Effects of FGF9 on embryonic Sertoli cell proliferation and testicular cord formation in the mouse

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ABSTRACT    Proliferation and cord formation by embryonic Sertoli cells are pivotal events involved in testis morphogenesis. A number of growth factors have been implicated in mediating these events. However, the exact level of involvement and importance of each as yet remains elusive. We have adopted an in vitro approach to assess developing mouse Sertoli cells, whereby they are cultured in the presence or absence of fibroblast growth factor (FGF9) and/or extracellular matrix ( ECM ) gel, since previous studies have shown that ECM gel aids Sertoli cell differentiation. The present findings corroborate this effect, but in addition demonstrate that in the presence of FGF9 (10 ng/ml), cells undergo greater proliferation than those cultured on gel alone. They also display a differentiated epithelial phenotype, with appositional contact of cell membranes in cord-like aggregations. In addition we have shown that cultured Sertoli cells generally express a smaller truncated, nuclear form of the FGFr3, although in the presence of FGF9 and absence of gel, the larger, cytoplasmic form of the receptor is also expressed. Immunolocalisation of FGFr3 in Sertoli cells of whole testes revealed a temporal expression pattern profile, with high levels being abundant in the embryonic testicular cords and at puberty, but an absence in adult Sertoli cells. Our findings suggest that FGF9 plays an important role in proliferation and organisation of embryonic Sertoli cells during testis morphogenesis.

KEY WORDS: Sertoli cell, FGF9, cell culture, gonadal differentiation, testicular cord

Introduction

Many factors contribute to male infertility. Some disorders of testicular function may arise due to abnormal organisation or proliferation of the embryonic Sertoli cells (reviewed by Sharpe et al., 2003 ), which can result in cellular dysfunction, defective morphology and reduced cell number. Sertoli cells play a pivotal role in testis morphogenesis as they are the first cell type to differentiate, an event which enables seminiferous cord formation. Germ cell entry into meiosis is prevented as the embryonic Sertoli cells aggregate and enclose the newly arrived germ cells (reviewed by Mackay 2000 ). There is a significant relationship between the number of Sertoli cells and sperm density, thus more Sertoli cells means numerically the potential for greater sperm production ( Orth et al., 1988; Sharpe 1994, 1999 ). Proliferation of the Sertoli cells during development therefore is vital for normal adult fertility.

Specification and commitment of a bipotential gonad to differentiate into a testis is dependent on the occurrence of time-specific key events at both the molecular and cellular level. The discovery of the testis-determining gene, Sry, in the 1990s has directed attention to the Sertoli cells, since Sry appears to act in a cell-autonomous fashion. Development diverges when it is expressed during a narrow window of time, between embryonic day (E)10.5-12.5 in the mouse XY gonad. SRY initiates several other downstream targets, including Sox9, another transcription factor, which is also expressed in the Sertoli cells (reviewed by Swain and Lovell-Badge 1999 ). Upon induction by Sry, a cascade of molecular signalling occurs, resulting in the male pathway of development.

Abbreviations used in this paper: AMH, anti-Müllerian hormone; DAB, diaminobenzidine; DMEM, Dulbecco’s Modified Eagle’s Medium; DPC, days post coitum; E, embryonic; ECM, extracellular matrix; EGF, epidermal growth factor; FGF9, fibroblast growth factor 9; FGFr3, fibroblast growth factor receptor 3; FSH, follicle stimulating hormone; GDNF, glial-derived neurotrophic factor; ICC, immunocytochemistry; NT3, neurotrophin 3; PBS, phosphate buffered saline; PCNA, proliferating cell nuclear antigen; PCR, polymerase chain reaction; PDGF, platelet derived growth factor; SRY, sex determining region of the Y chromosome; TGFβ, transforming growth factor beta.
Increased cellular proliferation is triggered by \textit{Sry} expression. This is a crucial step in testis morphogenesis and is critical for establishment of the male differentiation pathway. The increased proliferation occurs within an 8 hour time window whereby the male gonad doubles its size relative to the female (Schmahl \textit{et al.}, 2000; Schmahl and Capel 2003). One of the first morphologically distinct events in testis development is the aggregation and rearrangement of pre-Sertoli cells and germ cells to form cords (Pelliniemi \textit{et al.}, 1993). As the cords mature they become surrounded by peritubular myoid cells, which migrate into the gonad from the mesonephros from around E11.5 in the mouse (Buehr \textit{et al.}, 1993). These cells interact with the Sertoli cells and a basement membrane is formed between the two cell types (Tung and Fritz 1980; Skinner \textit{et al.} 1985).

Many factors, involved in signalling pathways downstream of \textit{Sry}, have been implicated in controlling the processes of cell migration, proliferation and differentiation and underlie mammalian gonadal development (see Mackay 2000). An important early signal in the developing testis, prostaglandin D2, is produced by the primordial germ cells and has been proposed to act as a paracrine factor to induce Sertoli cell differentiation (Adams and McLaren 2002).

Upon commitment of the somatic cells to the Sertoli cell line, cord formation proceeds. Several mediating factors are proposed to have a role in this event. One study has implicated platelet-derived growth factor (PDGF) as being a regulatory candidate. This hypothesis was tested by Uzumcu \textit{et al.}, (2002), who cultured E13 rat testes in the presence of a PDGF inhibitor (tyrphostin), demonstrating that inhibition of PDGF action did not prevent cord formation but altered normal cord development and morphology (Uzumcu \textit{et al.}, 2002). Tyrphostin-treated testes formed “swollen cords”. This phenomenon tends to occur when there is a significant decrease in the number of cords per testis area and increased cord diameter due to fusion of cords. Platelet-derived growth factor and its receptor were found to be temporally expressed in the rat testes, with highest levels being expressed just after birth (Gnessi \textit{et al.} 1995). In the mouse, the PDGF receptor–α has been proposed to have an organisational role in endothelial cells, partitioning of testis cords, proliferation, cell migration and Leydig cell development (Brennan \textit{et al.}, 2003). Other factors which have been implicated to be involved in cord formation include glial-derivived neurotrophic factor (GDNF) (Hu \textit{et al.}, 1999) and neurotrophin 3 (NT3) (Levine \textit{et al.}, 2000; Cupp \textit{et al.}, 2003).

Recent studies by Colvin and co-workers (2001), have suggested that one of the crucial factors involved in cord formation is fibroblast growth factor 9 (FGF9). This is a member of the fibroblast growth factor family, which consists of 23 currently...
identified members. They are intercellular signalling molecules with multiple and varied roles in animal development. (Burgess and Maciag 1989; Crumley et al., 1991). Knockout mice studies demonstrated that in the absence of FGF9, gonadal development is directed along the female pathway of differentiation. This occurred in the majority of XY individuals, although a few animals did display male traits in the form of hypoplastic testes (Colvin et al., 2001). The specific role of FGF9 in Sertoli cell proliferation, organisation and differentiation however remains elusive (Adams and McLaren 2002); therefore, an in vitro approach has been utilised in the present study, to determine more fully the effect of FGF9 on isolated developing Sertoli cells in culture.

Results

Phase contrast microscope observations

In all experimental regimes (i.e. in the presence of FGF9 and/or ECM gel) the embryonic Sertoli cells displayed flattening and attachment to substrate after 24hrs in culture. In the control wells (i.e. DMEM only), cells proliferated, but possessed a mesenchymal morphology (larger, stellate and with more cytoplasmic processes), indicative of an undifferentiated state (Fig. 2A). The cells cultured on ECM gel without growth factor assumed an epithelial-like phenotype (Mackay et al., 1999) and clustered together, with close apposition of neighbouring cell membranes, to form cord-like structures (Fig. 2 B,D). Cells cultured with FGF9 in absence of gel demonstrated rapid proliferation after 48hrs in culture compared to the other wells (Fig. 2C). The cells in experimental wells containing both gel and FGF9 began to form cord-like aggregations after 48hrs, which were abundant after 4 days.

Monitoring of isolation procedure

Investigations of the cultures of supernatants revealed that germ cells were largely removed in the first centrifugation, while fibroblasts, interstitial cells and peritubular myoid cells (Fig. 3A) were contained in subsequent supernatants. Sertoli cells, as identified by granular-like immunostaining of AMH in the cytoplasm (Fig. 3B), were found in the cultures prepared from the final pellet of the isolation procedure. No signal was detected in negative controls lacking the AMH primary antibody (Fig. 3C).

PCNA expression in cultured embryonic Sertoli cells

Mitotic cells with PCNA immuno-positive nuclei were present in control and experimental wells (Fig. 4A). Significantly more PCNA-positive cells were present in cultures supplemented with FGF9 compared to controls in medium alone (Fig. 4 B,C) at the two time points assessed (3 and 7 days).

FGF3 expression in cultured embryonic Sertoli cells

Negative ICC staining controls lacking primary antibody demonstrated little immunoreactivity (Fig. 5A). Immunolocalisation of FGF3 was detectable in cells from all culture regimes (Fig. 5 B-
L. Willerton et al.

However, in the presence of FGF9 more positively-stained cells were apparent. Staining was predominantly nuclear but, in the absence of ECM gel, cytoplasmic staining was also observed (Fig. 5 C, E).

**FGFr3 expression in the testes in vivo**

FGFr3 expression was detected in the testicular cords of the E14.5 urogenital complex, but was absent from testicular interstitial tissue and from the mesonephros and metanephros (Fig. 6A). All the cell types of the seminiferous tubules of postnatal Day 15 testes showed abundant FGFr3 staining (Fig. 6B). By Day 25 expression was more localised to the spermatagonia and spermatocytes (Fig. 6C), while in the adult expression was restricted to the spermatogonia (Fig. 6D). FGFr3 expression was absent in the interstitial compartment of the testes of all ages and in the staining control without primary antibody (Fig. 6E).

**Discussion**

It is widely accepted that proliferation and aggregation of somatic cells during development are crucial events in establishing the fate of the gonad, committing it to form a testis (Schmahl and Capel 2003). In the current study we have demonstrated that addition of FGF9 to embryonic Sertoli cells in culture induces enhanced proliferation with an approximately two-fold increase in cell number after 7 days with exogenous FGF9 in the absence of gel. Cells cultured on ECM gel formed cord-like aggregations, displaying an epithelial-like phenotype. These observations are consistent with our previous findings that ECM gel enhances morphological maturation of the embryonic Sertoli cells in culture (Mackay et al., 1999). Functional maturity of the cultured Sertoli cells was confirmed by immunocytochemistry for the presence of AMH (Mackay et al., 2004).

The presence of FGF9 and ECM gel together increased proliferation and induced cord-like aggregation of the developing Sertoli cells. Control cells in DMEM alone however, remained randomly distributed with only minor aggregations, despite attaching and showing proliferation. These results support our previous findings that ECM gel promotes maturation of an epithelial-like phenotype (Mackay et al., 1999). Extra-cellular matrix is known to have an inductive effect on epithelial cells via integrin signalling (reviewed by Giancotti and Ruoslahti 1999). Our findings for the effect of FGF9 are also consistent with those of Colvin and co-workers (2001), who found that functional testes were absent in the FGF9 knock-out mouse demonstrating the key role of the growth factor in embryonic testis cord morphogenesis.

Fibroblast growth factor receptor 3 localisation was detected predominantly in the nucleus of all the cultured cells, with some cytoplasmic staining also observed (Fig. 5 C, E). However, in the presence of FGF9 more positively-stained cells were apparent. Staining was predominantly nuclear but, in the absence of ECM gel, cytoplasmic staining was also observed (Fig. 5 C, E).
It is unclear however, whether this increased expression applies to both the cytoplasmic and nuclear isofoms of the receptor and quantitative analysis is required to clarify this.

Immunolocalisation of FGFR3 in testes in vivo has a temporal pattern of expression. In the E14.5 urogenital complex, FGFR3 immunostaining is abundant in most of the cells within the seminiferous tubules reflecting much mitotic activity occurring at this time. By puberty, FGFR3 immunostaining is generally localised in spermatagonia and Sertoli cells closest to the basement membrane of the tubule. Receptor expression is restricted in the adult to the spermatogonia which remain mitotically active.

In future studies, effects of FGFR9 on whole gonad explants will be investigated and the functional status of the developing Sertoli cells will be determined by molecular methods. However, the present study, using our in vitro approach, provides evidence of the role of FGFR9 in embryonic testis development, especially in early proliferation.

Materials and Methods

Animals

CBA mice maintained on a reversed lighting regime were used for matings and the presence of a vaginal plug was designated day 0 of pregnancy. The total number of pregnant females used for culture experiments was 20 (i.e. usually 1-2 per experiment, yielding 7-10 pups per litter). Pregnant females were CO2 asphyxiated at 14.5 days post coitum (day of finding a plug = day 0 post coitum) and embryos were dissected from uterine horns and transferred to Hanks buffer (Gibco). The developmental stage of each embryo was determined according to Theiler (1972). Testes were identified by their larger size and striped appearance due to early vascularisation and cord development (Fig. 1A), whilst ovaries were smaller with a granular appearance (Fig. 1B). Once the urogenital complex was removed from the embryo, the attached mesonephros was separated and the testes from each individual were transferred to different Eppendorf tubes containing Hanks buffer, so that the control and experimental cultures contained tissue from the same individuals, thus reducing variation.

Cell culture

Embryonic Sertoli cells were isolated from E14.5 testes using a modification (Mackay et al., 1999) of the method of Chapin and co-workers (1987). This stage was chosen as a compromise between developmental state and size of testis, so that a sufficient number of developing Sertoli cells could be obtained. Briefly, the embryonic testes were subjected to enzymatic treatment at 37°C with 0.25% collagenase and 0.025% trypsin in Hanks buffer, each for 10 mins. The enzymatic digestion was stopped by transfer to Dulbecco’s modification of Eagle’s medium (DMEM) supplemented with 10% fetal calf serum and 0.1% gentamycin. Tissue was then disrupted further by mechanically tearing with Microlance needles and aspirating 10 times. Finally, the suspensions were transferred to microcentrifuge tubes and subjected to a series of centrifugations at 1000 – 4000 rpm for 1 min each in a Microcentaur. The supernatant was removed from each control and experimental preparation and replaced with fresh Dulbecco’s modified Eagles medium (DMEM) supplemented with 10% fetal calf serum and 1% gentamicin sulphate (ICN Biomedicals Inc.). After each spin the pellet was re-suspended. This method produces a gradual separation of Sertoli cells with germ cells, peritubular and Leydig cells lost in successive supernatants. Finally cells were plated out into Nunclon multiwell dishes at equal plating densities (5 x 10^5 cells/ml) with viability estimated at 80-90% by Trypan Blue exclusion (Smith 1984). The wells were either coated with ECM gel (Sigma Aldrich cat. no. E1270) or left uncoated and in experimental wells DMEM was supplemented with 10 ng/ml FGF9 (Sigma Aldrich cat. no. F1168) which, in pilot studies, was found to be effective. In order to monitor cell types separated throughout the isolation steps, supernatants were cultured for 4 days in DMEM and screened for specific markers.

All cultures were incubated at 37°C in 5% CO2 / 95% air for 3-7 days. Medium was changed every 48 hours and cultures monitored by daily

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**Fig. 6. Immunolocalisation of FGFR3 in vivo.** (A) Low power view of the E14.5 urogenital complex with testis (T), mesonephros (Mes) and metanephros (Met). Note that positive immunofluorescence is restricted to the developing testicular cords (C). (B) Arrows highlight representative immunostained cells in the seminiferous tubules of the postnatal day 15 testis. (C) Sertoli cells (SC) and spermatogonia (SG) are both positively stained at postnatal day 25. (D) In the adult testis, staining is confined to spermatogonia (arrows) with absence of any staining in the interstitial tissue (IT) or in the lumen of the seminiferous tubule (STL). (E) Staining control of adult testis with primary antibody omitted. Bars, 100 µm (A), 20 µm (B, C and E), 50 µm (D).
phase contrast microscopy. Culture experiments were repeated at least ten times for proliferation studies and receptor localisation assessed for at least 4 experiments with all culture conditions.

**Immunocytochemistry**

At the end of the culture period cells were fixed in 4% paraformaldehyde for immunocytochemistry (ICC), using the following antibodies purchased from Santa Cruz Biotechnology, Inc. UK: FGFfr3 (cat. no. sc-123), Anti-Müllerian Hormone (AMH) (cat. no. sc-6886) and proliferating cell nuclear antigen (PCNA) (cat. no. sc-56). AMH was used both as a Sertoli cell-specific marker and also to assess functional status of the cells (Mackay et al., 2004), PCNA as a proliferative marker and FGFfr3 as the receptor for FGF9. Smooth muscle α-actin antibody, a known smooth muscle specific marker (Skalli et al., 1986), was purchased from Sigma Aldrich (cat.no. A-2547) and used to identify peritubular cells in the cultured supernatants.

In initial pilot experiments, a range of antibody dilutions was evaluated: 1 in 200 proved to be the optimal dilution for FGFfr3, 1 in 100 for PCNA and smooth muscle α-actin and 1 in 50 for AMH. All antibodies were diluted with 0.3% Triton in phosphate buffered saline (PBS) containing 1% normal goat serum. This was incubated at 4°C overnight, followed by two washes in phosphate buffered saline (PBS). Secondary antibodies: anti-goat (AMH), anti-mouse (PCNA and smooth muscle α-actin) and anti-rabbit (FGFfr3) were then added (1/100) for one hour. Binding was visualised by either the avidin-biotin/DAB method (reviewed by Beck 1998), (Vectastain, Vector laboratories Ltd, Peterborough, UK) for PCNA and FGFfr3 or by fluorescence detection (AMH, smooth muscle actin). Negative controls, omitting the primary antibody, were included for each.

Proliferating cell nuclear antigen (PCNA) is a protein which is commonly used as a mitotic marker (Kurki et al., 1988). Positive cells were counted and expressed as a mean percentage of the total cells for control and all experimental cultures in eight 450 µm fields per well in 4 repeat experiments. Statistical analysis was performed using analysis of variance and the Kruskall Wallis test with post hoc analysis.

**In vivo**

Tests were removed from mice of selected ages: embryonic (E14.5), pre-pubertal (day 15), pubertal (day 25) and adult (3 months of age). They were fixed in 4% paraformaldehyde and wax embedded. Sections were cut at 7 µm and mounted onto 3-Aminopropyltriethoxysilane (APES) coated slides. Immunocytochemistry was performed using FGFfr3 antibody and methods as described above.

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**References**


