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Xenografting of human fetal testis tissue: a new approach to study fetal testis development and germ cell differentiation

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Background: Abnormal fetal testis development can result in disorders of sex development (DSDs) and predispose to later testicular dysgenesis syndrome (TDS) disorders such as testicular germ cell tumours. Studies of human fetal testis development are hampered by the lack of an appropriate model, and intervention systems. We hypothesized that human fetal testis xenografts can recapitulate normal development.

Methods: Human fetal testes (at 9 weeks, n = 4 and 14–18 weeks gestation, n = 6) were xenografted into male nude mice for 6 weeks, with or without hCG treatment of the host, and evaluated for normal cellular development and function using immunohistochemistry, triple immunofluorescence and testosterone assay. The differentiation and proliferation status of germ cells within xenografts was quantified and compared with age-matched controls.

Results: Xenografts showed >75% survival with normal morphology. In the first-trimester xenografts seminiferous cord formation was initiated and in first- and second-trimester grafts normal functional development of Sertoli, Leydig and peritubular myoid cells was demonstrated using cell-specific protein markers. Grafts produced testosterone when hosts were treated with hCG (P = 0.004 versus control). Proliferation of germ cells and differentiation from gonocytes (OCT4⁺) into pre-spermatogonia (VASA⁺) occurred in grafts and quantification showed this progressed comparably with age-matched ungrafted controls.

Conclusions: Human fetal testis tissue xenografts demonstrate normal structure, function and development after xenografting, including normal germ cell differentiation. This provides an in vivo system to study normal human fetal testis development and its susceptibility to disruption by exogenous factors (e.g. environmental chemicals). This should provide mechanistic insight into the fetal origins of DSDs and TDS disorders.

Key words: fetal testis / disorders of sexual differentiation / testicular dysgenesis syndrome / xenografts / fetal germ cell differentiation

Introduction

Formation of a testis from the indifferent gonad is orchestrated by the SRY gene, resulting in differentiation of Sertoli cells (Wilhelm et al., 2007). These then enclose the fetal germ cells (gonocytes) to form seminiferous cords, starting at 7–9 weeks’ gestation (Wartenberg, 1981; Tilmann and Capel, 1999; Hanley et al., 2000; Gaskell et al., 2004; Ostrer et al., 2007). Fetal Leydig cells then differentiate and via hormone secretions, primarily testosterone, bring about bodywide masculinization (Scott et al., 2009).

Disorders of sex development (DSDs) may result from failure of normal gonadal development or subnormal androgen production/action (Hughes, 2008). Such disorders can predispose to development of testicular germ cell tumours (TGCTs) in young adulthood from pre-
malignant carcinoma in situ (CIS) cells (Skakkebaek, 1972; Rajpert-De Meyts, 2006). CIS cells are thought to originate because of failure of normal differentiation of fetal germ cells into prespermatogonia (Rajpert-De Meyts, 2006), a process that encompasses fetal and early post-natal life in humans and involves the loss of expression of pluripotency factors (OCT4 and NANOG) and expression of proteins indicative of differentiation (VASA and MAGE-A4) (Gaskell et al., 2004; Anderson et al., 2007; Mitchell et al., 2008). TGCT may be the most serious manifestation of a testicular dysgenesis syndrome (TDS), which also includes some cases of undescended testis, hypospadias and low sperm counts; these TDS disorders are all thought to result from maldevelopment of the fetal testis (Skakkebaek et al., 2001).

Much of our understanding of normal and disrupted testicular development derives from rodent models (Wilhelm et al., 2007; Gassei et al., 2008), because only retrospective studies are usually possible in patients with DSDs or TDS disorders, preventing identification of the mechanisms linking fetal testis maldevelopment to altered cell development/function. However, there are major fundamental differences in the pace and duration of fetal testis development between primates and rodents (Ehmcke et al., 2006), especially regarding fetal germ cell differentiation (Mitchell et al., 2008). Its long duration, in particular, limits the use of in vitro testis explants as an investigative approach. Such differences may explain why rodents do not develop TGCT equivalent to that in humans (Oosterhuis and Looijenga, 2005; Mitchell et al., 2008; Anderson et al., 2009).

Testicular xenografting has become increasingly utilized as a technique in germ/stem cell biology, fertility preservation and the production of transgenic sperm (Wistuba and Schlatt, 2002; Dobrinski, 2008; Ehmcke and Schlatt, 2008; Schlatt et al., 2010). Xenografting of immature testis tissue from several species, including the rhesus macaque, into a nude post-natal tissue is grafted (Geens et al., 2005; Mitchell et al., 2008). TGCT may be the most serious manifestation of a testicular dysgenesis syndrome (TDS), which also includes some cases of undescended testis, hypospadias and low sperm counts; these TDS disorders are all thought to result from maldevelopment of the fetal testis (Skakkebaek et al., 2001).

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Xenografting procedure

Xenografting was performed following ethical approval from Lothian Research Ethics Committee and animals were maintained in accordance with UK Home Office guidelines. Male CD1 nude mice (n = 18, Charles River UK, Margate, England) were anaesthetized by inhalation of isoflurane, castrated and small pieces (1 mm³ approx.) of donor human testis tissue were inserted subcutaneously under the dorsal skin using a 13G cancer implant needle (Popper and Sons, NY, USA). Between three and six testis grafts from a single fetus were inserted on either side of the midline. Mice were housed individually and received analgesia (Rimadyl LA, Pfizer, NY, USA) and antibiotics (Baytril, Bayer, Germany) in the drinking water for 5 days post-surgery. After 7 days, mice with second-trimester xenografts were randomly assigned to receive s.c. injections of either hCG (n = 6, 20 IU, three times a week, Pregnyl, Organon Laboratories, Cambridge, UK) in 0.9% saline containing 1% fetal bovine serum, or the vehicle. Grafts were retrieved after 6 weeks. Host mice were killed by cervical dislocation and blood obtained by cardiac puncture for measurement of testosterone. Seminal vesicles were removed and weighed, and xenografts were retrieved and weighed.

Tissue fixation and processing

Pre-graft, xenograft and equivalent age-matched control tissues were fixed for 2 h in Bouins, transferred to 70% ethanol and then processed into paraffin blocks using standard procedures.

Immunohistochemistry

Antibodies, dilutions and requirement for antigen retrieval are shown in Table I. Immunohistochemistry was performed with Tris-buffered saline (TBS, 0.05 M Tris and 0.85% NaCl, pH 7.6) washes between each step (Mitchell et al., 2008). Sections (5 µm) were subjected to heat-induced antigen retrieval in 0.1 M citrate buffer (pH 6) and endogenous peroxidase blocked with 3% (v/v) H₂O₂ in methanol for 30 min. Endogenous biotin was blocked using an avidin/biotin blocking kit (Vector Laboratories, Inc., Peterborough, UK), according to manufacturers’ instructions. Sections were incubated in appropriate normal serum diluted 1:5 with TBS

<table>
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<th>Table I Antibodies and conditions used for immunohistochemistry in xenograft study using human fetal testis tissue in nude mice.</th>
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<td><strong>Antigen</strong></td>
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<td>AR</td>
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<td>OCT 4</td>
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<td>3β-HSD</td>
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All antibodies were raised against human peptide sequences. AMH, anti-Müllerian hormone; AR, androgen receptor; SMA, smooth muscle actin; 3β-HSD, 3β-hydroxysteroid dehydrogenase. ⁴Santa Cruz Biotechnology, CA, USA. ⁵Abcam, Cambridge, UK. ⁶Dr Guilio Spagnoli, University Hospital, Basel, Switzerland. ⁷Prof. Ian Mason, The Queen’s Medical Research Institute, Edinburgh, UK. ⁸Chemicon/Upstate/Linco. ⁹Sigma, Poole, UK.
 containing 5% (w/v) bovine serum albumin (BSA) for 30 min. Sections were incubated overnight with primary antibody diluted in serum at 4°C in a humidified chamber and then incubated for 30 min with the appropriate biotinylated secondary antibody, diluted in normal serum, followed by 30 min incubation with Streptavidin-horse-radish peroxidase (DAKO, Ely, Cambridgeshire, UK) at 1:1000, diluted in TBS. Visualization was performed using 3,3′-diaminobenzidine tetrahydrochloride (DAB, DAKO) and sections were counterstained with haematoxylin before mounting in Pertex mounting medium (CellPath plc, Hemel Hempstead, UK). Negative controls were sections in which the primary antibody was replaced with the appropriate normal serum. Images were captured as previously described (Mitchell et al., 2008).

For double immunohistochemistry, the first primary antibody was detected using Streptavidin alkaline phosphatase (DAKO) at 1:400 for 30 min and visualized with 1 mg/ml Fast Blue (Sigma) in fast blue buffer, until desired staining was achieved (typically 10–15 min). The second primary antibody was then applied and subsequent steps were as described above for single immunostaining.

**Triple immunofluorescence**

Details of antibodies and detection reagents are shown in Table II. Antigen retrieval, blocking and incubation with the first primary antibody were performed as described for immunohistochemistry, with phosphate-buffered saline (PBS) replacing TBS. Sections were incubated with a peroxidise-conjugated secondary antibody diluted in normal serum/PBS/BSA for 30 min and kept in the dark thereafter. Slides were then incubated with labelled Tyramide diluted in buffer at 1:50 for 10 min before placing in citrate buffer and microwaving on full power for 2.5 min and left to cool for a further 30 min. The incubation of primary antibody, peroxidise-conjugated secondary and Tyramide (with different fluorescent labels), was repeated for the second and third primary antibodies, with a second microwave citrate retrieval prior to the third primary antibody. Sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma) diluted 1:1000 in PBS for 10 min. Slides were mounted using Permafluor (Immunotech, Marseille, France). Images were captured using an LSM 510 Meta Confocal microscope (Carl Zeiss, Hertfordshire, UK).

**Quantification of germ cell differentiation and proliferation**

Quantification of germ cell subpopulations and proliferation indices were performed for the triple-stained sections. Images were obtained using an Axiosvert 200 M microscope with attached Axioimac HRc camera and Axiovision 4.6 software (all Carl Zeiss). All germ cells within each section were counted and quantified according to their protein expression profile and proliferation status using Adobe Photoshop 7.0 (Adobe, San Jose, CA, USA).

**Testosterone assay**

Host serum testosterone levels were measured by competitive radioimmunoassay using radiolabelled testosterone (I125, MP Biomedicals, UK) and a rabbit primary antibody (1:600000, AMS Biotech, Abingdon, UK). Residual I125 was measured with a gamma counter (WIZARD 1470, Perkin Elmer, Turku, Finland) and testosterone levels were expressed as ng/ml.

**Statistics**

Statistical analysis was performed using Graphpad Prism 5 software (La Jolla, CA, USA). Multiple groups were analysed using one-way analysis of variance, while comparisons between pre-graft control and xenografted material taken from the same fetus were compared using a paired t-test. Statistical significance was set at $P < 0.05$.

**Results**

**Retrieval rates, growth and gross morphology of human fetal testis xenografts**

Surviving grafts were easily identifiable as a distinct mass on the under surface of the dorsal skin. Retrieval rates were similar with 9/12 (75%) first-trimester and 50/65 (77%) second-trimester grafts recovered. Most xenografts had grown from a diameter of $\sim 1–3 \text{ mm}^3$ and their weights ranged from 0.1 to 5.2 mg (mean 2.1 mg). Grafts were enclosed by a capsule, separating the testis material from the surrounding s.c. tissue of the host (Fig. 1A). The recovered grafts exhibited normal gross appearance, with recognizable seminiferous cords (containing Sertoli cells and germ cells) surrounded by an interstitium containing Leydig cells in 9/9 (100%) of first-trimester and 47/50 (94%) of second-trimester grafts (Fig. 1A).

**Formation of seminiferous cords in first-trimester human fetal testis xenografts**

At the time of grafting, seminiferous cords had not fully formed in first trimester testes, but cords formed during the grafting period and exhibited a normal appearance (Fig. 1A). At grafting, Sertoli cells (identified by expression of SOX9) had aggregated but did not form obvious cords and many of the germ cells (identified by expression of OCT4) were still located in areas devoid of Sertoli cells (Fig. 1B). After grafting, Sertoli cells had enclosed the germ cells in seminiferous cords surrounded by a layer of cells expressing smooth muscle actin (SMA; presumptive peritubular myoid cells), which was not evident in pre-graft controls (Fig. 1C).

**Functional development of Sertoli and Leydig cells in first-trimester human fetal testis xenografts**

Androgen receptor (AR) immunoreexpression was not evident in first trimester pre-graft control testis tissue, but after grafting for 6 weeks AR expression was observed in peritubular myoid cells and in some interstitial cells, a pattern comparable with equivalent age-matched controls (Fig. 2). Anti-Müllerian hormone (AMH) was
expressed in presumptive Sertoli cells in the first trimester pre-graft control and expression was maintained in the cytoplasm of presumptive Sertoli cells after grafting, and was similar in localization to equivalent age-matched controls (Fig. 2).

**Steroidogenesis and testosterone production by human fetal testis xenografts**

Differentiation and functional activation of Leydig cells was assessed in the xenografts. In the absence of hCG treatment, Leydig cell cytoplasm was scanty compared with the conspicuous cytoplasm in Leydig cells from second-trimester grafts of hCG-treated animals (Fig. 3A). In vehicle-treated controls, the Leydig cells within grafts were mostly immunonegative for 3β-hydroxysteroid dehydrogenase (3β-HSD), but this enzyme was strongly expressed in Leydig cells from hCG-exposed grafts, indicating induction of steroidogenesis (Fig. 3A). Testosterone production was below the level of detection in (untreated) hosts bearing first-trimester xenografts, while low levels were detected (mean 0.07 ± 0.04 ng/ml) in vehicle-treated hosts bearing second-trimester grafts. Treatment of hosts bearing second-trimester xenografts with hCG resulted in nearly a 10-fold increase (mean 0.68 ± 0.23 ng/ml, *P* = 0.004) in serum testosterone levels (Fig. 3B). Seminal vesicle weight was also significantly (3-fold) increased in hCG-treated animals compared with vehicle-treated controls (Fig. 3B).

**Germ cell differentiation in human fetal testis xenografts**

The gonocyte pluripotency marker OCT4 was expressed in most germ cells of first trimester testes prior to grafting, while after grafting some of the germ cells had ceased to express this protein, coincident
with emerging expression of proteins associated with differentiation into pre-spermatogonia, such as MAGE-A4 and VASA (Fig. 4A). The latter were only rarely expressed in germ cells in first trimester testes prior to grafting. Similar results were obtained using second-trimester grafts. These results suggested that germ cells differentiate during the grafting period similar to that which occurs normally in the intact testis. To establish if this was the case, quantification of the change in germ cell differentiation marker expression was performed on sections co-stained for OCT4 and VASA (Fig. 4B). The results confirmed that the % of cells expressing OCT4 decreased during the grafting period while the % of cells expressing VASA increased. The % of cells expressing OCT4 after grafting was significantly reduced when compared with the pre-graft control tissue for both first and second trimester testes, while the % of cells expressing VASA significantly increased in the first-trimester grafts, compared with the pre-graft control. Further evidence for normal germ cell differentiation after grafting is that the % of cells expressing OCT4 or VASA in xenografts was not significantly different from that found in equivalent age-matched controls (Fig. 4B). Comparable results were also obtained when germ cells were co-stained for OCT4 and MAGE-A4 (not shown).

**Discussion**

The present study has demonstrated that human fetal testis development can be recapitulated using the xenografting approach and that overall testis (graft) growth and cellular proliferation/differentiation appear to progress normally. Graft survival rates were excellent (>75%) and the vast majority of retrieved grafts had normal testis structure with seminiferous cords of normal appearance.

Our study provides the first demonstration of ex situ seminiferous cord formation in the human testis as we have shown that first-trimester human fetal testis xenografts can form normal seminiferous cords, the timing of which matches that described previously for the normal intact human fetal testis (Wartenberg, 1981; Hanley et al., 2000; Ostrer et al., 2007). Xenografting of first-trimester fetal testes thus has potential for investigating the mechanisms of normal human fetal testis development, and may also be useful for studies that aim...
to assess the consequences of disrupting seminiferous cord formation (dysgenesis). However, in order to establish validity of the xenografting model for use in such studies, it was considered vital to first determine whether xenografts continue to develop normally from a structural, functional and hormonal perspective. The present studies have shown that testis structure is maintained throughout the first and second trimester and that expression of functional markers of Sertoli (AMH), Leydig (3β-HSD) and peritubular myoid cells (SMA)
are comparable with those of equivalent age-matched controls. AR and 3β-HSD were not expressed in first trimester testes at 9 weeks of gestation, but were expressed in the same testes 6 weeks after xenografting, indicating that steroidogenesis is beginning to occur and that the testes are becoming androgen responsive. SMA expression in peritubular myoid cells is considered to be androgen-dependent.
In addition to these changes, weak expression of AMH in first trimester testes became stronger within the Sertoli cells after xenografting. The change in expression patterns of AR, 3β-HSD and AMH in testis xenografts is comparable with that described previously for the human fetal testis between 7–9 and 14 weeks of gestation (Gaskell et al., 2004).

Production of androgens is crucial for other aspects of testis development such as Sertoli cell proliferation (Scott et al., 2007, 2008), as

(Schlatt et al., 1993). In addition to these changes, weak expression of AMH in first trimester testes became stronger within the Sertoli cells after xenografting. The change in expression patterns of AR, 3β-HSD and AMH in testis xenografts is comparable with that described previously for the human fetal testis between 7–9 and 14 weeks of gestation (Gaskell et al., 2004).

Production of androgens is crucial for other aspects of testis development such as Sertoli cell proliferation (Scott et al., 2007, 2008), as
well as for masculinization of the reproductive organs (Auchus, 2004; Scott et al., 2009). Our studies used measurement of serum testosterone and seminal vesicle weight in the host animal to determine the steroidogenic function of the xenografts and showed that second-trimester xenografts are capable of producing testosterone and that this could be significantly increased by hCG treatment. However, first-trimester xenografts did not produce detectable levels of testosterone in host serum, which may be related to the relatively small size and number of grafts in each host, or may be a result of age-related differences in the responsiveness of human grafts to host mouse LH, as has been described in Rhesus monkey testis xenografts (Rathi et al., 2008).

We anticipate that hCG treatment of host mice carrying first-trimester human fetal testis xenografts would result in the production of detectable levels of testosterone.

Previous studies have demonstrated that germ cells are present in second-trimester human fetal testis xenografts, but the identity and differentiation of these cells was not demonstrated (Povlsen et al., 1974; Skakkebaek et al., 1974; Yu et al., 2006). The present study has shown that germ cell differentiation from gonocyte (OCT4+/MAGE-A4+/VASA−) to pre-spermatogonium (OCT4−/MAGE-A4+/VASA−), a process that normally takes place during fetal and early post-natal life in the human, occurred normally in xenografts and was comparable with that of equivalent age-matched controls (Gaskell et al., 2004; Anderson et al., 2007; Mitchell et al., 2008). One minor exception was that OCT4+ germ cells in the first trimester xenografts had a significantly lower PI than did the pre-graft or equivalent age-matched controls, and this could artifically affect the proportion of germ cells expressing OCT4 in the xenografts. However, this could not account for the emergence of differentiated (VASA− or MAGE-A4+) germ cells in first-trimester grafts compared with pre-graft controls (most of which had no VASA or MAGE-A4 expressing cells prior to grafting) or for the increase in proportion of differentiated germ cells in the second-trimester grafts. Taken together these results indicate that germ cell differentiation occurs in the xenografts and that this is broadly comparable with the normal situation in vivo.

Having established xenografting as a suitable model for human fetal testis seminiferous cord formation and germ cell differentiation, we propose that it may be especially useful for studies investigating the origins of DSDs and TDS in humans. DSDs often result from genetic abnormalities involving the sex chromosomes or genes involved in gonad development (Hughes et al., 2006). Genetic manipulation of testis tissue or isolated cells prior to grafting has been shown to be possible in studies that introduced the backdoor pathway to dihydrotestosterone (Scott et al., 2009). For example, in vitro studies with first and second-trimester human fetal testis explants have shown no inhibition of testosterone production by either of two different phthalates (Hallmark et al., 2007; Lambrot et al., 2009), whereas in vivo studies have provided (indirect) evidence that such phthalates might impair androgen production (Swan et al., 2005). The present results show that testis xenografting would be a relevant approach via which to investigate the effects of these chemicals on the development of the seminiferous cords, germ cell development and steroidogenesis by the human fetal testis under conditions in which normal cell development and function appears to be occurring. Investigating the effects of phthalate esters in this system may help to resolve the uncertainty about whether these chemicals affect steroidogenesis (or other functions) of the fetal human testis (Scott et al., 2009).

In conclusion, we have demonstrated for the first time that human fetal testis xenografts are a comparable in vivo ex situ model of normal seminiferous cord formation, germ cell development and testosterone production. Germ cells within xenografts differentiate from gonocytes into pre-spermatogonia and proliferate in a manner similar to that in normal age-matched control testes. In addition, grafts are capable of producing testosterone, and an increase from basal levels can be induced by hCG treatment of the host animal. We propose that this system may help to dissect the cellular mechanisms that underpin normal human fetal testis development, the disruption of which can lead to common male reproductive disorders: these mechanisms are otherwise inaccessible for study in situ and may be compromised when studied in vitro. Genetic disruption of the initial stages of testis development or manipulation of testosterone production by proposed endocrine disruptors, using the xenograft approach, may be useful for studies relating to DSD or the development of TDS and TGCT.

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Conflict of interest: none declared.

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