Expression of *Cyp21a1* and *Cyp11b1* in the fetal mouse testis

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Abstract

Fetal Leydig cells and fetal adrenocortical cells may share a common progenitor cell. Both cell types show several similarities, particularly in relation to their primary steroidogenic function. Differences in steroid secretion are largely due to the expression of 21-hydroxylase (CYP21A1) and 11 β -hydroxylase (CYP11B1) activity in the adrenal. To determine whether expression of these enzymes defines a clear difference between adrenocortical and Leydig cells, or is further evidence of a link between the cell types, we have measured *Cyp21a1* and *Cyp11b1* expression and related enzyme activity in the fetal testis. Expression of both *Cyp21a1* and *Cyp11b1* was clearly detectable in the fetal testis by RT-PCR and Southern blotting. Real-time PCR studies showed that *Cyp11b1* was expressed only in the fetal/neonatal testis with no expression in the pubertal or post-pubertal animal. *Cyp21a1* was also predominantly expressed in the fetal testis although some lower expression was also seen in the adult. Expression of *Cyp21a1* and *Cyp11b1* in neonatal testicular cells was unaffected by incubation *in vitro* with human chorionic gonadotrophin or ACTH. Using immunohistochemistry, CYP21A1 was localised to a subset of interstitial steroidogenic cells in the fetal testis although CYP11B1 was not detectable. Incubation studies showed that 21-hydroxylase activity was present in the tissue although 11 β -hydroxylase activity could not be detected. Results indicate that a subpopulation of steroidogenic cells in the fetal testis express *Cyp21a1* and show 21-hydroxylase activity. This may provide further evidence of a link between fetal Leydig cells and adrenocortical cells but does not discount the possibility that these steroidogenic cells represent 'ectopic' adrenal cells.

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Introduction

During normal testis development in the mouse, two generations of Leydig cells arise sequentially. A fetal population appears shortly after testis differentiation and is responsible for masculinisation of the fetus while an adult population arises around day 7 after birth and is required for normal adult androgen production (Vergouwen *et al.* 1991, Baker *et al.* 1999, Nef & Parada 1999). The fetal Leydig cells differentiate from mesenchymal-like stem cells around 12.5 dpc in the mouse (Byskov 1986), although the origin of these stem cells remains uncertain with both the coelomic epithelium (Karl & Capel 1998) and mesonephros (Merchant-Larios & Moreno-Mendoza 1998, Val *et al.* 2006) as possible sources.

In support of a mesonephric origin of fetal Leydig stem cells, it has been suggested that fetal Leydig cells and adrenocortical cells share a common origin based on expression patterns of *Sf-1* in the developing gonadal ridge (Hatano *et al.* 1996). The adrenal gland develops adjacent to the gonad at the same time and these authors suggest that both cell types share a common precursor cell which arises around 10.5 dpc at the cranial end of the mesonephros. This hypothesis is supported by the

similarities that exist between fetal adrenal and fetal Leydig cells. Both are regulated by pituitary hormones and are primarily steroidogenic cells sharing a common steroidogenic pathway from cholesterol to progesterone. The major steroid secreted by the cells depends on the presence of 17 α -hydroxylase (CYP17A1) and 17 β -hydroxysteroid dehydrogenase (HSD17b3) in the testis and 21-hydroxylase (CYP21A1) and 11 β -hydroxylase (CYP11B1) in the adrenocortex although CYP17A1 has been shown to be expressed in the fetal adrenal (Heikkila *et al.* 2002). In further support of the concept that fetal adrenal and fetal Leydig cells may share a common precursor, we have shown recently that fetal Leydig cells express the melanocortin type 2 receptor (MC2R) and that they are sensitive to adrenocorticotrophic hormone (ACTH), the principal trophic stimulator of the adrenal cortex (Johnston *et al.* 2007). In addition, adrenocortical cells will express the luteinising hormone (LH) receptor and will respond to LH following hyperstimulation (Kero *et al.* 2000). One of the major differences between the two cell types, therefore, is the expression of CYP21A1 and CYP11B1 in the adrenal cortex which allows secretion of corticosteroids. In order to determine whether expression of these enzymes defines a clear

difference between adrenocortical and Leydig cells, we set out to establish whether *Cyp21a1* and *Cyp11b1*, and associated enzyme activity, are expressed in the fetal testis and to characterise that expression.

Results

Testicular mRNA expression during development

To determine whether *Cyp11b1* and *Cyp21a1* are expressed in the testis, cDNA prepared from fetal and adult testis was amplified by PCR and the presence of amplified product determined by Southern hybridisation. Results in Fig. 1 show that the full-length coding region of *Cyp11b1* was expressed in the fetal testis but not in the adult testis (Fig. 1A) while *Cyp21a1* was expressed in both fetal and adult testis (Fig. 1B). As expected, there was also clear expression of both *Cyp11b1* and *Cyp21a1* in the adrenal. While both fetal and adult testis expressed full-length *Cyp21a1*, a shorter transcript was also apparent in both. Sequencing of PCR products identified two alternate transcripts (Fig. 1C). Transcript C is the correct size for the major alternate transcript seen in Fig. 1B. Transcript B was not clearly seen on the Southern blots and is likely, therefore, to be a minor product.

The general steroidogenic pathways found in the adrenal and testis are shown in Fig. 2A. Real-time PCR was used to measure developmental changes in the expression levels of *Cyp11b1* and *Cyp21a1* in the testis (Fig. 2B) and, for comparison, expression of mRNA encoding the testicular steroidogenic enzymes HSD3B6, CYP11A1 and CYP17A1 was also measured. Data from the real-time studies show that *Cyp11b1* is only expressed in the fetal and neonatal testis and that expression is lost after day 10 (Fig. 2B). HSD3B6 is only expressed in the adult Leydig cell population (Baker *et al.* 1999) and expression of *Hsd3b6* is first seen as expression of *Cyp11b1* is lost. Expression of *Cyp21a1* was similar to *Cyp11b1* except that a low level of mRNA persisted into adulthood (Fig. 2B). Both *Cyp11b1* and *Cyp21a1* showed peak levels of mRNA at E18 which coincided with the fetal expression peak of the Leydig cell genes *Cyp11a1* and *Cyp17a1*.

Incubation of neonatal testicular cells *in vitro* with human chorionic gonadotrophin (hCG) or ACTH had no effect on expression of *Cyp21a1* or *Cyp11b1* (Fig. 3). In the same experiment, testosterone production by the cells was stimulated by both hormones (basal, 0.4 ± 0.1 pmol; hCG, 18.1 ± 2.1 and ACTH, 20.4 ± 3.3).

Enzyme immunolocalisation

Immunohistochemistry was used to try to identify cell types expressing *Cyp11b1* and *Cyp21a1* in the neonatal testis. Expression of CYP21A1 was restricted to the interstitial tissue and to cells that also express CYP11A1,

Figure 1 Expression of *Cyp11b1* and *Cyp21a1* in the fetal and adult testis. Total RNA from each tissue was reverse transcribed, amplified by PCR and visualised by Southern blot hybridisation. (A) Expression of *Cyp11b1* in fetal testis, adult testis and adrenal. The position of the expected full-length transcript (1704 bp) is shown with an arrow. (B) Results from two experiments showing expression of *Cyp21a1* in fetal testis, adult testis and adrenal. The position of the expected full-length transcript (1464 bp) is shown with an arrow labelled A. An alternate transcript is shown with an arrow labelled C which corresponds to structure C shown in part C. (C) Structure of alternate transcripts of *Cyp21a1* identified in fetal testis. Solid boxes represent exons while lines represent introns. (A) represents the full-length transcript while (B) and (C) show the structure of two different alternate transcripts.

a specific Leydig cell marker in the testis (Ikeda *et al.* 1994; Fig. 4). All cells that expressed CYP21A1 also expressed CYP11A1, although there were a number of cells expressing CYP11A1 which did not express CYP21A1 (Fig. 4). Expression of CYP11B1 was not detectable in the testis by immunohistochemistry.

Enzyme activity

To determine whether mouse testes show 21-hydroxylase and 11 β -hydroxylase activity, testicular homogenates were incubated with enzyme substrate and products isolated by thin-layer chromatography (TLC). **Figure 5**

Figure 2 (A) Steroidogenic pathways in the testis and adrenal. The enzymes which catalyse each step in the pathway are shown. HSD3B, 3 β -hydroxysteroid dehydrogenase (types 1 and 6); CYP11A1, cytochrome P450 side chain cleavage; CYP17A1, cytochrome P450 17 α -hydroxylase; CYP11B1, cytochrome P450 11 β -hydroxylase and CYP21A1, cytochrome P450 21-hydroxylase. (B) Expression levels of *Cyp11b1*, *Cyp21a1*, *Hsd3b6*, *Cyp11a1* and *Cyp17a1* mRNA in the mouse testis during development. Enzyme expression was measured by real-time PCR and bars represent the mean $\pm$ S.E.M. of data from four animals. Groups with at least one shared letter superscript are not significantly different. nd, not detectable.

shows a time-course of [3 H]progesterone metabolism by homogenate of neonatal testis. As expected, most of the substrate was metabolised to 17 α -hydroxyprogesterone and androgen (androstenedione plus testosterone). There was also, however, low but clear metabolism to deoxycorticosterone and 11-deoxycortisol which would both be formed by 21-hydroxylase activity. Confirmation of the identity of the 21-hydroxylated products was obtained through HPLC analysis following initial TLC separation. There was no clear further metabolism of these products to corticosterone or cortisol in this or similar experiments. To

determine whether the neonatal testis expresses 11 β -hydroxylase activity homogenate was incubated with [3 H]deoxycorticosterone. Under these conditions, we were unable to detect activity associated with [3 H]corticosterone following TLC and HPLC separation of products.

Discussion

The Leydig and adrenocortical cells share many similarities, not least of which are the primary steroidogenic function of the cells and their responsiveness to

Figure 3 Expression of *Cyp21a1* and *Cyp11b1* in neonatal testicular cell stimulated by ACTH or hCG. Cells isolated from mouse neonatal testes were incubated under basal conditions or in the presence of ACTH (10^{-7} M) or hCG (10^{-9} M) for 3 h. Expression of specific mRNA species was measured by real-time PCR as described in Material and Methods.

pituitary hormones. The nature of the steroid hormones generated by each cell type depends on the steroidogenic enzymes expressed and the predominant production of androgen by the Leydig cells depends largely upon expression of *Cyp17a1*. Expression of *Cyp21a1* and *Cyp11b1* in the fetal mouse testis means that both the fetal testis and adrenal express all genes which code for essential components of the corticosteroid synthetic pathway. Taking into account other similarities between the cell type (e.g. expression of *Mc2r* in the fetal Leydig cells (O'Shaughnessy *et al.* 2003), expression of *Cyp17* in the fetal adrenal (Heikkila *et al.* 2002) and LH receptor expression in the adrenal (Kero *et al.* 2000)) results described here would tend to support the hypothesis that both cell types originate from a common precursor (Hatano *et al.* 1996). It is also clear, however, from the immunohistochemical data that CYP21A1 is only expressed in a subpopulation of steroidogenic cells. A recent study by Val *et al.* (2006) has also shown by *in situ* hybridisation that *Cyp11b1* is only expressed in a subpopulation of steroidogenic cells in the fetal testis. Cells which express the 'adrenal' enzymes may, therefore, be a subpopulation of fetal Leydig cells or may represent ectopic adrenal cells which have migrated from the adrenal during early

differentiation (Heikkila *et al.* 2002, Jeays-Ward *et al.* 2003). Migration of cells from the adrenal to the gonad is normally inhibited by WNT4, however, and in the normal animal, the number of cells following this path is likely to be very few (Heikkila *et al.* 2002) and unlikely to explain the levels of gene expression and enzyme activity seen in this study. A definitive demonstration of a common origin of fetal Leydig cells and fetal adrenal cells is likely to require labelling and detailed tracking of the putative precursors.

CYP21A1 converts progesterone to deoxycorticosterone and is the essential first step in glucocorticoid synthesis. Our studies show that the enzyme is expressed in the fetal testis with expression declining markedly after birth. During development, two populations of Leydig cells arise sequentially. The fetal Leydig cells arise soon after testis differentiation in the mouse and are subsequently replaced, at least functionally, by the adult population which starts to develop in the post-natal, pre-pubertal period (Baker *et al.* 1999, Nef & Parada 1999). The pattern of expression of *Cyp21a1* determined by real-time PCR would be consistent, therefore, with expression in the fetal Leydig cells. Residual expression of *Cyp21a1* in the adult may be due to the persistence of fetal Leydig cells in the adult or to low expression in the adult cell population. It has been shown previously that CYP21A1 is not detectable by Western blotting in the adult testis which is consistent with the low levels of expression seen by real-time PCR (Hu *et al.* 2002). Interestingly, however, in *Cyp11a1*-null mice, there is detectable expression of CYP21A1 in the interstitial tissue probably due to increased levels of ACTH or LH in these mice (Hu *et al.* 2002). It has previously been reported that extra-adrenal 21-hydroxylase activity in the human and adult rat is not mediated through *Cyp21a1* (Mellon & Miller 1989). It is clear, however, from the PCR/Southern blots and from sequencing of PCR products that *Cyp21a1* is expressed in the fetal mouse testis. Failure to detect *Cyp21a1* in the adult rat testis is consistent with this enzyme being predominantly associated with the fetal testis, while lack of expression in the fetal human testis may indicate a species difference (Mellon & Miller 1989).

The final step in glucocorticoid biosynthesis is 11 β -hydroxylation of deoxycorticosteroids by CYP11B1. It has previously been reported that the fetal testis expresses *Cyp11b1* (Hatano *et al.* 1996, Val

Figure 4 Immunohistochemical localisation of CYP11A1 and CYP21A1 in the neonatal testis. Adjacent sections of testes (5 μ m) from a neonatal mouse were incubated with antibody to CYP11A1 or CYP21A1 and visualised following binding to a biotinylated secondary antibody. Cells expressing both CYP11A1 and CYP21A1 are indicated with a yellow arrow while cells expressing CYP11A1 alone are indicated with black arrows. The negative section had no primary antibody added.

Figure 5 Steroidogenic enzyme activity in the neonatal testis. The figure shows the time-course of progesterone metabolism by testicular homogenate. Neonatal testicular homogenates were incubated with [³H]progesterone for different times and products were separated by TLC. Results show steroids formed as a percent of the added substrate. To simplify the graph the products of 21-hydroxylase activity (11-deoxycortisol and deoxycorticosterone) are combined (labelled S + DOC) while the androgens, androstenedione and testosterone are also combined (labelled A). Beside each relevant steroid, the protein likely to be responsible for the illustrated conversion is shown in parentheses.

et al. 2006) and the results reported here confirm that full-length transcripts are expressed in the fetal mouse testis. Failure to detect CYP11B1 protein or 11 β -hydroxylase activity in the fetal testis suggests, however, that suppression of translation may occur. Regulation of translation through, for example, microRNA is an established developmental mechanism (Good 2003) which would, in this case, act to prevent significant corticosterone production by the testis. This is consistent with the very low levels of corticosteroids detected in medium from fetal testes incubated *in vitro* (O'Shaughnessy *et al.* 2003). Corticosteroids act to inhibit Leydig cell function and in the adult rat 11 β -hydroxysteroid dehydrogenase (11 β HSD) oxidises corticosteroids and thereby limits their effect (Hardy *et al.* 2005). Fetal and neonatal animals lack 11 β HSD (Phillips *et al.* 1989) and inhibition of *Cyp11b1* translation may, therefore, be a protective mechanism that prevents high levels of corticosteroid production by the testis. This hypothesis would suggest that CYP21A1 may not play a significant role in normal Leydig cell function and that expression of *Cyp21a1* may be a remnant of Leydig cell evolution (O'Shaughnessy *et al.* 2006).

Expression of *Cyp21a1* and *Cyp11b1* in the fetal testis is further evidence of a link between the developing testis and adrenal. It is likely that this expression is in a subpopulation of fetal Leydig cells and this subpopulation may form during Leydig cell differentiation from pluripotent stem cells that arise from the

coelomic epithelium (Yao *et al.* 2002, Cui *et al.* 2004) or may arise separately from the mesonephros towards the end of initial testis differentiation (Val *et al.* 2006). Thus, in the fetal testis, most or all fetal Leydig cells express MC2R and respond to ACTH (Johnston *et al.* 2007) while a subpopulation of cells also expresses genes encoding 'adrenal' steroidogenic enzymes.

Materials and Methods

Animals

The mice used in this study were bred at the University of Glasgow Veterinary School and were maintained as required under United Kingdom Home Office regulations. Normal mice, bred on a C3H/Heh-101/H genetic background, were derived from stock animals originally obtained from the MRC Radiobiology Unit (now the MRC, Mammalian Genetics Unit, Harwell, UK). To time fetal development, males and females were caged overnight and the morning was designated as fetal day 0.5 of pregnancy. The day of birth was designated as post-natal day 1.

Cell isolation and incubation

Dispersed testicular cells from neonatal mice were prepared by collagenase treatment of whole testes as previously described (Stalvey & Payne 1983). Testes from six animals were dispersed at 37 °C in DMEM/F12 containing 1 mg/ml collagenase (Worthington CLS type 4, purchased from Lorne Laboratories Ltd, Twyford, UK), and isolated cells were filtered through a nylon sieve with a pore size of 50 μ m. Aliquots of isolated cells (1 ml total) were incubated for 3 h at 37 °C in DMEM/F12 in an atmosphere of 5% CO₂ and in the presence or absence of hCG (10⁻⁷ M) or ACTH (1–24) (10⁻⁹ M; Sigma-Aldrich Co). At the end of the incubation period, cells and medium were separated by centrifugation at 150 g and the cell pellet stored in liquid N₂.

RT-PCR and Southern blotting

Total RNA was extracted using Trizol (Life Technologies). Isolated RNA was reverse transcribed using random hexamers and Moloney murine leukaemia virus reverse transcriptase (Superscript II, Invitrogen) as described previously (O'Shaughnessy & Murphy 1993, O'Shaughnessy *et al.* 1994). This cDNA was used as a template for subsequent PCRs. The PCRs were carried out in a total volume of 50 μ l using a 'hot-start' Taq polymerase (1.25 units AmpliTaq Gold, Applied Biosystems, Warrington, Cheshire, UK) in buffer (15 mM Tris-HCl (pH 8.0), 50 mM KCl and 2.5 mM MgCl₂) containing dNTPs (0.2 mM each), primers (200 nM each) and template. Reactions were started by 10 min at 95 °C followed by up to 35 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 2 min.

The primers used to amplify the full-length coding region of *Cyp21a1* and *Cyp11b1* were: *Cyp21a1*, forward: ATGCTGCTACCTGGGCTGCTG and reverse: TCAAGGAC-GCTCACCCTGGTCT; *Cyp11b1*, forward: GATGACAATG-CCTCTCAGGGTGAC and reverse: GAGAGGGCAATG-TGTCATCAGA.

An internal probe for Southern hybridisation of *Cyp21a1* was amplified by PCR from adrenal cDNA using primers TGCTGTTGCTGCTGCTAGCTGG and CAACGTGCTGTCC-TTGTCTCCAAA while a probe for *Cyp11b1* was amplified using TCAGGGTGACAACATATGTGTGGCT and CCATTCTG-GCCCATTTAGCAA. These primers generate amplicons of 511 and 411 bp respectively. The products of these reactions were gel-purified, sequenced and used for Southern hybridisation as described below.

Real-time PCR

Levels of specific mRNA species were measured by real-time PCR using the SYBR Green method following RT of the isolated RNA. Real-time PCRs were performed in a 96-well plate format using a Stratagene MX3000 cyclor. Reactions contained 5 µl 2×SYBR MasterMix (Stratagene, Amsterdam), primer (100 nM) and template in a total volume of 10 µl. The thermal profile used for amplification was 95 °C for 8 min followed by 40 cycles of 95 °C for 20 s, 63 °C for 20 s and 72 °C for 30 s. At the end of the amplification phase, a melting curve analysis was carried out on the products formed and gel electrophoresis was carried out on representative samples to confirm product size.

Primers for real-time PCR were designed using parameters previously described (Czechowski *et al.* 2004) and the amplicons all crossed at least one exon/exon boundary. The primers used were:

<i>Cyp21a1</i>	forward	CAAGAACTCTCTCGCTCAGCCCT
	reverse	CAACGTGCTGTCTTGTCTCCAAA
<i>Cyp11b1</i>	forward	TGCCCTTGAATCCTGGATAGT
	reverse	CCATTCTGGCCCATTTAGCAA
<i>Cyp11a1</i>	forward	CACAGACGCATCAAGCAGCAAAA
	reverse	GCATTGATGAACCGCTGGGC
<i>HSD3b6</i>	forward	GCTCCAGAGTGGGAGTGCTGACAC
	reverse	AATCCTCTGGCCCAAAAACCCTC
<i>Cyp17a1</i>	forward	TGGCCCCATCTATTCTCTTCGCCTG
	reverse	AGGCGACGCCTTTTCCTTGG.

Sequencing

PCR products were sequenced directly or were ligated into plasmid using TOPO TA cloning kits (Invitrogen) and sequence obtained from the cloned plasmid. Sequencing reactions were carried out using big dye terminator cycle sequencing kits (Applied Biosystems).

Southern hybridisation

For Southern hybridisation of PCR products, the DNA was transferred from agarose gels to nitrocellulose membranes and

hybridised with a [³²P]labelled cDNA probe prepared as above (O'Shaughnessy *et al.* 1994).

Enzyme activity

Steroidogenic enzyme activity was measured in homogenates of fetal testis and adrenal using tritiated substrate as described previously (O'Shaughnessy *et al.* 2000). Tissues were homogenised in PBS and incubated with [1,2,6,7-³H]progesterone (Amersham) or [1,2,6,7-³H]21-hydroxyprogesterone at 37 °C in the presence of 1 mM NADPH. The [³H]21-hydroxyprogesterone substrate was generated from [³H]progesterone by incubation with adult mouse adrenal homogenate. At the end of the incubation period, 50 µg non-radioactive carrier steroids were added to each sample along with [¹⁴C]testosterone and [¹⁴C]androstenedione (2000 d.p.m./sample) to measure recovery. Samples were extracted twice with 5 ml toluene and steroids were separated by TLC using polyester-backed silica gel plates (Whatman Ltd, Maidstone, UK). A two-step TLC - separation was used; initially, plates were developed in -chloroform/methanol (97/3) which separated cortisol, corticosterone, deoxycorticosterone, 11-deoxycortisol, testosterone and 17-hydroxyprogesterone. Progesterone and androstenedione were not separated in this system and they were eluted from the first TLC plate and separated by subsequent TLC in chloroform/ether (7/1). The identity of products was confirmed by reverse-phase HPLC using a C18 4 µm column as described previously (Mannan & O'Shaughnessy 1988).

Immunohistochemistry

Neonatal testes were fixed in 4% paraformaldehyde for 1 h and then washed in 70% ethanol, dehydrated and embedded in paraffin. Sections (5 µm) were mounted on glass slides, dewaxed and rehydrated. Endogenous biotin was blocked using an avidin/biotin blocking kit (R&D systems Europe Ltd, Abingden, UK) and sections were incubated with primary antibody overnight at 4 °C. The antibodies used were rabbit anti-human CYP21A1 (LAE Biotech International, Rockville, MD, USA), mouse anti-rat CYP11B1 monoclonal (Chemicon International, Temecula, CA, USA) and rabbit anti-bovine P450scc (gift from A H Payne). Sections were washed and incubated for 30 min with biotinylated secondary antibody (R&D systems Europe Ltd). Bound antibody was visualised using 3,3'-diaminobenzidine tetrahydrochloride (R&D systems Europe Ltd). Negative controls without the primary antibody were included in each experiment.

Statistical analysis

Data were analysed by ANOVA followed by Fisher's multiple comparison test.

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