue towards an ovary in a female or a testis in a male. By contrast, 'rightness' only allows testis development to continue. Furthermore, it seems likely that the ability of the left epithelium to respond to estrogens, made as a consequence of gene activity in the ovarian-determining pathway, is involved in promoting ovary development. It now seems clear that the two processes of sex determination and L-R asymmetry interact, rather than one being dependent on the other. This needs to be borne in mind when testing candidates for sex-determining genes in birds, and when exploring evolutionary relationships between species showing asymmetry in gonadal development and those that do not.

We thank M. Logan for sharing reagents and for stimulating discussions; W. Hatton for assistance with histology; the NIMR Imaging Laboratory for help with fluorescence microscopy; G. L. Greene for the anti-ERα antibody; DSHB for the anti-fibronectin antibody developed by D. M. Fambrough; and J. M. Turner and C. E. Scott for critical reading of the manuscript. The work was funded by the UK Medical Research Council.

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/134/23/4199/DC1

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