

Mesonephric cell migration induces testis cord formation and Sertoli cell differentiation in the mammalian gonad

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SUMMARY

In mammals a single gene on the Y chromosome, *Sry*, controls testis formation. One of the earliest effects of *Sry* expression is the induction of somatic cell migration from the mesonephros into the XY gonad. Here we show that mesonephric cells are required for cord formation and male-specific gene expression in XY gonads in a stage-specific manner. Culturing XX gonads with an XY gonad at their surface, as a 'sandwich', resulted in cell migration into the XX tissue. Analysis of sandwich gonads revealed

that in the presence of migrating cells, XX gonads organized cord structures and acquired male-specific gene expression patterns. From these results, we conclude that mesonephric cell migration plays a critical role in the formation of testis cords and the differentiation of XY versus XX cell types.

Key words: Cell migration, Mesonephros, Sex determination, Sex reversal, *Sry*

INTRODUCTION

The mammalian gonad is a unique model system for the study of organogenesis because it is bipotential: a single primordium, the urogenital ridge, can develop into one of two organs, a testis or an ovary, depending on the presence or absence of one Y-linked gene, *Sry* (Sex determining Region of the Y chromosome) (Gubbay et al., 1990; Sinclair et al., 1990). The bipotential nature of the gonadal primordium makes it possible to perform gain- as well as loss-of-function experiments. Accordingly, it has been shown that in XX mice carrying *Sry* as a transgene, the gonad develops as a testis (Koopman et al., 1991); whereas in XY individuals lacking *Sry* (Lovell-Badge and Robertson, 1990) or carrying mutations in the gene (Jager et al., 1990), the gonad develops as an ovary. The ability to perform loss- and gain-of-function experiments without losing the organ provides a means to link downstream signaling pathways and cellular events involved in testis formation to *Sry*.

The earliest clearly defined morphological change characterizing the onset of testis development is the initiation of testis cord formation, which occurs at approximately 12.0 days post coitum (dpc). Testis cords are composed of germ cells and epithelialized Sertoli cells, which are surrounded by a layer of peritubular myoid cells, a smooth muscle cell lineage. In the mouse, *Sry* expression occurs between 10.5 and 12.5 dpc in the cells of the XY gonad. One early consequence of *Sry* expression is the induction of somatic cell migration from the mesonephros via a long-range signal (Buehr et al., 1993; Merchant-Larios et al., 1993; Martineau et al., 1997; Capel et al., 1999). Migration of cells from the mesonephros brings at least three somatic cell types into the XY gonad: peritubular myoid cells, endothelial

cells and cells associated with the endothelium (Buehr et al., 1993; Merchant-Larios et al., 1993; Martineau et al., 1997). Migration of these cell types is consistently associated with testis cord formation in XY gonads (Buehr et al., 1993; Martineau et al., 1997), XX gonads expressing *Sry* as a transgene (Capel et al., 1999) and with testicular regions in ovotestes (K. H. Albrecht, B. C., L. L. Washburn and E. M. Eicher, unpublished data). These results suggest that cell migration from the mesonephros plays an important role in the formation of testis cords. Recruitment of mesenchymal cells to the basal side of an epithelium in this manner is known to be important for the organization and gene expression patterns of a number of developing organs (Streuli et al., 1991; Bostrom et al., 1996; Radice et al., 1997; Shannon et al., 1998). In vitro experiments using cells from neonatal rat testes have shown that peritubular myoid and Sertoli cells cooperate to produce basal lamina (Tung and Fritz, 1980; Skinner et al., 1985) and that the extracellular matrix may modulate gene expression in Sertoli cells cultured in vitro (Dym et al., 1991; Thompson et al., 1995).

Important experiments aimed at addressing the role of migrating cells in the early gonad showed that when migration of somatic cells from the mesonephros into an XY gonad was blocked using a membrane barrier, cord formation was compromised (Buehr et al., 1993). To further examine the role of mesonephric cell migration in cord formation, we assayed the effect of inducing migration of mesonephric cells into the bipotential XX gonad. Normally, these cells are recruited only into the XY gonad (Martineau et al., 1997; Capel et al., 1999). However, in previous experiments, we showed that signals from the XY gonad can induce mesonephric cells to migrate into the XX gonad when an 11.5 dpc XX gonad is cultured as

a sandwich between a ROSA26 mesonephros marked with β -galactosidase (β -gal) (Friedrich and Soriano, 1991), and a piece of an XY gonad (Martineau et al., 1997). The present experiments show that migration of mesonephric cells into an XX gonad leads to the organization of testis cords and male-specific gene expression in the XX tissue in a stage-dependent manner.

MATERIALS AND METHODS

Mouse strains, matings and organ culture

Embryos were collected at 11.5 dpc–13.5 dpc (where 0.5 dpc = noon of the day when a vaginal plug was detected) from ROSA26 mice (B6,129-TgR(ROSA26)26Sor) (Friedrich and Soriano, 1991) and/or random-bred CD-1 mice for experiments as specified. To sex embryos before sexual dimorphism was apparent, amnions from individual embryos were saved and stained as previously described (Palmer and Burgoyne, 1991b) for the presence of nuclei with a condensed chromatin body which identifies XX embryos. Whole genital ridges (gonad + mesonephros) were dissected from all embryos, and in some cases mesonephroi and gonads were separated. Mesonephroi and gonads were assembled in agar blocks as indicated for individual experiments (Fig. 1) and cultured as described (Martineau et al., 1997) for 30–44 hours in Dulbecco's Minimal Eagle Medium (DMEM)/10% fetal calf serum (FCS) (Gibco)/50 μ g/ml ampicillin at 37°C and 5% CO₂. In some cases, gonads were assembled in pairs in the absence of a mesonephros or with a 0.1 μ m nucleopore membrane positioned between the mesonephros and the gonad.

X-gal staining of organ cultures

Organ cultures were recovered from agar blocks, washed briefly with phosphate-buffered saline (PBS) (Gibco), fixed in 4% paraformaldehyde for 10 minutes at room temperature, washed in β -gal wash solution (2 mM MgCl₂, 0.2% Nonidet P-40, in PBS), and incubated in X-gal stain (1 mg/ml X-gal, 200 mM K₃Fe(CN)₆, 200 mM K₄Fe(CN)₆, in β -gal wash solution) overnight at 37°C to develop the blue color (Wasserman and dePamphilis, 1993). Samples were then washed in β -gal wash solution twice and post-fixed in 4% paraformaldehyde.

Cryosectioning, alkaline phosphatase staining, immunohistochemistry and in situ hybridization

For cryosectioning, samples were washed for 15 minutes in 10% sucrose, 15 minutes in 15% sucrose, 1 hour in 20% sucrose, and overnight at 4°C in 1:1 20% sucrose:OCT (cryo-embedding medium, Tissue Tek). Samples were embedded in molds in 20% sucrose:OCT (1:3), frozen on dry ice, cut into 12 μ m sections, and placed on Biobond-coated slides. For alkaline phosphatase staining of germ cells, 4.5 μ l/ml NBT and 3.5 μ l/ml BCIP were added to NTM (0.1 M NaCl/0.1 M Tris, pH 9.5/0.05 M MgCl₂) and incubated on sections for 5 minutes at room temperature. Sections were then washed in PBS to stop the reaction. For laminin staining, sections were blocked for 1 hour at room temperature in blocking solution (10% BSA/1% heat inactivated goat serum/0.1% Triton X-100). Primary antibody incubations were carried out overnight in blocking solution using a rabbit polyclonal antibody against laminin-1 (provided by Harold Erickson). Washes were performed in 1:10 dilution of blocking solution. Secondary incubations were carried out in blocking solution for 45 minutes at room temperature using an FITC-conjugated goat anti-rabbit secondary antibody (Jackson immunochemicals), 1:500 dilution. Samples were scored for organization as described in Table 1.

For RNA in situ hybridization, samples were fixed in 4% paraformaldehyde and in situ hybridizations were performed as described by Henrique et al. (1995) with probes to *Sox9* (kindly provided by Peter Koopman; Kent et al., 1996) and *Dax-1* (kindly provided by Robin Lovell-Badge; Swain et al., 1996).

RESULTS

Mesonephric cell migration into XY gonads is required in a stage-specific manner

Previous experiments showed that culturing 11.5 dpc XY gonads with a membrane between the mesonephros and gonad or without their adjoining mesonephroi resulted in a compromised ability to form cords (Buehr et al., 1993). To investigate the stage specificity of this effect, we extended these experiments using an improved culture system and samples that were carefully staged by tail somite (ts) counts. Cord formation in XY gonads was analyzed by processing samples with an antibody against laminin-1, which detects basal lamina surrounding testis cords.

When XY gonads were removed from their mesonephroi at 18ts (corresponding to 11.5 dpc) or older and cultured for 30–44 hours in agar blocks, cords formed in 100% (20/20) of the samples. Cords also formed in 100% (15/15) of unseparated 18ts cultured XY controls. The discrepancy between this and previous data might reflect a fine difference in staging or the mouse strain used. To determine if mesonephric cell migration is required at stages earlier than 18ts, XY gonads were removed from their mesonephroi at 16–17ts (2–4 hours earlier), or a membrane was included between the gonad and mesonephros. When such cultures were initiated at 17ts and cultured for 30–44 hours, 63% (8/12, 2/4 with membranes) of XY gonads formed cords as compared to 100% (14/14) of unseparated 17ts cultured XY controls. When 16ts XY gonads were separated from their mesonephroi (or a membrane barrier was included) and cultured for 30–44 hours, cords formed in 0% (0/10, 0/6 with membranes) of samples compared to 70% (7/10) of unseparated 16ts cultured XY controls.

These experiments were difficult to interpret for several reasons. It is never possible to determine how much migration has occurred prior to separation of the gonad from its mesonephros. This leads to an experimental plan in which gonads must be separated and cultured as early as possible (before 18ts). However, separating gonads from their mesonephroi at 16–17ts, when the gonad was only 8–10 cell layers thick, potentially caused damage to the tissue, which might have interfered with cord formation. Also, the viability of such gonads in culture and their ability to form cords even when unseparated (70% at 16ts) was lower than those at 18ts. Because of these technical limitations, we sought a better way to study the role of migration in cord formation and the differentiation of male cell types.

Migration from the mesonephros into XX gonads results in cord formation

Normally no mesonephric cell migration into the XX gonad occurs between 11.5 and 16.5 dpc. However, we previously showed that migration can be induced into the XX gonad by culturing it between a 'blue' ROSA26 mesonephros, ubiquitously expressing β -gal, and a piece of an XY gonad as a sandwich (Fig. 1A) (Martineau et al., 1997). This experiment raised the possibility that migration of mesonephric cells might influence the organization of the bipotential XX tissue and/or the differentiation of XX cells.

Other experiments using chimeric mice have shown that XY cells are capable of imposing a male program of development on XX cells in an XX \leftrightarrow XY mosaic gonad in which XX and

XY cells are randomly mixed (Burgoyne et al., 1988; Palmer and Burgoyne, 1991a). To better characterize the sandwich cultures, it was important to determine whether XX and XY cells were mixing in a mosaic manner, or were maintaining discrete populations. To test these possibilities, we assembled sandwich cultures with ROSA26 XY gonads and white CD-1 XX gonads and mesonephroi. Processing these cultures for β -gal activity revealed that cell mixing never occurred between the XY and XX gonads in sandwiches over a 44 hour culture period (0/8) (Fig. 1B).

To study the consequences of inducing mesonephric cell migration into an XX gonad, sandwich gonads were assembled using an XY gonad from a CD-1 or ROSA26 mouse and a whole CD-1 XX gonad + mesonephros complex (Fig. 1B). After culturing for 30-44 hours, morphology of samples was assayed by staining for the endogenous germ cell marker alkaline phosphatase and with an antibody against laminin-1 to detect basal lamina deposition. In XY control cultures initiated at 11.5 dpc and cultured for 30-44 hours, germ cells were aggregated inside testis cords, which were surrounded by laminin (Fig. 2A, 3A, Table 1). In XX control cultures initiated at 11.5 dpc, germ cells were randomly scattered throughout the gonad and laminin was not organized (Fig. 2B, 3B, Table 1). When migration was induced into 11.5 dpc XX gonads in culture, germ cell aggregation frequently resembled XY controls and laminin was deposited in a pattern characteristic of testis cords (Fig. 2C, 3C, Table 1). In Fig. 3C, the XY portion of the sandwich was determined by using an XY gonad fragment from a ROSA26 mouse (dark staining region at the top). However, processing samples for β -gal activity interfered with the clarity of laminin staining so all other experiments were performed without such processing. No organization occurred in the XY portion of this sample. This was often the case when the XY gonad was isolated from its mesonephros prior to 18ts (as previously described). However, the ability of XY gonads to organize had no effect upon their ability to induce organization in XX gonads. These results were

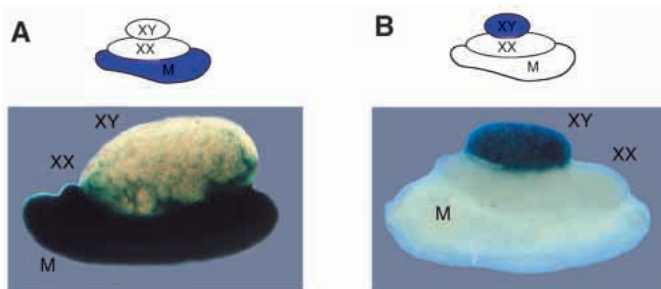


Fig. 1. (A) Mesonephric cell migration can be induced into an XX gonad by co-culture with an XY gonad apposed to its outer surface. Gonads were dissected from wild-type CD-1 mice and mesonephroi from ROSA26 mice ubiquitously expressing β -gal, and assembled as shown. Samples were then cultured in agar blocks for 30-44 hours and subsequently processed with X-gal to identify cells originating from the ROSA26 tissue. Sandwich gonads assembled with a blue mesonephros revealed that cell migration was induced into the XX portion of the sandwich. (B) Induction of cell migration into XX gonads was not due to cell mixing between the XY and XX tissues. Sandwiches were assembled with ROSA26 XY gonads and processed similarly to identify XY cells. No cell mixing occurred between the XX and XY portions of the sandwich (0/8 cases). M, mesonephros.

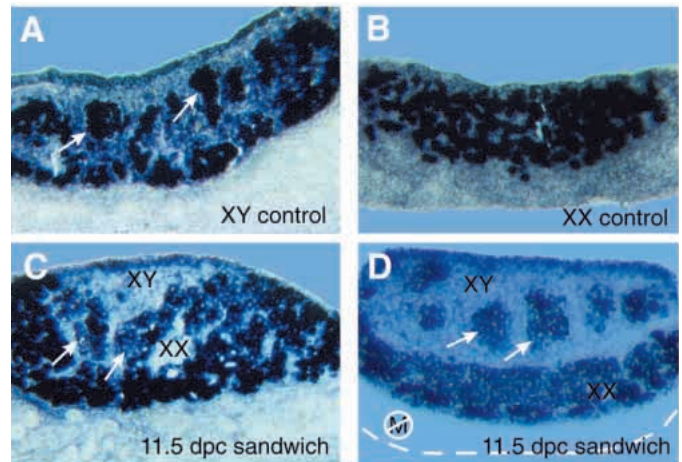


Fig. 2. Cell migration induces germ cell aggregation in XX gonads. (A-D) Alkaline phosphatase staining of germ cells. (A) In cultured XY gonads, germ cells aggregated inside testis cords (arrows), whereas no germ cell aggregation occurred in XX cultured controls (B). (C) Induction of migration into XX gonads resulted in germ cell aggregation in the XX portion of sandwich gonads (arrows). (D) When migration was blocked by removing the mesonephros or by including a 0.1 μ m membrane barrier between the mesonephros and XX gonad (the original position of mesonephros is indicated by dashed line), no germ cell aggregation occurred in the XX portion of the graft. Organization of germ cells sometimes occurred in the XY portion (arrows) depending upon its stage. M, mesonephros.

independent of the sex of the mesonephros (data not shown). As seen in Fig. 2C, germ cell staining was frequently lost from the XY portion of sandwich gonads for unknown reasons.

Even though no cell mixing occurred between the XX and XY portions of the sandwich, factors produced by the XY gonad at this time, such as Müllerian Inhibiting Substance (MIS), have been shown to have a sex-reversing effect on ovaries in culture, and could be influencing organization of the XX tissue (Vigier et al., 1987). Such a substance produced by the XY gonad could promote cord formation in the absence of a mesonephros. To address this possibility, sandwiches were assembled without mesonephroi or with a 0.1 μ m membrane barrier between the XX gonad and mesonephros. In either case, cords never formed in the XX portion of the graft (0/14 and 0/10 samples, respectively), showing that contribution of mesonephric cells was required for the organization of XX gonads into cord-like structures (Fig. 2D, 3D, Table 1). In Fig. 2D and 3D, organization of germ cells and laminin in the XY portion of the sandwich still occurred independently of a mesonephros because these gonads were separated from their mesonephroi after 18ts.

We tested whether another source of mesenchyme, the limb bud, was capable of supporting testis cord formation in sandwich gonads. In agreement with Moreno-Mendoza et al. (1995), we saw migration of cells from the limb bud into sandwich gonads (data not shown). However, cord formation was not initiated, in agreement with Buehr et al. (1993) (0/20 samples, Table 1).

Induction of organization in XX gonads is stage specific

Experiments have suggested that there is a window between

11.5 and 12.5 dpc in which *Sry* must initiate testis formation, otherwise ovarian development ensues (Eicher and Washburn, 1986; Burgoyne and Palmer, 1991; McLaren, 1991). Sensitivity to the timing and level of *Sry* expression during this period has been demonstrated in transgenic mice (Swain et al., 1998), mice with mutations affecting *Sry* expression levels (Capel et al., 1993), and in certain strains of mice that undergo frequent male to female sex reversal (Palmer and Burgoyne, 1991b; Eicher et al., 1995). In earlier studies, we showed that migration into XY gonads begins near 11.5 dpc and continues until at least 16.5 dpc, long after *Sry* expression has ended (Martineau et al., 1997). Despite ongoing migration, the competency to form cords might be limited.

We tested whether cord formation was still induced in the XX gonad when mesonephric cell migration was initiated at later stages of development. When sandwiches were assembled using 12.5 dpc components and cultured 30-44 hours,

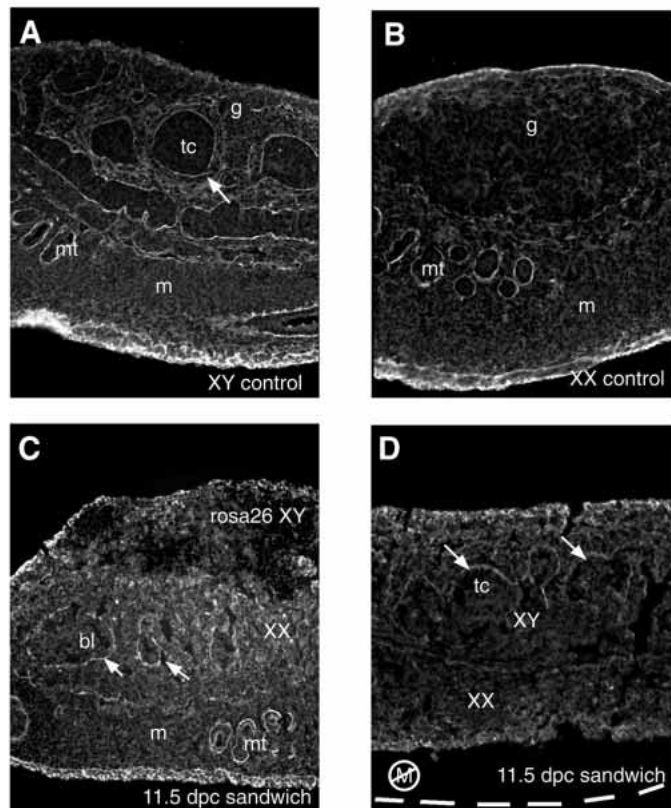


Fig. 3. Cell migration induces laminin deposition in XX gonads. (A-D) Anti-laminin-1 staining to detect basal lamina deposition. (A) Laminin surrounded testis cords (tc, arrow) in XY cultured controls initiated at 11.5 dpc, whereas (B) no such organization occurred in XX cultured controls. (C) Basal lamina (bl, arrows) was deposited in the XX portion of sandwich gonads in a pattern resembling XY controls. In this sample, no organization occurred in the ROSA26 XY portion of the sandwich (dark area, see text). (D) When migration from the mesonephros was blocked by removing the mesonephros or by including a 0.1 μ m membrane barrier between the mesonephros and XX gonad, no organization of laminin was seen in the XX portion of sandwich gonads (the original position of mesonephros is indicated by dashed line). Organization of laminin sometimes occurred in the XY portion (arrows), depending its stage. g, gonad; m, mesonephros; mt, mesonephric tubule.

mesonephric cell migration occurred into the XX gonad (data not shown). However, XX gonads failed to become organized, as indicated by the pattern of germ cells and laminin-1 (Fig. 4A, Table 1). These results can be explained by one or more of the following hypotheses: (1) at stages 12.5 dpc and older, XX gonads cannot be induced to organize; (2) when XY gonads are 12.5 dpc or older, they are not capable of inducing cord formation in XX tissue; or (3) older mesonephroi are not capable of supporting cord formation.

First, to determine the window of competence in which the XX gonad can form cords, 12.5 dpc or older XX gonads were assembled with 11.5 dpc mesonephroi and XY gonads and cultured for 30-44 hours. In this experiment, both the mesonephros and XY inducing tissue were at a stage already shown to support cord formation. Experiments set up in this manner showed that at 12.5 dpc, XX gonads were not capable of organizing cord structures (0/17 samples) (Fig. 4B, Table 1). In a few samples, some aggregation of germ cells was seen, but basal lamina was never produced. The same was true for 13.5 dpc XX gonads (data not shown).

Second, to determine if XY gonads at 12.5 dpc were capable of inducing organization in XX gonads, heterochronic sandwiches using whole 11.5 dpc XX gonads and mesonephroi were assembled with 12.5 dpc XY inducing gonads and cultured 30-44 hours. These samples also failed to organize cord structures (Fig. 4C, Table 1) suggesting that the 12.5 dpc XY gonad does not signal properly to induce cord formation in 11.5 dpc XX gonads.

Finally, sandwiches assembled with 12.5 dpc XX mesonephroi, 11.5 dpc XX gonads and 11.5 dpc XY gonads, did form cords (Fig. 4D,E, Table 1). Thus, only the gonad and not the mesonephros regulated this stage-specific effect. When this experiment was performed using 12.5 dpc XY mesonephroi, cord formation was compromised compared to XX mesonephroi (Table 1).

Migration induces Sertoli cell differentiation in XX gonads

Mesonephric cell migration can reorganize XX cells into testis cord-like structures. In XY gonads, testis cords are composed of germ cells and epithelial Sertoli cells surrounded by a basal lamina and a layer of peritubular myoid cells, one of the migrating cell types. It seemed possible that organization of XX tissue into cords could also induce Sertoli cell differentiation. To test this possibility, we performed RNA *in situ* hybridization on sandwich gonads with probes specific for the sexually dimorphic markers, *Sox9* and *Dax-1*. In the developing gonad, *Sox9* expression begins in XY and XX gonads near 10.5 dpc. By 11.5 dpc, *Sox9* expression ends in XX gonads, while in XY gonads it is upregulated and specific to Sertoli cells (da Silva et al., 1996; Kent et al., 1996). Conversely, *Dax-1* expression is upregulated in XX gonads and declines in XY gonads after 11.5 dpc (Swain et al., 1996). XY control cultures initiated at 11.5 dpc and cultured for 30-44 hours showed strong expression of *Sox9*, whereas cultured XX controls showed no expression of *Sox9* (Fig. 5A,B). When migration was induced into 11.5 dpc XX gonads, Sertoli cells began to differentiate as evidenced by the upregulation of *Sox9* expression (Fig. 5C). This effect was not seen when migration was blocked or the mesonephros component was removed (Fig. 5D). In Fig. 5D, *Sox9* was strong in the XY portion of the

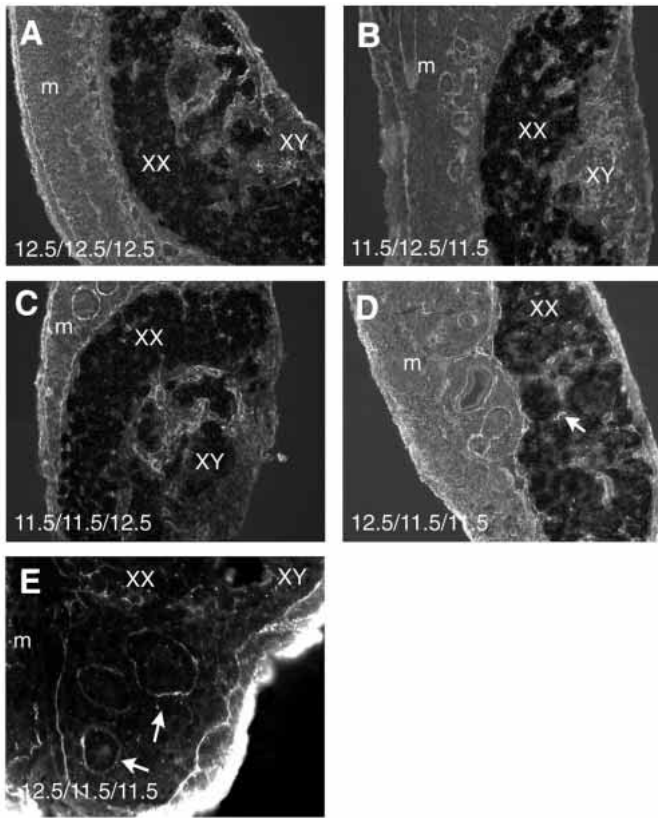


Fig. 4. Organization of laminin and germ cells in sandwich gonads is dependent upon the stage of the XX and XY gonad. (A-E) Samples were processed for alkaline phosphatase activity (dark cells) and subsequently with an antibody against laminin-1 (bright staining). Stage of each component at the time of assembly is indicated at the bottom left of each panel as mesonephros/XX gonad/XY gonad. (A) No laminin deposition or germ cell aggregation occurred in the XX portion of sandwiches when all parts were 12.5 dpc. (B) No laminin deposition or germ cell aggregation occurred in the XX portion of sandwiches when the XX gonad was 12.5 dpc and all other parts were 11.5 dpc. No organization occurred in the XY portion of this sample. (C) No laminin deposition or germ cell aggregation occurred in the XX portion of sandwiches when the XX gonad and mesonephros were 11.5 dpc and the XY gonad was 12.5 dpc. (D,E) Two examples of laminin deposition and germ cell aggregation within the XX portion of sandwiches when the mesonephros was from a 12.5 dpc XX embryo and all other parts were 11.5 dpc. (D) Intermediate organization within the XX portion (arrow). (E) A high magnification view of cord structures within the XX portion of a sandwich (arrows). m, mesonephros.

sample. This was the case when the XY gonad was isolated from its mesonephros after 18ts.

In older and heterochronic sandwiches in which the XX gonad was 12.5 dpc, a patch of *Sox9* expression was seen in the XX tissue in cells near the XY border of a few samples (Fig. 5E), but not in most (Fig. 5F). This effect was independent of the presence of a mesonephros (Fig. 5G). When a 13.5 dpc XX gonad was used, *Sox9* expression was never seen within the XX portion (Fig. 5H). This expression data correlates with the loss of the ability to induce cord structures in XX gonads after 12.5 dpc. When 16-17ts XY gonads were removed from their mesonephroi, apposed along their cut edges and cultured without mesonephroi, *Sox9* expression was

Table 1. Summary of data

Experiment	Organization		
	-	±	+
11.5 XY control	0	0	15
11.5 XX control	11	0	0
11.5 XY G 11.5 XX G 11.5 M Sandwich	5	5	9
11.5 XY G 11.5 XX G Sandwich, no mesonephros	14	0	0
11.5 XY G 11.5 XX G 11.5 M Sandwich + membrane	11	0	0
11.5 XY G 11.5 XX G 11.5 Limb bud Limb Sandwich	20	0	0
12.5 XY G 12.5 XX G 12.5 M 12.5 Sandwich	17	0	0
11.5 XY G 12.5 XX G 11.5 M Heterochronic Sandwich	13	0	0
12.5 XY G 11.5 XX G 11.5 M Heterochronic Sandwich	14	0	0
11.5 XY G 11.5 XX G 12.5 XY M Heterochronic Sandwich	11	1	1
11.5 XY G 11.5 XX G 12.5 XX M Heterochronic Sandwich	2	5	4

Samples were scored for their organization in the XX portion of the tissue by comparison with controls.

-, no evidence of organized laminin and germ cells (these samples were indistinguishable from cultured XX controls).

+, clear aggregates of germ cells formed and circles of basal lamina were produced, as would occur in XY controls.

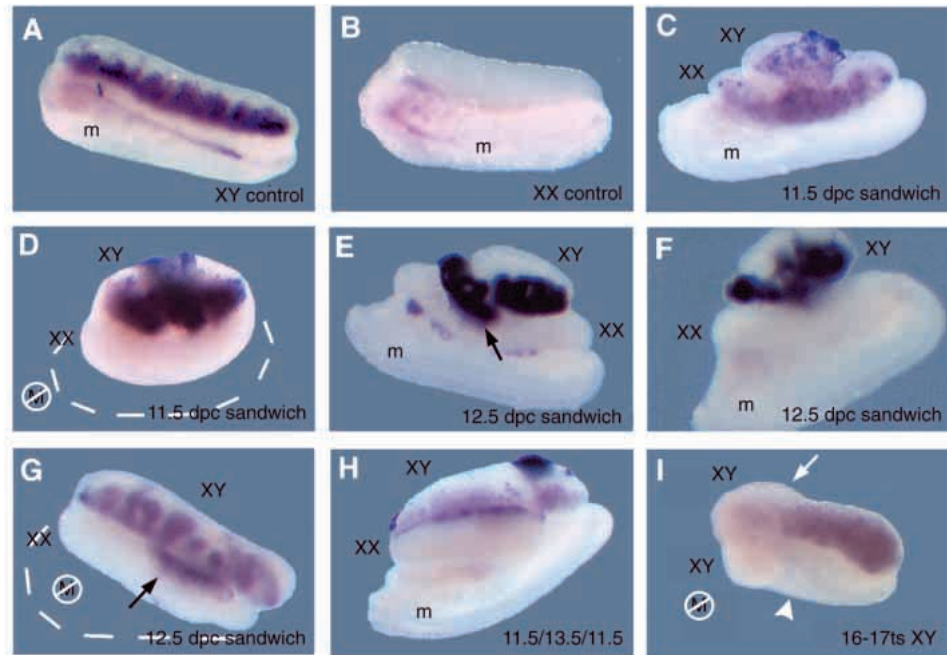
±, samples that had an ambiguous appearance: faint circles of laminin were seen enclosing germ cells, which were slightly aggregated in some regions.

Light gray, 11.5 dpc; dark gray, 12.5 dpc at the time of assembly.

severely compromised (Fig. 5I) compared to unseparated controls at the same stage (data not shown).

We also tested the expression of the female-specific marker, *Dax-1*, in sandwich gonads. As shown in Fig. 6A,B, *Dax-1* expression was strong in XX compared to XY control cultures initiated at 11.5 dpc and cultured for 30-44 hours. When migration was induced into 11.5 dpc XX gonads in sandwich cultures, *Dax-1* expression was downregulated (Fig. 6C). The down-regulation of *Dax-1* was also dependent upon the presence of a mesonephros as *Dax-1* expression was

Fig. 5. Mesonephric cell migration is required for *Sox9* expression in sandwich gonads. (A) *Sox9* was expressed inside testis cords in cultured XY controls, whereas no expression was seen in cultured XX controls (B). (C) Strong *Sox9* expression was seen in the XX portion of sandwich gonads assembled at 11.5 dpc. (D) No expression of *Sox9* was detected in the XX portion of sandwich gonads when the mesonephros was removed (original position of mesonephros is indicated by dashed line). *Sox9* expression was maintained in the XY portion of this sample, which was separated after 18ts. (E,F) Two samples of sandwiches assembled at 12.5 dpc showing slight *Sox9* expression near the XX/XY boundary (E, arrow) and a case where no *Sox9* expression was detected in the XX portion (F). (G) In sandwiches assembled at 12.5 dpc and cultured without mesonephroi, *Sox9* expression was occasionally detected near the XX/XY boundary (the original position of mesonephros is indicated by dashed line) (arrow). (H) When the XX gonad was 12.5 dpc or older and all other parts were 11.5 dpc, little to no *Sox9* expression was seen in the XX gonad. (I) *Sox9* expression was severely compromised in XY gonads when they were cultured without adjoining mesonephroi at 16-17ts. To prevent curling of the tissue, both XY gonads from the same embryo were apposed in culture. One gonad showed decreased *Sox9* expression in one half (arrow), and the other gonad showed no *Sox9* expression (arrowhead). m, mesonephros.



maintained in the XX portion when samples were cultured without a mesonephros (Fig. 6D). In sandwiches assembled at 12.5 dpc and cultured for 44 hours, *Dax-1* expression remained high in the XX portion (data not shown).

DISCUSSION

In this report, we show that somatic cell migration from the mesonephros plays a pivotal role in the formation of testis cords and differentiation of Sertoli cells. Migration, beginning

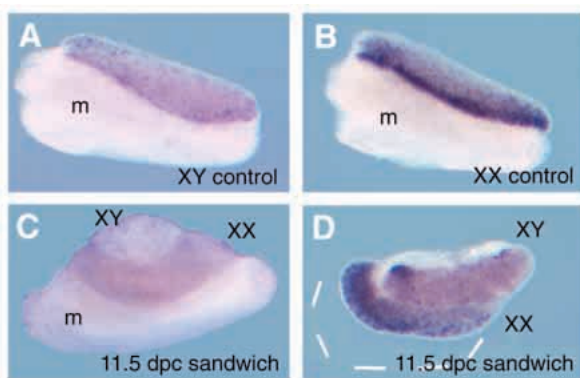


Fig. 6. Mesonephric cell migration downregulates *Dax-1* expression in sandwich gonads. (A) *Dax-1* expression levels were low in cultured XY controls whereas in cultured XX controls, expression was strong (B). (C) In the XX portion of 11.5 dpc sandwich gonads, expression of *Dax-1* resembled XY controls. (D) In sandwiches cultured without adjoining mesonephroi, expression of *Dax-1* was similar to XX controls (original position of mesonephros is indicated by dashed line). m, mesonephros.

prior to 18ts, is required for the initiation of cord formation in XY gonads. Furthermore, ectopic cell migration into XX gonads results in organization of XX tissue into testis cords. Mesonephric cell migration is required for the differentiation of Sertoli cells in XY gonads, and is capable of initiating this event in XX gonads in the absence of *Sry* expression within the XX cells.

Migration prior to 18ts is required for cord formation in XY gonads

Our experiments using an improved organ culture system and carefully staged XY gonads show that mesonephric cell migration prior to 18ts is required for cord formation in XY gonads. These results are consistent with the idea that cell migration begins before 18ts (11.5 dpc). Our results also show that continuing migration (at stages after 18ts) is not necessary to maintain cords, at least over the time course of these experiments (2 days).

Induction of migration into XX gonads results in cord formation

The role that mesonephric cell migration plays in cord formation was further investigated by inducing cells to migrate into bipotential XX gonads. This experiment has advantages over previous experiments. Male signaling events, including the male-specific migration of the mesonephric cell population, can be initiated under experimental control. Therefore, the gonad can be separated from its mesonephros after 18 tail somites (ts) when potential damage to the tissue is significantly reduced. In these experiments, both germ cells and somatic cells organized in a testis-like fashion, showing that migrating mesonephric cells can actually initiate cord formation in the absence of cell-autonomous *Sry* expression.

Formation of cord structures in XX gonads was dependent upon the migrating mesonephric cells. However, these experiments do not rule out the possibility that secreted substances produced by the XY gonad are also required for the induction of cord formation in a sandwiched XX gonad. However, if such substances are present, they alone are not sufficient to drive cord formation in the absence of migrating mesonephric cells. Our results with limb bud mesenchyme are contradictory to those of Moreno-Mendoza et al. (1995). An explanation for this, in light of our other results, is that in these earlier experiments, XY gonads were separated from their mesonephroi at stages when sufficient mesonephric cell migration had already occurred to initiate cord formation.

Induction of cord formation is stage restricted

Our experiments with heterochronic sandwiches show that after 12.5 dpc, XY gonads do not provide the proper signals to initiate cord formation in 11.5 dpc XX gonads, and that 12.5 dpc XX gonads are no longer capable of responding to induction. Two explanations for this effect in the inducing tissue are that (1) the XY gonad does not signal the proper cells to migrate or (2) a secreted substance required for cord formation is absent from the 12.5 dpc XY gonad. Of the migrating cell types, peritubular myoid cells are the most likely candidate to drive cord formation because of their close association with Sertoli cells. An explanation of these results, which is consistent with the failure of limb bud mesenchyme to induce cord formation, is that myoid cell precursors are specific to the mesonephros, and their migration occurs between approximately 11.5 and 12.5 dpc. However, a marker that distinguishes this cell type between 11.5 and 12.5 dpc is not yet available.

At 12.5 dpc and after, XY mesonephroi are less effective cell donors than XX mesonephroi. The reason for this is unclear; however, one possibility, consistent with the myoid cell hypothesis, is that 12.5 dpc XY mesonephroi are depleted of this cell population because they have donated cells to an XY gonad for approximately 24 hours prior to separation.

We have also shown that XX gonads are no longer able to form cords after 12.5 dpc (at least over the course of this assay). One possibility for this result is that there are important interactions established between XX somatic cells and/or germ cells that interfere with signals to form cords at 12.5 dpc and later.

Cell migration is required for the maintenance of Sertoli cell differentiation

The upregulation of *Sox9* and the downregulation of *Dax-1* in XY gonads are among the earliest known transcriptional events in testis development after the expression of *Sry* itself (da Silva et al., 1996; Kent et al., 1996; Swain et al., 1996). From these results, we conclude that migration of cells from the mesonephros plays a crucial role in controlling this event and/or the maintenance of Sertoli cell differentiation. Whether Sertoli cell differentiation in XX gonads is initiated by contact between basal lamina components and XX cells that act as Sertoli precursors or by some factor brought in by the migrating cells is unknown. Other experiments have shown that the basal lamina can play a role in the differentiation of epithelia (Streuli et al., 1991). Likewise, direct interactions between signaling molecules may occur between migrating

cells and cells within the gonadal tissue. This kind of cell induction has been shown in many systems where one cell or tissue type is required for the differentiation of another (Tanabe and Jessell, 1996; Shannon et al., 1998).

Recruitment of XX cells into the Sertoli lineage and XY cells into the follicle cell lineage has been shown previously in XX \leftrightarrow XY and XO \leftrightarrow XY chimeras by Palmer and Burgoyne (1991a,c). These results suggest that *Sry* is not solely responsible for the decision to become a Sertoli or follicle cell and that other cellular interactions are important. In XX \leftrightarrow XY chimeras, 10% of Sertoli cells were XX, showing that XX cells can differentiate as Sertoli cells without *Sry*. However, in these experiments, 90% of Sertoli cells were XY, demonstrating a strong bias for the XY cells to differentiate as Sertoli cells. Given our results, the bias for Sertoli cells to develop from XY precursors deserves consideration. First, expression of *Sry* in XY cells is likely to initiate Sertoli cell differentiation, giving XY cells a head start over XX cells. Second, the proliferation rate of XY cells could be faster. Experiments in our laboratory indicate that XY cells do proliferate faster than XX cells in the early stages of gonad development (J. Schmahl, E. M. Eicher, L. L. Washburn and B. C., unpublished data). Finally, XY cells might have a chemoattractive effect on migrating cells because it is the XY cells that are producing the signal(s) for migration (Martineau et al., 1997). Our results suggest that, in a mosaic gonad which forms an ovary or ovotestis, due to insufficient numbers of XY cells, XY supporting cells not coming into close proximity with migrating cells would become follicle cells.

Other cases of experimental sex reversal have been reported. Culturing fetal ovaries in the presence of MIS (Vigier et al., 1987) or under an adult kidney capsule (Takeeto et al., 1984; da Silva et al., 1996) has been shown to result in cord formation in XX tissue. The predominant idea for the mechanism of sex reversal in these cases is that follicle cells, which lose their meiotic germ cells, rearrange to form cords (Burgoyne et al., 1988; McLaren, 1990; Whitworth et al., 1996). This mechanism for sex reversal probably has little relevance to cord formation in vivo. It is important that in sandwich cultures in this study, germ cells within the XX tissue were not lost; therefore, this cannot account for the XX somatic and germ cell reorganization. Loss of germ cells may be necessary to induce cord formation after 12.5 dpc or once the XX follicle structure is established. The fate of XX germ cells within induced cords is under investigation.

The mechanism of sex reversal described here has strong implications for the early events in testis cord formation in vivo. Here we have defined the stage at which mesonephric cell migration is required in the normal XY gonad and shown that migration from the mesonephros, which is normally a male-specific event, is actually capable of driving cord formation and Sertoli cell differentiation in the bipotential XX gonad where cords would not normally form and *Sry* is not expressed.

One of the fundamental goals in developmental biology is to understand how regulatory genes initiate cellular processes, which in turn control the patterning and differentiation of tissues. Cell movements and cell interactions play important roles in the development of organs. In the XY gonad, migration of mesonephric cells is induced by the regulatory gene, *Sry*. From the results presented here, we conclude that cell migration initiates testis cord formation, which plays a critical role in

Sertoli cell versus follicle cell differentiation. These results provide a clear example of how a regulatory gene, *Sry*, initiates a cellular process, cell migration, which in turn influences the patterning and differentiation of cells within an organ.

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