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The mouse forkhead gene *Foxc1* is required for primordial germ cell migration and antral follicle development

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Abstract

Foxc1 encodes a forkhead/winged helix transcription factor expressed in many embryonic tissues. Previous studies have investigated defects in the urogenital system of *Foxc1* null mutants, but the mechanisms underlying the abnormal development of the gonad have not been explored. From earliest stages, the mutant ovaries are smaller than normal, with fewer germ cells and disorganized somatic issue. No bursa membrane is formed, and the oviduct remains uncoiled. Although germ cells are specified correctly, many of them do not migrate to the gonadal ridge, remaining trapped in the hindgut. Consequently, the number initially reaching the gonad is less than 25% of normal. Once in the ovary, germ cells proliferate normally, but the supporting somatic cells are not organized correctly. Since mutant embryos die at birth, further development was followed in ovaries grafted underneath the kidney capsule of ovariectomized females. Transplanted ovaries display normal folliculogenesis up to preantral stages. However, no follicles develop beyond early antral stages. Mutant follicles are often polyovulatory and have disrupted theca and granulosa cell layers. We conclude that alongside its previously known roles in kidney, cardiovascular and eye development, *Foxc1* has essential functions during at least two stages of gonad development—germ cell migration and folliculogenesis.

Keywords: Forkhead; Gonad; Primordial germ cell; Folliculogenesis; TGFβ

Introduction

The differentiation of the mammalian gonad requires multiple, highly coordinated interactions between germ cells, including primordial germ cells (PGCs) and somatic cells. For example, the initial migration of PGCs along the hindgut and into the genital ridge requires adhesive interactions between PGCs, the extracellular matrix and somatic cells (McLaren, 2003; Molyneaux and Wylie, 2004). Once the PGCs have entered the genital ridges, they influence the differentiation of the supporting somatic cell lineage. For example, if only a few PGCs enter the female genital ridge, the somatic cells fail to differentiate and a streak ovary is formed (Merchant, 1975). Bi-directional communications between PGCs and somatic cell lineages are also essential for follicle development and oocyte maturation within the adult ovary (Gilchrist et al., 2004). Gap junctions and transzonal projections between adjacent granulosa cells, and between granulosa cells and the oocyte, provide a physical basis for a network of intercellular communication (Kidder and Mhawi, 2002).

Many signaling pathways have been identified that mediate these reciprocal interactions between cell types in both the ovary and testis. In particular, members of the transforming growth factor-β (TGFβ) superfamily play important roles in PGC and gonad development and maturation (Drummond, 2005; Findlay et al., 2002). For example, the initial specification of PGCs requires bone morphogenetic proteins (BMPs) (de Sousa Lopes et al., 2004; Lawson et al., 1999; Ying et al., 2000), while growth of preantral and antral follicles is dependent upon local activity of activin, inhibin and TGFβ (Brown et al., 2000; Liu et al., 1999; Matzuk et al., 1995a,b). The precise mechanisms through which these and other signaling molecules act in gonad development are being investigated through a combination of genetic, in vivo transplantation and in vitro

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culture methods (Juengel and McNatty, 2005; Pangas and Matzuk, 2004; Phillips, 2005; Ross and Capel, 2005).

Along with secreted signaling factors, transcription factors also play an important role in the interactions between somatic and germ cells during gonad development. Forkhead/winged helix proteins constitute one such family of transcription factors that, besides playing numerous roles in embryonic development, including cell fate determination, proliferation and differentiation, also function during gonadogenesis (Accili and Arden, 2004; Coffer and Burgering, 2004; Katoh and Katoh, 2004; Lantz and Kaestner, 2005; Lehmann et al., 2003; Prueitt and Zinn, 2001). Fox proteins share an evolutionarily conserved DNA-binding domain and have been identified as components of different signal transduction pathways, including those downstream of TGFβ-related ligands (Rice et al., 2005; Seoane et al., 2004; Zhao et al., 1998; Zhou et al., 2002). Several mammalian forkhead family members have been associated with gonad development and folliculogenesis. *Foxo3a* null mice show age-dependent reduced fertility due to global follicular activation and early depletion of functional follicles. This suggests that *Foxo3a* is normally involved in suppressing the initiation of follicular growth (Hosaka et al., 2004). *Foxl2* is expressed in both somatic and germ cells in ovaries around the time of sex determination (Schmidt et al., 2004). In goats, *Foxl2* is involved in repressing male sex determination. Sex reversed XX individuals with polled intersex syndrome (PIS) have a deletion that encompasses elements regulating the transcription of at least two genes, including *Foxl2* (Pailhoux et al., 2001; Pannetier et al., 2005). In mice, *Foxl2* is required for initiating folliculogenesis beyond primary stages and for repressing Sertoli cell fate within the granulosa cells of the postnatal ovary (Loffler et al., 2003; Ottolenghi et al., 2005). In *Foxl2* homozygous mutants, granulosa cells within primordial follicles do not undergo transition to cuboidal cells, leading to an absence of follicles beyond the primordial stage and eventual oocyte atresia (Schmidt et al., 2004). One hypothesis for the action of *Foxl2* is that it regulates TGFβ-related signaling pathways, such as those downstream of activin/inhibin (Prueitt and Zinn, 2001).

In her description of the congenital hydrocephalus phenotype in mice homozygous for the null allele *Foxc1*^{ch}, Green (1970) reported a more anterior position of the gonads, along with kidney and ureter abnormalities. *Foxc1*, together with the closely related gene *Foxc2*, have since been shown to be essential for many aspects of mesoderm development, including patterning of the intermediate mesoderm, from which the mesonephros, gonad and metanephros develop (Kume et al., 2000; Kume et al., 1998; Wilm et al., 2004). *FOXC1* has also been shown to be a TGFβ1-responsive gene in human ovarian, endometrial and cervical cancer cell lines and is inactivated in a significant fraction of endometrial and ovarian cancers (Zhou et al., 2002). Taken together, these results suggest that *Foxc1* is required for normal gonad development and that it acts through TGFβ-mediated signals. In this study, we investigated the role of *Foxc1* in the development of the germ cells and gonads in the mouse and have identified two processes in particular that are affected. PGCs are initially specified correctly in *Foxc1* null mutants but do not migrate efficiently to the genital ridges, with many PGCs remaining trapped in the hindgut. In addition, the gonads of homozygous mutants are smaller than normal and disorganized, particularly in posterior regions. Ovaries from E18.5 *Foxc1* mutants transplanted into adult ovariectomized hosts display normal folliculogenesis up to preantral stages. However, no follicles develop beyond early antral stages. These results suggest that *Foxc1* is required for signaling between somatic and germ cells at two stages of PGC and gonad development. Firstly, for the migration of PGCs out of the hindgut and into the genital ridges and, secondly, for the maturation of follicles beyond the early antral stage.

Materials and methods

Mice

Mice heterozygous for the null mutation *Foxc1*^{lacZ} were maintained by interbreeding on the Black Swiss background and genotyped as previously described (Kume et al., 1998). Homozygous Oct4ΔPE:GFP^{+} mice (Anderson et al., 1999) were obtained from Christopher Wylie (Cincinnati Children's Hospital Medical Center) and crossed onto heterozygous *Foxc1* mutant mice. Genotyping primers for Oct4ΔPE:GFP^{+} were F-5′GGA GAG GTG AAA CCG TCC CTA GG3′ and R-5′GCA TCG CCC TCG CCC TCG C3′ producing a 250 bp fragment (Yeom et al., 1996). Fox Chase Outbred SCID mice were purchased from Charles River Laboratories.

Tail somite (ts) stages were counted as previously described (Hacker et al., 1995), where 18 ts corresponds to E11.5 and 30 ts corresponds to E12.5. Noon on the day of plug was E0.5.

X-gal staining

Whole embryos at E10.5 and genital ridges from E11.5 to E14.5 were dissected in PBS and transferred to 4% paraformaldehyde (PFA) for 1 h at 4°C, as described (Kume et al., 1998). Samples were then rinsed in wash buffer (2 mM $MgCl_2$, 0.02% Nonidet P-40 in PBS) and incubated in lacZ stain (1 mg/ml X-gal, 200 mM $K_3Fe(CN)_6$, 200 mM $K_4Fe(CN)_6$) at 37°C overnight. After staining, samples were rinsed in wash buffer and post-fixed in 4% PFA overnight at 4°C.

Histological analysis

Gonads and whole embryos were fixed in 4% PFA at 4°C overnight and embedded in paraffin wax. Samples were sectioned at 7 μm and stained with hematoxylin and eosin.

PGC detection in embryos and gonads

Embryos were obtained from pregnant females at E8.5–E11.5. PGCs were detected using alkaline phosphatase assays as described (Lawson et al., 1999). Briefly, whole embryos or genital ridges were fixed in 4% PFA at 4°C for 1 h then washed in PBS with 0.1% bovine serum albumin (BSA). Samples were incubated in 70% ethanol overnight at 4°C and washed three times for 10 min with distilled H_2O. To detect alkaline-phosphatase-positive (AP) cells, samples were stained with Fast Red TR and α-napthyl phosphate (Sigma) in malate buffer and washed with PBS. Samples were then embedded in OCT and sectioned at 7 μm. Germ cells positive for AP were counted in every fifth section.

RT-PCR

RNA was extracted from gonads and mesonephric regions of E11.5, E12.5, E13.5 and adult mice using the RNeasy kit (Qiagen). The RNA was reverse transcribed using Superscript II (GibcoBRL), and Foxc1 cDNA was amplified using primers F-5′TTT GGC ATC TGG CTC AAG G 3′ and R-5′GC GAA TT GTA GGA GTT CCC TAG 3′. Foxc2 cDNA was amplified using primers F-5′