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Developmental Biology 284 (2005) 337 – 350

DEVELOPMENTAL BIOLOGY

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Male-specific cell migration into the developing gonad is a conserved process involving PDGF signalling

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Received for publication 14 April 2005, revised 23 May 2005, accepted 24 May 2005
Available online 7 July 2005

Abstract

Male-specific migration of cells from the mesonephric kidney into the embryonic gonad is required for testis formation in the mouse. It is unknown, however, whether this process is specific to the mouse embryo or whether it is a fundamental characteristic of testis formation in other vertebrates. The signalling molecule/s underlying the process are also unclear. It has previously been speculated that male-specific cell migration might be limited to mammals. Here, we report that male-specific cell migration is conserved between mammals (mouse) and birds (quail–chicken) and that it involves proper PDGF signalling in both groups. Interspecific co-cultures of embryonic quail mesonephric kidneys together with embryonic chicken gonads showed that quail cells migrated specifically into male chicken gonads at the time of sexual differentiation. The migration process is therefore conserved in birds. Furthermore, this migration involves a conserved signalling pathway/s. When GFP-labelled embryonic mouse mesonephric kidneys were cultured together with embryonic chicken gonads, GFP+ mouse cells migrated specifically into male chicken gonads and not female gonads. The immigrating mouse cells contributed to the interstitial cell population of the developing chicken testis, with most cells expressing the endothelial cell marker, PECAM. The signalling molecule/s released from the embryonic male chicken gonad is therefore recognised by both embryonic quail and mouse mesonephric cells. A candidate signalling molecule mediating the male-specific cell migration is PDGF. We found that PDGF-A and PDGF receptor-α are both up-regulated male-specifically in embryonic chicken and mouse gonads. PDGF signalling involves the phosphotidylinositol 3-kinase (PIK3) pathway, an intracellular pathway proposed to be important for mesonephric cell migration in the mammalian gonad. We found that a component of this pathway, PI3KC2α, is expressed male-specifically in developing embryonic chicken gonads at the time of sexual differentiation. Treatment of organ cultures with the selective PDGF receptor signalling inhibitor, AG1296 (tyrphostin), blocked or impaired mesonephric cell migration in both the mammalian and avian systems. Taken together, these studies indicate that a key cellular event in gonadal sex differentiation is conserved among higher vertebrates, that it involves PDGF signalling, and that in mammals is an indirect effect of *Sry* expression.

Keywords: Cell migration; Gonad; PDGF signalling; Testis development; Sex determination; *Sry*

Introduction

The differentiation of embryonic gonads into either testes or ovaries provides an ideal model system for studying organogenesis. In mammals, gonadal sex differentiation is governed by *SRY*, the so-called master sex-determining gene, which initiates a cascade of molecular and cellular events leading to testis formation in male embryos (Sinclair et al., 1990; Koopman et al., 1990, 1991). In the absence of *SRY*, as in XX embryos, ovarian differentiation occurs. In the mouse model, *Sry* expression between 10.5 and 12.5 days post coitum (dpc) leads to a number of distinct male-specific cellular events. These events include an increase in cell proliferation during a critical developmental window (Schmahl and Capel, 2003; Schmahl et al., 2000), differentiation of Sertoli cells and their organisation into seminiferous cords (Magre and Jost, 1991; Tilmann and Capel,

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doi:10.1016/j.ydbio.2005.05.030

1999), establishment of male-specific vasculature, including a prominent coelomic blood vessel (Brennan et al., 2002), and recruitment of mesenchymal cells from the neighbouring mesonephric kidney (Merchant-Larios et al., 1993; Buehr et al., 1993; Martineau et al., 1997; Capel et al., 1999; Albecht et al., 2000). These male-specific processes occur shortly after *Sry* expression in the embryonic mouse gonad and they do not occur in embryonic female gonads over the same time period.

Cellular events that have been identified in presumptive male gonads appear to be vital for proper testis organisation. In the mouse embryo, migration of cells from the neighbouring mesonephros is a key process required for normal testis development. Embryonic gonads cultured in the absence of a mesonephros still show Sertoli cell differentiation, but the cells fail to organise into seminiferous cords (Buehr et al., 1993; Merchant-Larios et al., 1993). Factors that inhibit migration of mesonephric cells in vitro can disrupt normal cord organisation (Uzumcu et al., 2002a,b; Cupp et al., 2003). Furthermore, when an embryonic XX gonad is cultured between an XY gonad and mesonephros, the XY gonad can induce mesonephric cell migration through the XX gonad, which then shows cord organisation (Tilmann and Capel, 1999). Although cells recruited into the male gonad under the influence of *Sry* seem to be required for proper cord formation, they do not contribute structurally to the cords. Immigrating cells surround the cords, differentiating into interstitial myoid and endothelial cells, and probably also the testosterone-producing Leydig cells (Buehr et al., 1993; Martineau et al., 1997; Merchant-Larios and Moreno-Mendoza, 1998; reviewed in Brennan and Capel, 2004). It is thought that the myoid population interacts with differentiating Sertoli cells to produce basement membrane and organise cord structure (Tung and Fritz, 1980; Skinner et al., 1985). Studies on mouse strains with aberrant *Sry* alleles, or those carrying an *Sry* transgene, show that this gene is both necessary and sufficient for mesonephric cell migration into the mammalian gonad (Capel et al., 1999; Albecht et al., 2000). Therefore, one of the key cellular events initiated by *Sry* is male-specific mesonephric cell migration.

The importance of male-specific mesonephric cell migration has been clearly established in the mouse model, where it depends on *Sry* expression. However, conservation of this process among other vertebrates has not been demonstrated. Embryonic turtle gonads of either sex cultured with embryonic mouse mesonephric kidneys do not induce any significant mesonephric cell migration at the time of sexual differentiation (Yao et al., 2004). One interpretation of these findings is that male-specific cell recruitment could represent a mammalian-specific process, regulated by *Sry*, which is restricted to mammals. This would represent a fundamental difference between gonadal development in mammals and that in other vertebrates. However, here we report that the key process of male-specific mesonephric cell migration is conserved in avian embryos (co-cultured embryonic chicken gonad–quail mesonephros). In addition, male-specific cell migration also occurs in chicken–mouse co-cultures, demonstrating that the paracrine signalling mechanism involved is at least partially conserved between mammals and birds. Lastly, inhibition of platelet-derived growth factor receptor (PDGFR) tyrosine kinase disrupts male-specific migration in both the mammalian and avian systems, pointing to a conserved pathway in both groups. These results have implications for our understanding of mesonephric cell migration generally, because migration occurs in the avian system despite the absence of the *Sry* gene.

Materials and methods

Chicken and quail embryos

Fertilised eggs of the chicken (*Gallus gallus*) and quail (*Coturnix coturnix japonica*) were obtained from a local breeder and incubated at 37.8°C/70% relative humidity until required. All embryos were staged using the morphological criteria of Hamburger and Hamilton (1951). Chicken embryos were sexed by PCR, using W (female)-specific *Xho1* primers and autosomal *GAPDH* primers as internal controls, as described previously (Smith et al., 1997). Quail embryos were also sexed by PCR, but using quail-specific *WPKCI* (W-linked) primers (Hori et al., 2000) together with chicken GAPDH primers, which recognised quail *GAPDH*. Female embryos showed a strong 229-bp *WPKCI* PCR product, while males showed no product or weaker related background bands.

Mouse embryos

Swiss white HSD/OLA wild type and GFP-Lac Z mice were used (Tam et al., 1994; Hadjantonakis et al., 1998). Day 0 of embryogenesis was taken from midnight on the evening of mating, confirmed by vaginal plugging. Embryos of the required age were dissected from uteri and the gonads and/or mesonephroi removed with the aid of a dissecting microscope. Tissues were held at 37°C in culture medium prior to the assembly of co-cultures (described below). Mouse embryos were sexed using *Sry* PCR primers, with *myogenin* used as internal control (McClive and Sinclair, 2001).

Antibodies and immunofluorescence

Polyclonal antibodies were raised in rabbits against chicken aromatase, SOX9 and DMRT1 proteins. For chicken DMRT1, an internal sequence was used, predicted to be superficial and antigenic. This antibody has been described previously (Smith et al., 2003). For aromatase and SOX9, sixteen amino acids at the C-terminal were chosen as immunogens, conjugated to diphtheria toxoid. These C-terminal regions have previously been used to successfully

raise antibodies. All antibodies were purified by protein-A sepharose chromatography. The aromatase antibody detected a single band of the predicted size on Western blots of urogenital tissue, which was abolished when the antibody was pre-absorbed against the immunising peptide. For SOX9, two bands were detected by Western blotting, but the cognate band was abolished with antigen pre-adsorption prior to blotting (not shown). These antibodies were used as markers of chicken testicular and ovarian differentiation in vivo and in vitro. For the in vivo studies, urogenital systems were dissected from precisely staged chicken embryos prior to and during gonadal sex differentiation (stages 27, 28, 29, 30, 31 and 33; corresponding to embryonic days 5.0, 5.5, 6.0, 6.5, 7.0 and 7.5, respectively). All embryos were staged using the morphological criteria of Hamburger and Hamilton (1951).

Tissues were briefly fixed in 4% paraformaldehyde in PBS at room temperature, equilibrated overnight at 4°C in 20% sucrose in PBS, and snap frozen in OCT embedding compound (Tissue Tek). Ten-micron frozen sections were mounted onto silane-treated slides and briefly air-dried. For urogenital tissues dissected from staged chicken embryos, transverse sections were cut. For all tissues used in the organ co-cultures, longitudinal sections were cut. Sections were firstly treated with 1% Triton X-100 in PBS, blocked for 1 h in 5% BSA/PBS, and incubated overnight at 4°C with primary antibodies diluted in 1%BSA/PBS. The aromatase, DMRT1 and SOX9 in-house antibodies were used at 1 μg/mL. The SOX9 antibody cross-reacted with both chicken and mouse SOX9. Mouse anti-chicken fibronectin (Serotec) was used at 1:200, rat anti-PECAM (Abcam) at 1:100, rabbit anti-laminin (Sigma) at 1:200, and mouse anti-GFP (Sigma) at 1:500. To detect quail cells, a mouse anti-QCPN primary antibody was used at 1:50 (Developmental Studies Hybridoma Bank). This antibody detects a nucleolar antigen present in all quail cells. It does not recognise chicken cells. Following incubation with primary antibodies, sections were washed in PBS and incubated for 1 h at room temperature with the appropriate secondary antibodies linked to red or green Alexa fluors (Alexa 594 or Alexa 488; Molecular Probes). Sections were washed again, mounted in Fluorosave (Calbiochem) and examined under a fluorescence compound microscope. Control sections lacked primary antibody or were incubated instead with pre-immune serum.

Expression analysis of PDGF-A, PDGF receptor-α and phosphotidylinositol 3-kinase, C2 domain containing, alpha polypeptide (PI3KC2α) by whole mount in situ hybridisation

Urogenital systems were dissected from staged chicken and mouse embryos and fixed overnight at 4°C in 4% paraformaldehyde/PBS. Tissues were then dehydrated through increasing concentrations of methanol and stored at −20°C in 100% methanol prior to analysis. Whole mount in situ hybridisation was carried out using digoxygenin-labelled RNA probes as previously described (Andrews et al., 1997). The cDNA templates for RNA probe synthesis were generated by PCR, cloned into plasmid vectors and confirmed by sequencing. These cDNA templates were *G. gallus* platelet-derived growth factor alpha (PDGF-A; NM_204306; bases 159–562) and *G. gallus* PDGF receptor alpha (PDGFR-α; NM_204749, bases 168–568). The 800-bp *PI3KC2α* fragment isolated by subtractive hybridisation (Hudson et al., 2005) was cloned into pGEM®-T easy vector. Following probe hybridisation, and incubation with alkaline phosphatase-conjugated DIG antibody, tissues were left in the NBT/BCIP chromogen to allow staining to develop. Staining was only present in tissues exposed to the antisense probe, with no staining observed in the sense probe negative control.

Chicken–quail interspecific organ cultures

Chicken and quail eggs were incubated to the onset of gonadal sex differentiation, stage 29–30 (day 6–6.5 for chicken, day 5.5–6 for quail). Under sterile conditions, stage 29–30 embryonic chicken gonads were carefully dissected away from the adjoining mesonephric kidneys. Similarly, quail gonads and mesonephroi were separated and the mesonephroi retained. Stage-matched chicken gonads and quail mesonephroi were placed together within grooves on agar slabs [1.5% agar (Difco) in DMEM culture medium]. Only the left chicken gonad was used, because the right gonad in females does not differentiate into a normal ovary. Tissues were placed together such that the chicken gonad was abutting the surface of the quail mesonephros in the region where the quail gonad had been removed. At the time of explantation, a small amount of tissue was digested in PCR-compatible buffer (McClive and Sinclair, 2001), and 1 μL was taken for PCR sexing as described above. Following assembly of organ co-cultures, agar slabs were placed into 35-mm tissue culture dishes (Falcon) containing 0.5 mL DMEM culture medium supplemented with 10% foetal calf serum and ampicillin at 50 μg/mL. DMEM lacking phenol red was used, as previous studies have shown that phenol red can act as a weak estrogen. Estrogen (normally produced only by female gonads) can potentially feminise male gonads. Pilot experiments were also conducted using charcoal-stripped foetal calf serum to remove any potential steroid hormones such as estrogen, but gonadal sex differentiation proceeded normally in vitro with either stripped or nonstripped serum. Nonstripped serum was therefore used. Co-cultures were incubated under humid conditions in a 37°C/5% CO_2 incubator for 3 days (equivalent to day 9, stage 35, in vivo). The culture medium was changed each day. For each sex of chicken gonad, several co-cultures with either male or female quail mesonephros were set up. After 3 days, tissues were processed for immunofluorescence as described above.

Chicken–mouse interspecific organ cultures

Left gonads were isolated from stage 29–30 embryonic chicken embryos as described above. Individual gonads were assembled together with individual mesonephroi from 15.5 dpc mouse embryos ubiquitously expressing GFP. Previous studies have shown that embryonic mouse mesonephroi of either sex are competent to contribute mesenchymal cells to mouse gonads from 11.5 to 16.5 dpc (Martineau et al., 1997). Chicken gonad–mouse mesonephroi were co-cultured on agar slabs as detailed above. Organs were cultured for 3 days, with medium being changed each day. At the end of the culture period, co-cultures were examined and photographed under a dissecting microscope equipped with fluorescence optics to view overall GFP-labelled cell migration. Tissues were then fixed in 4% paraformaldehyde and processed for immunofluorescence as described above.

Inhibition of PDGF signalling in vitro

Interspecific chicken gonad–quail mesonephros and chicken gonad–mouse mesonephros co-cultures were assembled as described above. A selective inhibitor of PDGF receptor signalling, AG1296 (tyrphostin; Calbiochem), was added to the agar slabs and to the culture medium. AG1296 specifically blocks PDGF receptor tyrosine kinase function, effecting both the PDGFα and PDGFβ receptors. AG1296 was dissolved in dimethylsulfoxide (DMSO) and added at a final concentration of 10 μM. Uzumcu et al. (2002a,b) carried out a dose–response study and found that AG1296 used at 10 μM was the optimal concentration for disturbing cord formation in embryonic rat gonads cultured in vitro. Control co-cultures received vehicle (0.1% DMSO) dissolved in culture medium. For mouse gonad–mesonephros co-cultures, DMEM with phenol red + 10% FCS and ampicillin was used. 12.5 dpc tissues were assembled for the mouse studies. For the chicken–quail co-cultures, DMEM-phenol red + 10% FCS and ampicillin was used, and stage 29/30 (day 5.5–6.0) tissues were used. Organs were co-cultured for 2 days (mouse) or 3 days (chicken quail), with a fresh medium change each day. For both the chicken–quail and chicken–mouse experiments, sufficient numbers of co-cultures were set up so that at least four samples were included in each treatment or control group. Chicken–quail co-cultures were fixed and processed for immunofluorescence, while mouse tissues were firstly viewed under a dissecting microscope to assess GFP+ cell migration before fixing and processing.

Results

Gonadal differentiation in the chicken embryo

To firstly characterise the normal course of gonadal sex differentiation in the avian gonad, urogenital systems were dissected from chicken embryos before, during and after the time of histological differentiation (Carlon and Stahl, 1985; Ebenspeger et al., 1988). The first unambiguous markers of chicken gonadal sex differentiation are SOX9 (male) and aromatase (female). Previous studies have shown that the *SOX9* and *aromatase* genes are expressed from at least as early as day 6.5 (stage 30) in embryonic chicken gonads. These two genes are known or inferred to be required for male and female sex determination in the avian model. However, all previous studies have assessed the onset of chicken *SOX9* and *aromatase* expression at the transcriptional level only. Here, we studied expression at the protein level in carefully staged embryos, as a prerequisite for the organ culture studies.

In both sexes, gonads appeared morphologically undifferentiated at stage 28 (day 5.5) (Fig. 1). At this stage, no SOX9 or aromatase protein could be detected by immunofluorescence. Fibronectin immunostaining revealed unorganised cords of cells already present in the gonads of both presumptive males and females (Figs. 1A and B). SOX9 protein was first detectable by immunofluorescence in stage 29 (day 6.0) male embryos. Approximately 50% of the somatic cells within the inner part of the gonad (the medulla) showed positive nuclear staining for SOX9 at this stage. Expression became more widespread at stage 30, when visibly organised seminiferous cords became apparent, as assessed by fibronectin immunofluorescence. From stage 31 (day 7.5), male-specific SOX9 expression was detectable in all somatic cells of the organising cords (Fig. 1). No SOX9 protein was ever detected in female gonads.

Aromatase protein expression was also first detected in stage 29 (day 6.0) embryos. Aromatase was only seen in female gonads, where its initial expression in the somatic cells of the medullary cords was higher in the left compared to the right gonad (Fig. 1B). By stage 30, expression was strong in both the left and right gonads, a trend maintained over stages 31–33. Over stages 30–33, the outer cortical layer of the right gonad became diminished, while the same region of the left gonad thickened and proliferated, consistent with previous reports showing that only the left gonad develops into a functional ovary in the chicken embryo.

These in vivo expression profiles provided a benchmark for the in vitro cell migration experiments. Pilot studies showed that embryonic chicken urogenital systems (gonad + mesonephros) dissected at stage 29 or 29/30 and placed in organ culture grew and differentiated according to their genetic sex. Male tissues expressed SOX9 while female tissues expressed aromatase markers, as occurs in vivo. Gonads dissected one stage earlier (stage 28) did not differentiate as well as those explanted at stage 29, while those dissected at stage 27 (day 5.0) failed to develop in vitro. This is reminiscent of the findings in mouse embryos, where gonads explanted at 11.5 dpc differentiate in vitro, but differentiation is compromised when explants are taken at 10.5 dpc.

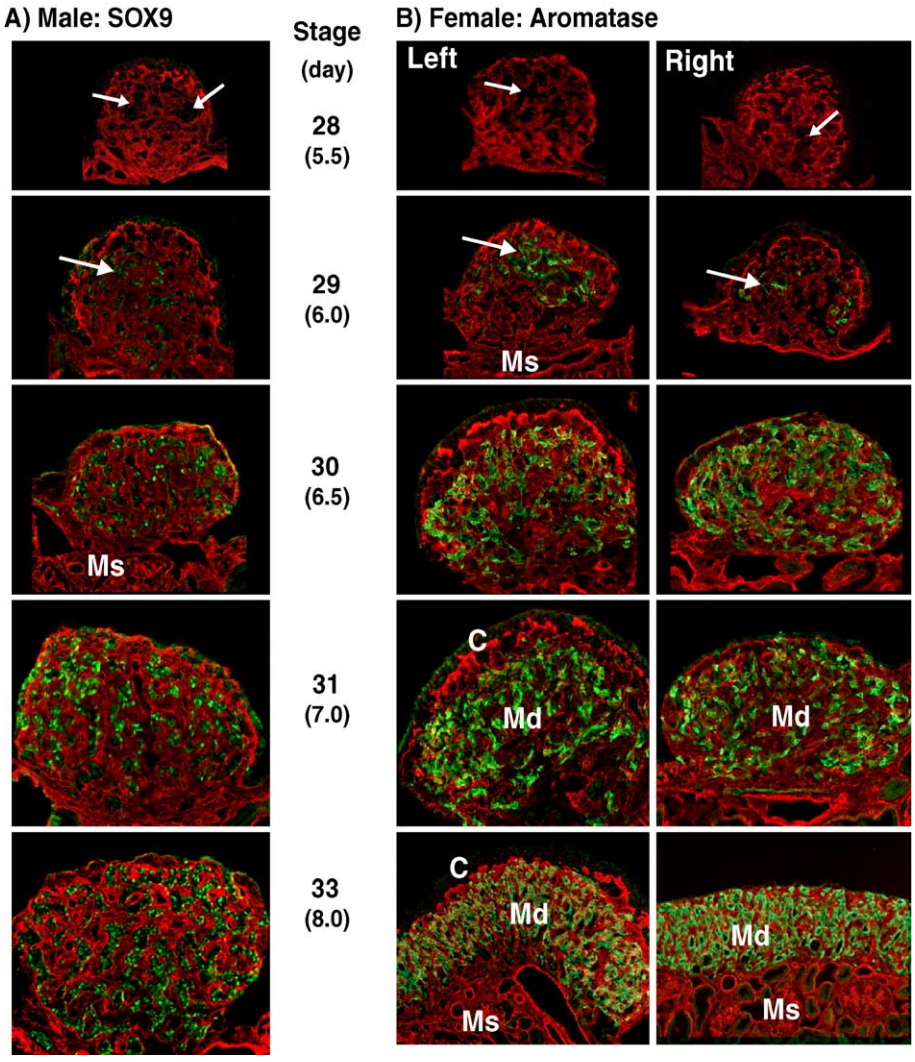

Fig. 1. Expression of SOX9 and aromatase proteins during gonadal sex differentiation in embryonic chicken gonads, determined by immunofluorescence. SOX9 is only detectable in male gonads (A; green), while aromatase is only detectable in female gonads (B; green). General organisation of the gonads is revealed by fibronectin immunofluorescence (red), which stains the interstitium but not the gonadal cords. At developmental stage 28 (day 5.5), the gonads of both sexes are morphologically undifferentiated. Fibronectin immunofluorescence (red) outlines cords of cells in the interior (medulla) of the gonad in both presumptive males and females (arrows). At stage 29 (day 6.0), some cells within the cords of male gonads show positive nuclear staining for SOX9 (green; arrows). At the same stage, some cells in female cords become positive for aromatase, with more positive cells in the left compared to the right gonad (green; arrows). By stage 30 (day 6.5), fibronectin negative cords are becoming organised into uniformly sized seminiferous cords in male gonads, and SOX9 expression is widespread within the cords. In stage 30 female gonads, the outer cortex of the left gonad is thickening, separated from the underlying medulla by a strongly fibronectin positive basement membrane. The cortex does not thicken in the right gonad. In both left and right female gonads, aromatase protein expression is now very strong. After stage 30, SOX9 and aromatase expression remain strong, and, by stage 33 (day 7.5–8.0), the testis and (left) ovary are well-developed. C = gonadal cortex; Md = gonadal medulla; Ms = mesonephric kidney.

Male-specific mesonephric cell migration is conserved in the avian gonad

Embryonic quail mesonephric kidneys were cultured together with embryonic chicken gonads to determine whether male-specific cell migration is conserved in avian embryos. Chickens and quails are very closely related birds within the order Galliformes. Tissues were explanted at stage 29 or stage 29/30, that is, at the onset of gonadal differentiation. Immigration of quail mesonephric cells was assessed by immunofluorescent detection of the ubiquitous quail-specific marker, QCPN. These studies showed that there was male-specific recruitment of quail cells into the embryonic chicken gonad after 3 days in culture (Fig. 2). In stage 29 embryonic female chicken gonads cultured together with stage 29 embryonic quail mesonephroi of either sex, there was no immigration of $QCPN^{+}$ quail cells (Fig. 2A). In these co-cultures, the female embryonic chicken gonad strongly expressed the aromatase protein (Fig. 2B), demonstrating sexual differentiation in vitro. Double labelling showed mutually exclusive staining for quail QCPN positive mesonephric cells (green) and chicken gonadal aromatase positive cells (red) (Fig. 2C).

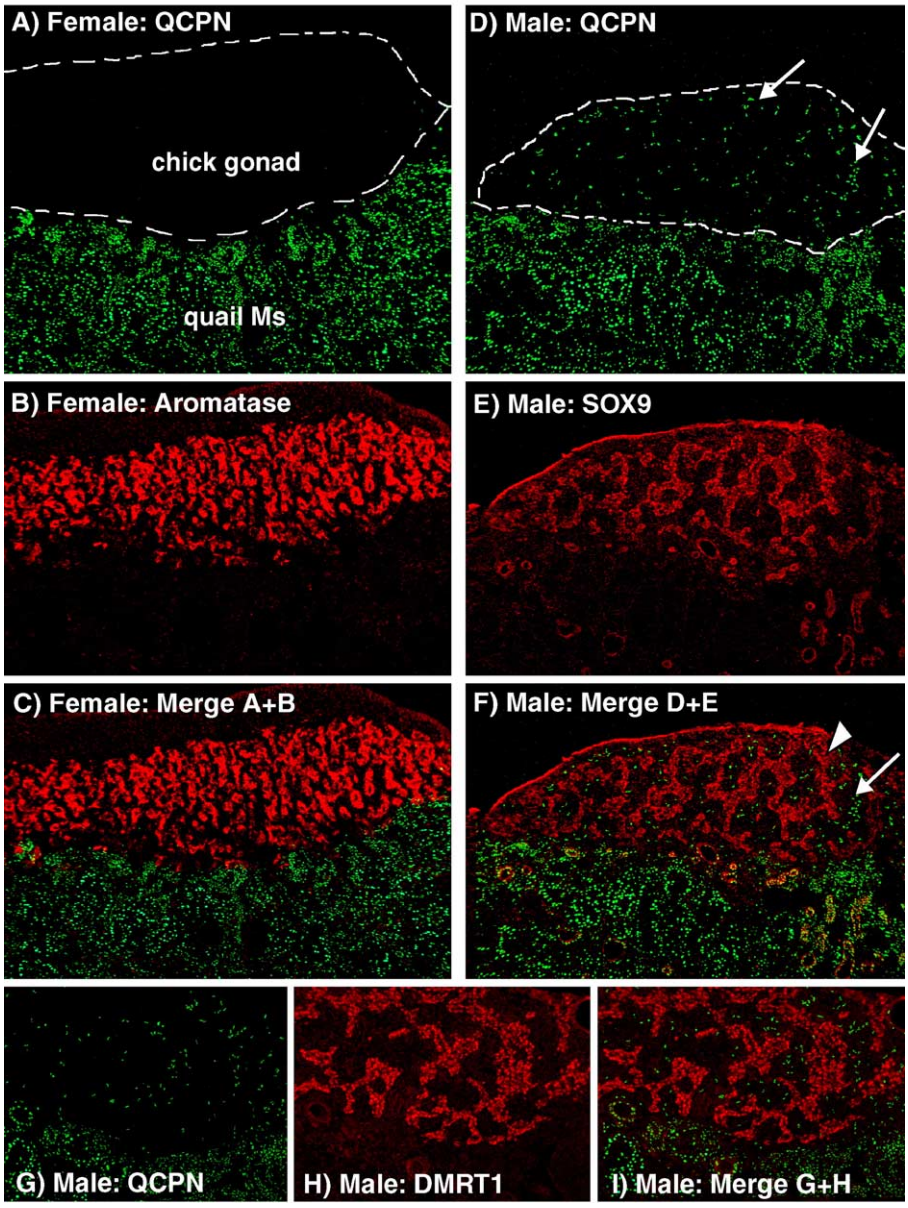

Fig. 2. Chicken–quail gonad–mesonephros co-cultures, showing male-specific migration of mesonephric cells. Stage 29 embryonic chicken gonads co-cultured for 3 days with stage-matched embryonic quail mesonephric kidneys. Quail QCPN (green), aromatase in female (red), and SOX9 or DMRT1 in males (red). (A) Female chicken gonad cultured with female quail mesonephros, showing green QCPN+ quail mesonephros and no migration into the gonad. (B) Aromatase expression in female gonad. (C) Merged images of panels A and B. (D) Male chicken gonad cultured with female quail mesonephros, showing migration of quail cells into the gonad (arrows). (E) SOX9 expression in the male gonad, showing gonadal differentiation in vitro. (F) Merged images of panels D and E, showing immigration of quail cells (arrows) between the cords of the male gonad (arrowhead). (G) High-power view of green QCPN+ quail cells in a male chicken gonad. (H) DMRT1 expression in the same male gonad, delineating well-formed cords. (I) Merged images of panels G and H.

In contrast, stage 29 male embryonic chicken gonads cultured with quail mesonephroi of either sex induced migration of QCPN positive mesenchymal cells (Fig. 2D). Male gonadal sex differentiation in vitro was assessed by the expression of SOX9 protein, which delineated well-developed seminiferous cords after 3 days in culture, as expected (Fig. 2E). Double labelling for SOX9 and QCPN showed that the immigrating cells were distributed in the interstitium between the cords, with no SOX9 co-localisation (Fig. 2F). Some sections were also double-stained for DMRT1 protein and QCPN. *DMRT1* is a Z-chromosome linked candidate male determining gene in birds. It shows strong up-regulated expression in developing male embryonic chicken gonads, where it is expressed in Sertoli and germ cells. In co-cultures, DMRT1 showed strong expression in Sertoli and germ cells within developing cords, with no overlap of expression with QCPN. (Figs. 2G–I). These data indicate that the cells migrating into the male embryonic chicken gonad from stage 29 do not contribute to Sertoli or germ cell populations. Rather, they

contribute exclusively to the interstitial cell population (myoid and/or endothelial and/or steroidogenic Leydig cells).

A conserved signalling mechanism induces male-specific mesonephric cell migration in mammals and birds

To determine whether male-specific mesonephric cell migration into the developing gonad involves a conserved signalling mechanism, we cultured embryonic chicken gonads together with GFP-expressing mouse mesonephroi (Fig. 3). The embryonic chicken gonads were at stage 29 or 29/30, while the mouse mesonephroi were at 15.5 dpc. Previous studies have shown that the mouse mesonephros is competent to contribute cells from 11.5 to 16.5 dpc, at least in mouse–mouse co-cultures (Martineau et al., 1997). When female chicken gonads were used in the gonad–mesonephros co-cultures, no immigration of GFP^+ mouse mesonephric cells could be seen after 3 days in vitro (Fig. 3A). In contrast, presumptive male chicken gonads induced migration (Figs. 3B and C). In some cases, isolated groups of GFP^+ mouse cells were seen throughout the male chicken gonad (Fig. 3B), while in other cases, a connecting network of cells was present (Figs. 3B and C).

Chicken–mouse co-cultures were sectioned and stained for the expression of marker proteins. In male chicken gonads, DMRT1 expression revealed normal seminiferous cord organisation, with the GFP+ mouse cells distributed between the cords, as in the chicken–quail studies (Fig. 3D). Immigrating GFP+ mouse cells were double labelled with the mammalian endothelial cell marker, PECAM, which does not detect avian PECAM. This showed that most immigrating mouse cells had an endothelial-like phenotype (yellow fluorescence in merged images; Figs. 3E–G). (PECAM also labels mammalian germ cells, but no germ cells are expected to be present among the mouse meso-

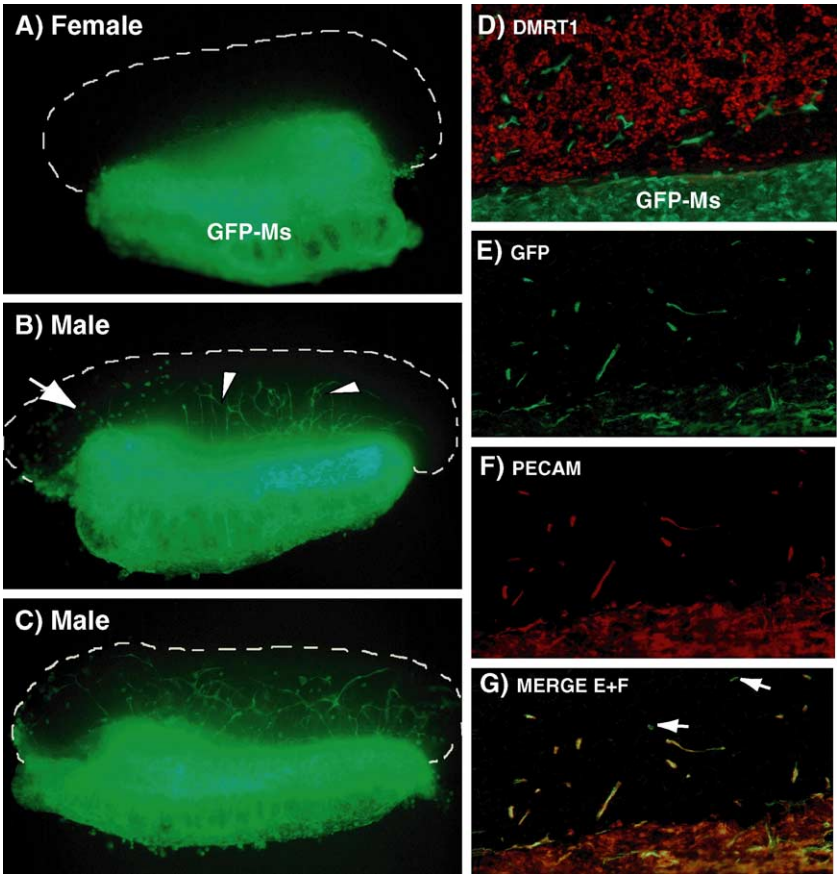

Fig. 3. Embryonic chicken gonad–mouse mesonephros co-cultures, showing conserved male-specific cell migration. Chicken gonads were taken from stage 29 embryos, and mouse mesonephroi were taken from 15.5 dpc embryos expressing GFP. Tissues were co-cultured for 3 days. (A) Female chicken gonad co-cultured with a female mouse mesonephros. No migration of GFP+ mouse mesonephric cells is seen. (B) Male chicken gonad induces migration of GFP+ mouse cells. In some areas, isolated groups of cells are detected (arrow), and in other areas, a network of connecting cells is apparent (arrowheads). (C) Another male chicken gonad–mouse mesonephros co-culture, showing an extensive network of migrating mouse cells. (D) Section taken through a co-culture of male chicken gonad–female mouse mesonephros double stained for DMRT1 and GFP. The GFP+ mouse cells have migrated between the organising seminiferous cords. (E) Distribution of GFP+ mouse cells in a male chicken gonad. (F) Same section as panel E above, showing expression of (mouse) PECAM in immigrating cells. (G) Merged image of panels E and F, showing that most immigrating mouse cells express PECAM (yellow). However, some GFP+ cells do not express PECAM (green only; arrows).

nephric cells.) A small number of GFP+ cells did not stain for PECAM, suggesting that they were destined to become other interstitial cell types, namely, myoid or embryonic Leydig cells. Since the mouse mesonephric cells could migrate into embryonic chicken gonads, the signalling mechanism must be at least partly conserved between the two groups.

Male up-regulated expression of PDGF-A and PDGFR-α genes in embryonic chicken gonads

A candidate growth factor regulating male-specific migration in both mammalian and avian systems is platelet-derived growth factor (PDGF) and/or its receptors. PDGFs are paracrine growth factors, mediating proliferation and migration of mesenchymal cells in a variety of developmental contexts. There are currently four known PDGFs, -A, -B, -C, and -D. Their receptors are members of the receptor tyrosine kinase family, and there are currently two known forms, α and β. PDGFs can bind the receptors as homo- or heterodimers, and different combinations of dimers have different affinities for the two receptors (Heldin and Westermark, 1999). Previous studies have suggested that platelet-derived growth factor receptor-α (PDGFR-α) plays a role in mesonephric cell migration and the organisation of seminiferous cords during mouse testicular differentiation (reviewed in Brennan and Capel, 2004).

PDGF-A and *PDGFR-α* mRNA expression were examined in stage 30, 33, and 35 chicken embryos (during the time of gonadal sex differentiation). In whole urogenital tissues, *PDGF-A* expression was detected in the gonads of both sexes, but was stronger in males (Fig. 4). Similarly, *PDGFR-α* was up-regulated in males but was only weakly detectable in females (Fig. 4). For *PDGFR-α*, expression was also seen in the Müllerian ducts (again, higher in males).

A component of the phosphotidylinositol 3-kinase pathway, PI3KC2α, is expressed male-specifically in embryonic chicken gonads

The effects of PDGF and other growth factors are mediated through a number of intracellular pathways, including phosphotidylinositol 3-kinase (PI3K). In a PCR-based screen for genes differentially expressed in embryonic chicken gonads (Hudson et al., 2005), we identified the phosphatidylinositol 3-kinase, C2 domain containing, alpha polypeptide (PI3KC2α). The expression profile of *PI3KC2α* mRNA was examined in embryonic chicken urogenital systems, using whole mount in situ hybridisation. PI3KC2α was expressed male-specifically between stages 25 (day 4.5) and stage 30 (day 6.5) (Fig. 5). This coincides with the onset of gonadal sex differentiation (Fig. 1) and the time of mesonephric cell migration demonstrated in vitro (Fig. 2). Male-specific expression of PI3KC2α may be involved in the conserved male-specific immigration of mesonephric cells seen in the embryonic urogenital system, potentially mediating the involvement of PDGFR.

Conserved mesonephric cell migration involves platelet-derived growth factor (PDGF) signalling

To assess whether PDGF signalling has a conserved role in mesonephric cell migration, PDGFR signal transduction was blocked in vitro, using the specific inhibitor, AG1296 (tyrphostin). This inhibitor blocks both α and β PDGF receptor functions. This experiment was firstly conducted in the mammalian system, using GFP^+ mesonephroi (15.5 dpc) cultured with 12.5 dpc wild type embryonic mouse gonads. When female embryonic mouse gonads were cultured with GFP-labelled mesonephroi, no mesonephric cell migration was detected after 2 days in culture (Fig. 6A). In contrast, male wild type gonads induced GFP^+ mesonephric cell migration (Fig. 6B). In the presence of AG1296 PDGFR inhibitor, no migration was seen into female gonads, as in control females (Fig. 6E). However, AG1296 disrupted cell migration into male gonads after 2 days in culture (Fig. 6F). In sections of control male gonads, seminiferous cords showed normal organisation in vitro, outlined by the basement membrane marker, laminin (Fig. 6C). In double-labelled sections, SOX9 immunofluorescence revealed well-formed cords, surrounded by PECAM positive interstitial (endothelial) cells. These cells partitioned the cords into uniform regions (Fig. 6D). In sections of co-cultures treated with the PDGFR inhibitor, AG1296, laminin staining revealed large, abnormally swollen cords (Fig. 6G). In these gonads, SOX9 was widely expressed, and $PECAM^+$ endothelial cells did not partition the cords normally, but encircled single swollen cords (Fig. 6H). The abnormal structure of the seminiferous cords in the presence of AG1296 is consistent with previous reports on cord morphology, demonstrating that the inhibitor was functional in organ culture, and recapitulated the phenotype of the Pdgfra mutant mouse (Brennan et al., 2003).

In chicken–quail co-cultures, PDGFR inhibition also disrupted mesonephric cell migration (Fig. 7). When female embryonic chicken gonads (stage 29) were cultured with quail mesonephroi, no migration of $QCPN^+$ quail cells was seen in either the presence or absence of AG1296 PDGFR inhibitor. Aromatase protein was expressed normally in the female gonads. (Figs. 7A–F). When male chicken gonads were used, extensive immigration of QCPN+ cells occurred in controls (Figs. 7G–I), but migration was completely blocked in the presence of AG1296 (Figs. 7J–L). DMRT1 immunofluorescence was used to mark seminiferous cord development in the treated male co-cultures. DMRT1 expression was somewhat weaker than in control male gonads, but expression still delineated forming seminiferous cords (Figs. 7K and L). Unlike the mouse gonads treated with PDGFR inhibitor,

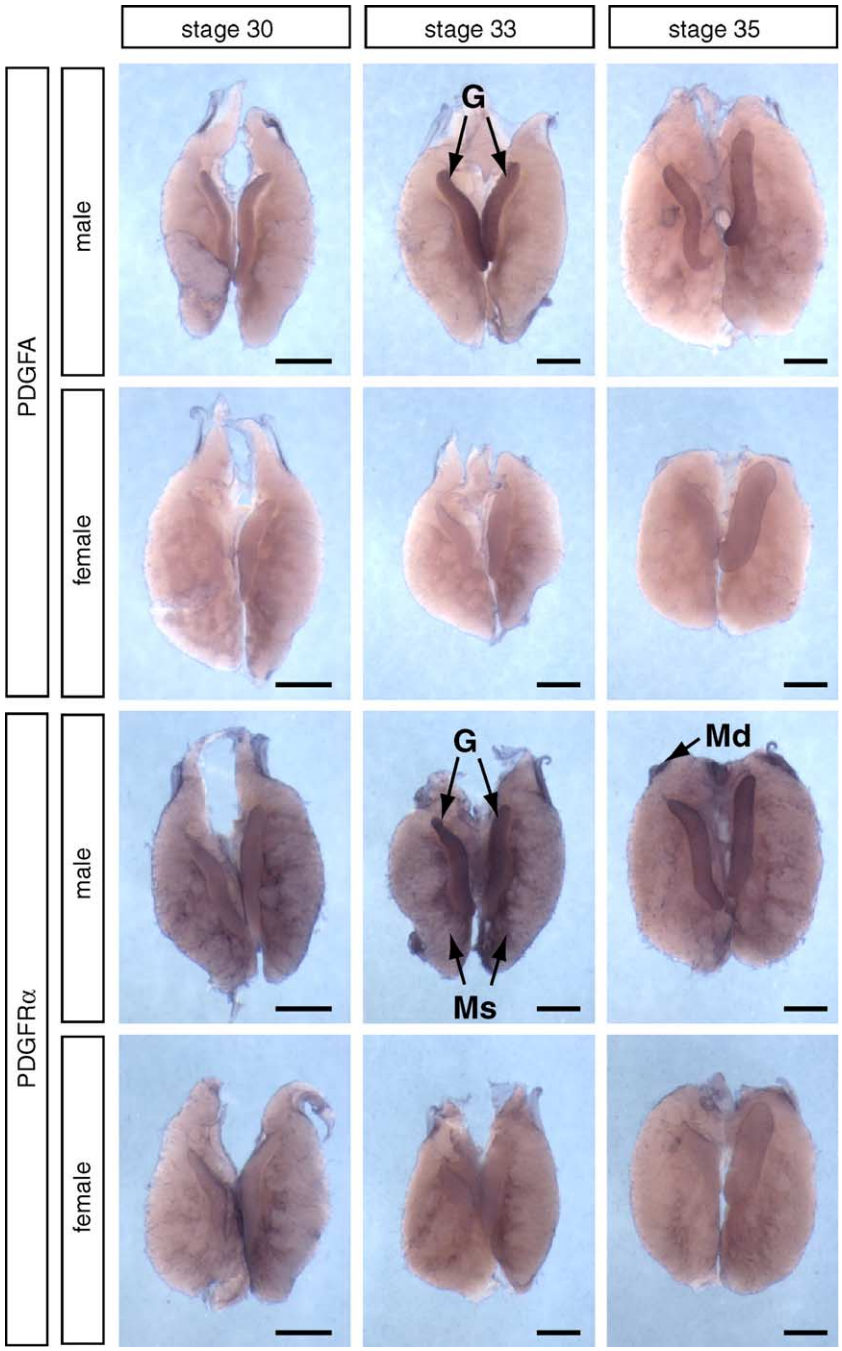

Fig. 4. Expression of chicken PDGF-A and PDGFRα mRNA during urogenital development. (Whole mount in situ hybridisation of stages 30, 33, and 35.) PDGF-A is up-regulated in the gonads (G) of male embryos, but is only weakly expressed in females. PDGFR-α is also more strongly expressed in male gonads, while some weaker expression is detectable in the mesonephroi (Ms), and in Müllerian ducts (Md). Scale bar = 500 μm.

the male chicken gonads did not show large swollen cords.

Discussion

In the mouse embryo, mesonephric cell migration is either a direct or indirect effect of *Sry* gene expression in the XY gonad (Capel et al., 1999; Albecht et al., 2000). However, in the chicken–mouse interspecific co-cultures, mouse cells migrated in the absence of any gonadal *Sry*, which does not exist in the chicken (Fig. 3). In the quail–chick co-culture studies, cell migration also occurs in the absence of *Sry*. This suggests that *Sry* indirectly induces mesonephric cell migration in the mouse via a pathway that is shared with birds.

The chicken–quail co-cultures showed that male-specific mesonephric cell migration can occur into the

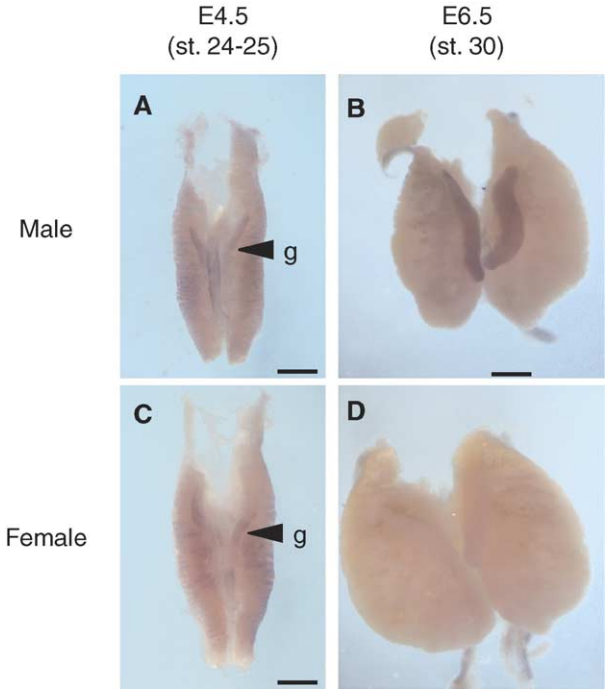

Fig. 5. Gonadal expression of chicken *phosphotidylinositol 3-kinase, C2 domain containing, alpha polypeptide* (*PI3KC2α*). Whole mount in situ hybridisation of male urogenital tissues at stage 25 (A) and stage 30 (B), and female tissues at stage 24 (C) and stage 30 (D). Low-level expression is initially detectable in the gonads (g) of both sexes prior to sexual differentiation (day 4.5; stage 24/25) (A and C), but expression becomes restricted to male gonads at the onset of morphological differentiation (day 6.5; stage 30; arrows) (B versus D). No expression is detectable in the mesonephric kidneys. Scale bar = 500 μm.

avian gonad after stage 29 of development (Fig. 2). While this coincides with the onset of gonadal sex differentiation (Fig. 1), consistent with the mouse studies (Martineau et al., 1997), it is possible that immigration of cells could begin prior to stage 29 in vitro. This is the first report of male-specific cell migration during gonadal development in a non-mammalian model, and it highlights the conservation of the process. While no quail cells were seen to migrate into the female chicken gonads between day 6 (stage 29) and day 9 (stage 35) in vitro (Fig. 2), it is possible that mesonephric cells contribute to female gonadal development before or after the period of sexual differentiation studied here.

Recent experiments have utilised embryonic turtle gonads co-cultured with embryonic mouse GFP+ mesonephroi to explore the potential conservation of male-specific cell migration (Yao et al., 2004). These studies revealed only minimal mouse mesonephric migration into turtle gonads and it was concluded that either no sex-specific migration occurs in turtles, or that there is tissue/signal incompatibility between the two species. Given the male-specific migration seen here in chicken–quail and chicken–mouse co-cultures, it is possible that migration does also occur in turtles, but it may not be apparent when a mammalian mesonephros is co-cultured due to signal/receptor divergence. This might reflect the greater evolutionary distance between mammals and turtles.

A number of different growth factors have been suggested to play a role in mesenchymal cell migration associated with tissue morphogenesis. In the embryonic gonad, platelet-derived growth factors may play a role in the male-specific migration reported here. Male up-regulation of Pdgfa and PDGFr-α in embryonic mouse gonads has been previously reported (Brennan et al., 2003); however, the chicken expression profiles reported here are new and they confirm conservation of this signalling pathway in the gonads.

Our studies and those of others indicate that PDGFR-α is strongly expressed in the gonads themselves rather than the mesonephroi (Fig. 4). This suggests that the Pdgf signalling required for proper mesonephric cell migration may be indirect. Indeed, co-culture studies using wild type and mutant tissues have shown that the defect causing disrupted mesonephric cell migration seen in Pdgfr-$\alpha^{-/-}$ lies with the gonads, not the mesonephroi (Brennan et al., 2003). This indirect induction of mesonephric cell migration shown by the mouse studies appears to be conserved in avians (Fig. 7). The identity of the chemotactic factor responding to PDGFR and directly responsible for inducing male-specific migration is still unknown. However, strong expression of PDGFR-α in the interstitial cells of the mouse testis suggests that these cells play a role via their response to the PDGF signal. Under this scenario, differentiating Sertoli cells express PDGF, which then binds receptor in the neighbouring interstitial cells, which then send a chemotactic signal to the mesonephros, inducing cell migration.

An alternative approach to analysing the role of growth factors in vitro is to specifically block their receptors or signal transduction pathways. This strategy does not rely on artificial doses of exogenous growth factor, but blocks endogenous growth factor function. Here, we specifically blocked PDGFR function. This resulted in reduced (mouse) or abolished (avian) mesonephric cell migration (Figs. 6 and 7), implicating PDGFR signalling in a conserved pathway involved in testicular differentiation. The same blocking compound used at the same concentration (10 μM AG1296) has been shown to induce abnormal seminiferous cord formation in male embryonic rat gonads cultured in vitro (Uzumcu et al., 2002a,b). The cords were fewer in number and swollen, as found here in the mouse cultures. Similarly, Brennan et al. (2003) demonstrated impaired mesonephric cell migration and abnormally partitioned cords in Pdfgr-α null mutant mouse embryos. In contrast, Pdfgr-β mice show no defects in testis development (Brennan and Capel, 2004). Taken together, these data indicate that Pdfgr-α signalling is required for proper mesonephric cell migration into embryonic gonads, facilitating cord organisation.

Our inhibition studies indicate that PDGFR signalling is involved in male-specific mesonephric cell migration in both the mammalian and avian systems. However, in contrast to findings in the mouse, there was no obvious

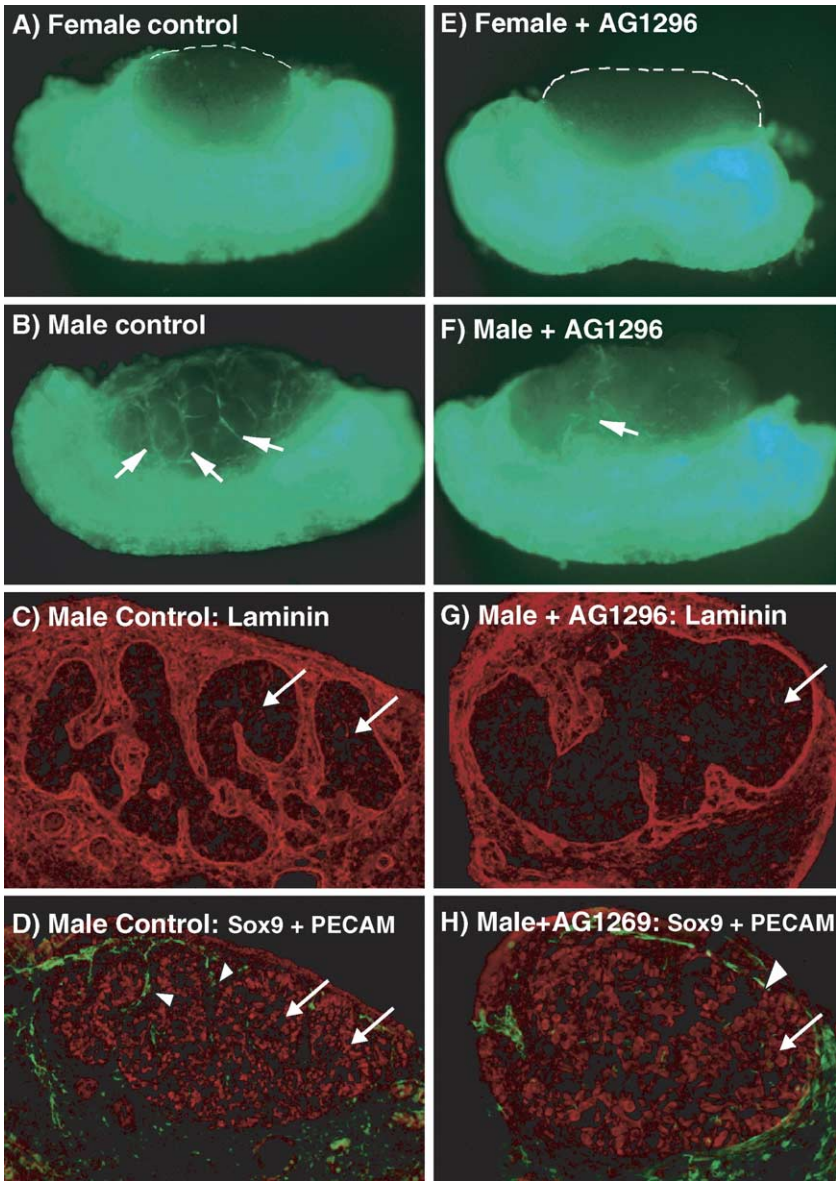

Fig. 6. The PDGFR inhibitor, AG1296, disrupts cell migration in mouse gonad–mesonephros co-cultures. 12.5 dpc wild type mouse gonads co-cultured for 2 days with 14.5 dpc GFP+ mesonephroi. (A–D), controls; (E–H), treated cultures. (A) Control female gonad shows no induction of mesonephric cell migration. (B) Control male gonad induces migration of GFP+ mesonephric cells. (C) Laminin immunofluorescence in a control male co-culture, showing well-developed laminin-negative seminiferous cords (arrows). (D) Double-labelled control male co-culture, showing SOX9+ seminiferous cords (red; arrows) partitioned by PECAM+ endothelial cells (green; arrowheads). (E) Female gonad co-culture treated with AG1296 shows no induction of cell migration. (F) Male gonad co-culture treated with AG1296 shows disrupted migration of mesonephric cells (arrow). (G) Laminin immunofluorescence of a male co-culture treated with AG1296, showing a large, swollen cord (arrow). (H) Double labelled male co-culture after treatment with AG1296. A single swollen cord containing SOX9+ cells (red; arrow) is bordered by PECAM+ cells (green; arrowhead). Note the lack of normal cord formation and partitioning by PECAM-expressing cells.

effect on general cord organisation or partitioning in the male chicken gonad following PDGFR inhibition, as assessed by DMRT1 expression (Fig. 7). This suggests that immigration of mesonephric cells may not be required for normal cord partitioning in the avian system. Indeed, Haffen (1975) reported that embryonic male chicken and duck gonads explanted before sexual differentiation can still form seminiferous cords and differentiate in the absence of a mesonephric kidney, as assessed by standard histology. Similarly, the gonads of both sexes can apparently differentiate normally when the presumptive mesonephros has been microsurgically removed in ovo (Merchant-Larios et al., 1984). These observations are in agreement with our finding that cord formation is not overtly impaired when mesonephric cell migration is inhibited in the avian system. Promotion of cord organisation may not be the primary function of cell migration in all vertebrates, or cell migration may not be necessary for organisation.

Other functions of immigrating mesonephric cells in avian gonads could include recruitment of peritubular

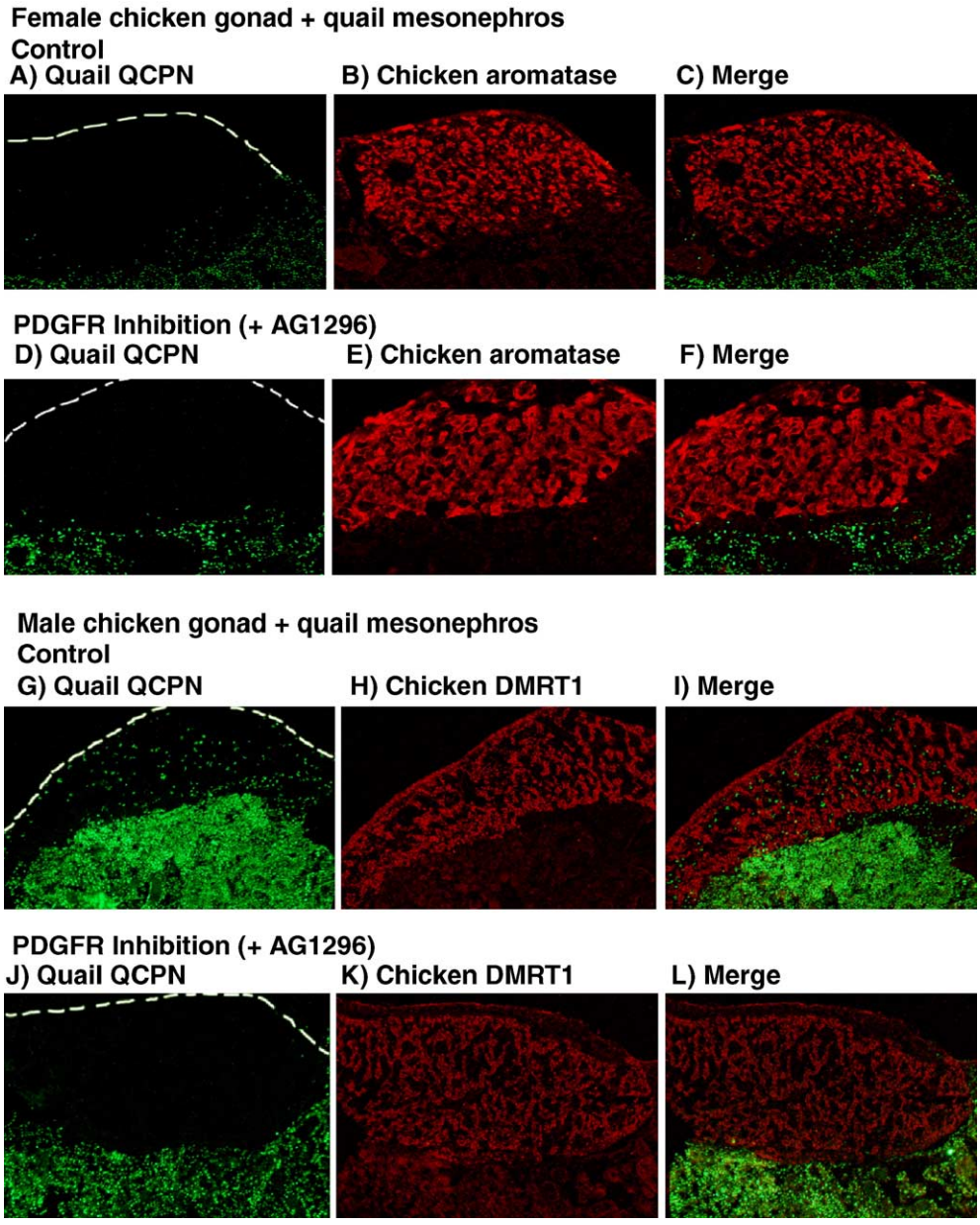

Fig. 7. The PDGFR inhibitor, AG1296, blocks cell migration in chicken–quail co-cultures. Stage 29/30 embryonic chicken gonads co-cultured for 3 days with stage-matched quail mesonephroi. (A–F) Female chicken gonads, (G–I) male chicken gonads. (A) Control female chicken gonad co-cultured with a female quail mesonephros, shows no migration (green QCPN). (B) Aromatase expression in control female chicken gonad (red). (C) Merged image of panels A and B confirms no migration. (D) Female chicken gonad co-cultured with female quail mesonephros and treated with AG1296 shows no migration. (E) Aromatase expression in treated female is normal (red). (F) Merged image of panels D and E, showing no migration. (G) Control male chicken gonad co-culture shows migration of QCPN+ quail mesonephric cells (green). (H) Normal DMRT1 expression in organising seminiferous cords of male gonad (red). (I) Merged image of panels G and H, showing (green) quail cell migration. (J) Male chicken gonad co-cultures treated with AG1296, showing abolition of migration (green). (K) DMRT1 expression is maintained in AG1296-treated male chicken co-cultures (red) with normal cordal expression. (I) Merged image of panels J and K, showing no migration.

myoid cell, vascular endothelial cell, and steroidogenic Leydig cell precursors. In the mouse, Pdgfr-α null mutants have impaired Leydig cell differentiation in conjunction with disrupted mesonephric cell migration (Brennan et al., 2003). Lack of suitable antibody markers precluded the clear identification of immigrating quail cells in our chicken–quail co-cultures. However, PECAM was expressed in migrating mouse cells that entered male chicken gonads. This indicates that most cells were becoming endothelial, as they would in a normal male mouse gonad, pointing to a conserved pathway of differentiation. In the mouse embryo, immigrating PECAM positive cells contribute to the arterial system, in particular the formation of a prominent coelomic blood vessel, not seen in females (Brennan et al., 2002). It has been suggested that this might facilitate the export of

testosterone produced in the embryonic male gonad. (Compared to the male, the female mammalian gonad is steroidogenically quiescent at this time.) Interestingly, no such coelomic blood vessel is seen in the developing male chicken gonad, and it is the female gonad that is more steroidogenically active.

The fact that many growth factors can influence mesonephric cell migration in vitro suggests that there may be functional redundancy among these factors. Greater specificity might be conferred downstream, within the intracellular signal transduction pathway. We have found male-specific expression of PI3KC2α, a key signal transducing kinase used by PDGFR, during gonadal sex differentiation in the chicken embryo. Male-specific PI3KC2α expression (Fig. 5) corresponds to the time of mesonephric cell migration demonstrated in vitro (Fig. 2). This kinase may play a regulatory role in transducing the action of Pdgf receptor mediated mesonephric cell migration. It should be noted that, like Pdgf and Pdgfr-α, PI3KC2α is male up-regulated in the gonad rather than the mesonephros (Fig. 5). One recent study found that inhibition of the phosphotidylinositol 3-kinase pathway with the drug LY294002 blocked mesonephric cell migration and normal testis cord organisation in E13 embryonic rat gonads in vitro (Uzumcu et al., 2002b). While this drug inhibits all classes of PI3 kinases, it may be targeting PI3KC2α. It will be of interest to examine the expression of PI3KC2α during mammalian gonadal development to assess conservation.

In this study, we provide evidence that the mesonephric migration process is not restricted to mouse embryos, and that PDGFR may be a key conserved element in the pathway leading to testicular differentiation, at least in higher vertebrates. The question remains as to the nature of the direct chemotactic signal released from the gonad and received by the mesonephros. It is possible that several factors are released from the developing male gonad in response to PDGFR signalling.

Acknowledgments

We thank Dr. Peter Farlie for providing the QCPN antibody. This work was supported by an Australian National Health and Medical Research Council grant to AS, CS and PM^{c}C.

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