Characterisation of Urogenital Ridge Gene Expression in the Human Embryonal Carcinoma Cell Line NT2/D1

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Key Words

Gene expression • Gonad development • Intersex • NT2/D1 • Sex determination • SRY

Abstract

The study of the mammalian sex-determining pathway has been hampered by the lack of cell culture systems to investigate the underlying molecular relationships between sex-determining genes. Recent approaches using high-throughput genome-wide studies have revealed a number of sexually dimorphic genes expressed in the developing mouse gonad. Here, we investigated a human testicular cell line in terms of its expression of known sex-determining genes and newly identified candidates. The human embryonal carcinoma cell line NT2/D1 was screened for the expression of 46 genes with known or potential roles in the sex-determining and differentiation pathway. Forty genes tested were expressed in NT2/D1 cells including the testis-determining genes *SRY*, *SOX9*, *SF-1*, *DHH* and *FGF9*. Genes not ex-

pressed included *WT1*, *DAX1* and the ovary-specific genes *FOXL2* and *WNT4*. Cell-specific markers demonstrate that NT2/D1 cells reflect a number of cell types in the gonad including Sertoli, Leydig and germ cells. Our results suggest that male pathways initiated by *SRY*, *SOX9* and *SF-1* remain intact in these cells. Lack of expression of ovary-specific genes is consistent with a commitment of these cells to the male lineage. Manipulation of gene expression in this cell line could be an important new in vitro tool for the discovery of new human sex-determining genes.

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Sex determination in mammals is chromosomally controlled and governs whether a testis or an ovary forms in the embryo. The discovery of the testis-determining gene *SRY* on the Y chromosome gave an entry point into this molecular pathway [Gubbay et al., 1990; Sinclair et al., 1990]. The *SRY* gene encodes a 204 amino acid protein containing a high mobility group (HMG) domain which binds and bends DNA [Harley et al., 1992; Pontiggia et al., 1994]. *SRY* is expressed in pre-Sertoli cells of the genital ridge and initiates testis determination (7 weeks in humans and 10.5 dpc in mice) [Hanley et al., 2000; Bullejos and Koopman, 2001], however, no direct *SRY* target

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gene has been defined. Therefore, our current view of the molecular genetics of sex determination and differentiation is based on the roles of a handful of genes isolated through clinical cases of sexual ambiguities or through mouse transgenic/knockout studies. Most of these genes encode transcription factors (*SRY*, *SOX9*, *WT1*, *SF-1*, *DAX1* and *GATA4*) or secreted factors (*AMH*, *WNT4*, *FGF9* and *DHH*).

To identify new candidate genes required for gonad development, a number of groups have investigated the sexually dimorphic expression pattern of genes during the gonad development phase using screening approaches. Such approaches include whole mount in situ hybridization screening using probes from 11.5 dpc mouse cDNA libraries [Wertz and Herrmann, 2000]; subtractive differential display between male and female gonads [Nordqvist and Tohonen, 1997; Bowles et al., 2000; Menke and Page, 2002; McClive et al., 2003]; differentiation screens between 12 and 12.5 dpc Sertoli cells [Boyer et al., 2004]; and microarray screening of the expression profile of genes during gonad development in males and females [Grimmond et al., 2000; Small et al., 2005]. Recent approaches have also screened for differential expression patterns utilizing transgenic mouse models that allow for the selection of somatic cells within the sex determination window [Nef et al., 2005; Beverdam and Koopman, 2006]. Such techniques have given rise to a large number of known and novel genes whose cell specificities and possible roles in sexual development are now being further investigated. In order to decipher the molecular relationships between candidate genes and already established sex-determining genes, it is becoming increasingly evident that there is a need for a simplified, in vitro model system.

A number of gonad cell lines have been produced, characterised and used with varying success to study molecular interactions of sex-determining genes. These include the murine TM3 (Leydig-like) and TM4 (Sertoli-like) cell lines [Mather, 1980]; Leydig-like clonal cell lines [Ascoli, 1981]; primary cultured cells from the 11.5 dpc gonad [Nishino et al., 2004]; and immortalized gonadal cell lines from transgenic mice or embryos that over-express the SV40 T-antigen [Hofmann et al., 1992; Peschon et al., 1992; Haqq et al., 1994; Lebel and Mes-Masson, 1994; Capel et al., 1996]. Many of these immortalized primary cell lines have been derived from rodent immature or adult testis, hence, due to the temporal expression pattern of *Sry* in these species, where *Sry* is switched off after the sex determination period (12.5 dpc), these lines would not be expected to express *Sry* or contain other stage-spe-

cific factors involved in the differentiation of the testis. For example, the cell lines TM3 and TM4 were not found to express *Sry* [Beverdam et al., 2003].

Immortalized primary cell lines have also been established from cell types isolated from the genital ridge of rodent gonads during the sex determination period [Haqq et al., 1994; Capel et al., 1996; Nishino et al., 2004]. In each instance, *Sry* transcripts were expressed in these cell lines; however, continued culture of these cells led to a progressive loss in *Sry* expression after 48 hours [Nishino et al., 2004] and after 6 months [Capel et al., 1996]. These results correlate with the eventual loss of *Sry* expression observed in vivo and also suggest that the gonad-specific factors involved in *Sry* regulation are predominantly lost over time. Furthermore, in order to define downstream targets of SRY, transient over-expression of SRY was carried out in these gonadal cell lines producing mixed results. In one case, endogenous *Sox9* was not found to be up-regulated, a process believed to be initiated by SRY, in the BCG cell line over-expressing SRY [Beverdam et al., 2003].

Given the major differences in human and rodent SRY proteins [Bowles et al., 1999], a human cell line would serve a more desirable choice for studying the action of human SRY compared to the rodent lines mentioned above. NT2/D1, a human pluripotent embryonal carcinoma (EC) cell line, has the ability to differentiate into different cell types upon different stimuli [Andrews, 1984; Andrews et al., 1984; Caricasole et al., 2000] and therefore may have the potential to differentiate as Sertoli cells. In their undifferentiated state, NT2/D1 cells have proven an invaluable in vitro tool to groups studying aspects of sex determination. These include cloning of the human SRY transcript from NT2/D1 [Clepet et al., 1993], the regulation of the *AMH* gene [De Santa Barbara et al., 1998; Nachtigal et al., 1998], the regulation of SRY transcription by SF-1 and WT1 [Shimamura et al., 1997; De Santa Barbara et al., 2001; Hossain and Saunders, 2001] and more recently, the effect of acetylation/deacetylation on SRY sub-cellular localisation [Thevenet et al., 2004]. Other studies of SRY and SOX9 protein partners have also been reported [Poulat et al., 1997; Desclozeaux et al., 1998; Ohe et al., 2002; Malki et al., 2005]. Furthermore, a screen of 17 human cell lines for the expression of SRY showed that expression was limited to NT2/D1 and only one other cell line [Clepet et al., 1993].

To further characterise this cell line for its use in the study of sex determination, we investigated the expression profile of genes expressed in the urogenital ridge po-

tentially involved in mammalian sex determination/differentiation. We identified 40 genes expressed in NT2/D1 cells including the testis-determining genes *SRY*, *SOX9*, *SF-1*, *DHH* and *FGF9*. Genes not expressed included *WT1*, *DAX1* and the ovary-determining genes *FOXL2* and *WNT4*. Our results suggest that testis-determining pathways initiated by genes such as *SRY*, *SOX9* and *SF-1* are likely to be intact while those initiated by the ovary-determining genes *WNT4* or *FOXL2* are not.

Materials and Methods

Cell Culture

NT2/D1 (ATCC CRL-1973) cells were grown in vitro in Dulbecco's minimal essential medium with high glucose, supplemented with 10% (v/v) fetal calf serum at 37°C under humidified atmosphere containing 5% CO₂ in air. The molecular phenotype of NT2/D1 cells was routinely monitored for changes to its differentiation state with evaluation of expression levels of the EC cell marker *PDGFRA* [Mosselman et al., 1996] and the absence of early neuronal markers such as *NES* [Pleasure and Lee, 1993] by RT-PCR.

RNA Isolation and RT-PCR

We examined the gene expression profile of 35 genes. The selection of these genes was based on (i) whether the gene had previously been demonstrated to play a role in the determination or differentiation of the XY gonad into a testis (from clinical or mouse transgenic/knockout studies) and (ii) whether the expression of the candidate gene occurs in the male gonad during the sex determination window (10.5–12.5 dpc). Where genes have been chosen from mouse studies, the human homolog was identified for amplification by BLAST. Total RNA was isolated using the TRIzol Reagent (Life Technologies) from NT2/D1 cells according to the manufacturer's instructions. Human testis RNA was purchased (Sigma) and human ovary RNA was a kind gift of Dr. Peter Fuller (PHIMR, Melbourne). RNA was treated with DNase I (Ambion) and RNA quality was checked by 1.5% agarose gel electrophoresis and RNA quantitated by spectrophotometry at 260 nm. 0.5 µg of total RNA was reverse-transcribed in a 20-µl reaction using Superscript II (Promega) according to manufacturer's guidelines. 1 µl of the cDNA product was used for PCR amplification. RT-PCR oligonucleotides for each gene were designed to span introns to exclude genomic contamination in PCR products. Oligonucleotides were designed using the online design program Primer3 (<http://www.virus.kyoto-u.ac.jp/cgi-bin/primer2.cgi>) (table 1). PCR conditions were optimized for annealing temperature, MgCl₂ concentration and limited cycle number to ensure that product formation was in the responsive range. Positive controls used in this study were either human testis RNA or human ovary RNA. As an RNA loading control, the house keeping gene β_2 -microglobulin (*B2M*) was amplified.

Immunohistochemistry

SRY and *SOX9* immunohistochemistry was carried out as described previously [Argentaro et al., 2003]. The primary antibodies

Fig. 1. Localisation of endogenous *SRY* and *SOX9* proteins in NT2/D1 cells. Upper panel: *SRY* protein appeared both nuclear and cytoplasmic with a higher proportion of *SRY* visualized in the nucleus. *SOX9* was detected predominantly in the nucleus, although faint cytoplasmic staining was observed in some cells. Lower panel: immunostaining of NT2/D1 cells with pre-immune sheep and rabbit IgGs (negative control). Scale bar: 10 µm.

ies used include the affinity-purified sheep anti-human *SRY* (1:400) [Sim et al., 2005] and affinity purified rabbit anti-*SOX9* (1:400) antibodies. The secondary antibodies used include Alexa 488-conjugated donkey anti-sheep IgG and Alexa 569-conjugated donkey anti-rabbit IgG antibodies (Molecular Probes). DNA was stained with 0.1 µg/ml of 4',6-diamidino-2-phenylindole (DAPI). Immunofluorescence was visualized using an Olympus FV500 confocal microscope.

Results and Discussion

SRY and *SOX9* Protein Expression in NT2/D1 Cells

NT2/D1 cells were first evaluated for the presence of *SRY* and *SOX9* protein. We raised antibodies to *SRY* and *SOX9*, and immunohistochemistry revealed that both proteins were expressed (fig. 1). The immunostaining of endogenous *SRY* in NT2/D1 cells is consistent with that observed in Sertoli cells of human gonads during sex determination [Hanley et al., 2000]. NT2/D1 cells show predominantly nuclear *SRY* localisation which correlates with nuclear localisation of *SRY* in the human testes. The exclusively nuclear staining of *SOX9* in NT2/D1 cells reflects staining observed in the human gonad. In Sertoli cells of the developing gonad *SOX9* is cytoplasmic at 6 weeks and then becomes nuclear by 6.5 weeks upon the onset of *SRY* expression [De Santa Barbara et al., 2000]. Therefore the exclusively nuclear localisation of *SOX9* is consistent with a cell that has expressed *SRY*.

Gene Expression Profiling of NT2/D1 Cells Using RT-PCR

RT-PCR was undertaken with primers against the human homologues of mouse urogenital ridge genes (table 1). Agarose gel electrophoresis revealed that a product was formed for all genes using control RNA. The sizes of the products were as predicted (table 1). 35 genes were analysed. For 31 genes, RT-PCR products were observed by agarose gel electrophoresis (representative examples in fig. 2). What follows is a description of the genes that we analysed.

Genes Essential for Testis Formation and SRY Regulation

The transcription factors Wilms' Tumour 1 (*WT1*) and steroidogenic factor 1 (*NR5A1*, commonly referred to as *SF-1*) play numerous roles in sexual development, with mutation of either gene resulting in XY male-to-female sex reversal [Pelletier et al., 1991; Barbaux et al., 1997; Achermann et al., 1999]. Both genes are detected in both sexes early in mouse gonad development (9.5 dpc), however *Sf-1* expression only persists in male gonads from 12.5 dpc [Pelletier et al., 1991; Ikeda et al., 1994]. Targeted disruption of either gene in mice results in a blockage of gonad development [Kreidberg et al., 1993; Luo et al., 1994]. Transfected cell studies carried out in NT2/D1 cells have shown that exogenous *WT1* is capable of activating endogenous *SRY* [Hossain and Saunders, 2001] and that both *WT1* and *SF-1* are capable of binding and activating the *SRY* promoter [Shimamura et al., 1997; De Santa Barbara et al., 1998; Hossain and Saunders, 2001]. *WT1* can also synergize with *SF-1* to activate *AMH* transcription required for regression of the Müllerian (female) duct system [Nachtigal et al., 1998]. *SF-1* has also been demonstrated to play an important role in the transcriptional activation of the *DAX1* promoter [Kawabe et al., 1999]. RT-PCR showed the presence of *SF-1* mRNA in NT2/D1 cells; however, *WT1* mRNA was not detected (fig. 2). The absence of *WT1* expression correlates with previous observations [Hossain and Saunders, 2001].

GATA4 and Friend of *GATA2* (*FOG2/ZFPM2*) genes have also been described as playing a role in *SRY* regulation [Tevosian et al., 2002]. Mice with targeted disruption of these two genes exhibit abnormalities in gonadogenesis, possibly due to decreased levels of *Sry*, *Dhh*, *Sox9* and *Amh* expression [Tevosian et al., 2002]. As neither *Wt1* nor *Sf-1* expression is altered in these knockout mice, it is thought that *GATA4* and *FOG2* act in a separate pathway to regulate *Sry* expression. Furthermore, *GATA4* has

been implicated in the regulation of *Amh* through co-operation with *SF-1* [Viger et al., 1998] and also in the regulation of the testis-determining gene *Dmrt1* [Lei and Heckert, 2004]. RT-PCR revealed that both *GATA4* and *FOG2* genes are expressed in NT2/D1 (fig. 2).

Downstream Targets of SRY and SOX9

The human *SRY* transcript was first cloned from NT2/D1 cells [Clepet et al., 1993], therefore, the *SRY* mRNA detected by RT-PCR correlated with previous studies (fig. 2). The presence of *SRY* expression in NT2/D1 cells in the absence of *WT1* also supports a role for independent positive regulators such as *SF-1*, *FOG2* and *GATA4*. *SOX9* is one of the earliest known markers of differentiating Sertoli cells and may therefore be an immediate downstream target of *SRY*. Mutations in the *SOX9* gene cause Campomelic Dysplasia/Autosomal Sex Reversal 1 (CD/SRA1) [Foster et al., 1994]. RT-PCR results showed that *SOX9* is expressed in NT2/D1; however, levels of *SOX9* transcript are quite low in comparison to testis (fig. 2).

Experiments conducted in NT2/D1 cells have also shown that *SOX9* is capable of activating *AMH* transcription [De Santa Barbara et al., 1998]. The expression of *AMH* may be a direct consequence of the continued regulation by *SOX9* and other co-factors responsible for *AMH* regulation expressed in NT2/D1 cells (fig. 2). Furthermore, other studies have also demonstrated that another *SOX* transcription factor, *SOX8*, can activate *AMH* in vitro [Schepers et al., 2003]. The expression of *SOX8* was also detected in NT2/D1 cells (fig. 2).

SOX9 was also shown to be capable of activating a novel gene, *KIAA0800* [Zhao et al., 2002]. This gene is highly conserved between human and mouse and is preferentially expressed in the testis, however its function downstream of *SOX9* is yet to be elucidated. *KIAA0800* was also found to be expressed in NT2/D1 cells (fig. 2).

Signals Required for Gonad Formation

Signalling molecules play a number of roles in both sex determination and differentiation via cell-to-cell interactions in the gonad. Sertoli cells act as the core organizers of testis development by playing numerous roles in the gonad. Not only are Sertoli cells the source of *AMH*, these cells are also important for inducing the migration of cells into the gonad from the neighbouring mesonephros [Buehr et al., 1993], for producing signals that inhibit germ cells entering meiosis [McLaren, 2003], and for signalling to cells in the genital ridge assisting in the differentiation of Leydig cells [Rouiller-Fabre et al., 2003].

Table 1. Oligonucleotides used for the RT-PCR amplification of urogenital ridge genes

Gene symbol	Gene name	Accession No.	RT-PCR primers	Annealing temp., °C	Product size, bp
<i>SRY</i>	sex determining region on Y	NM_003140	F 5'-TTCCCGCAGATCCCGCTTCGGTACTCTG-3' R 5'-(T) ₁₅ GAAATGAATAAG-3'	60	329
<i>WT1</i>	Wilms' Tumor 1	BC032861	F 5'-GATAACACACAACGCCCATC-3' R 5'-CACACGTCGCACATCCTGAAT-3'	55	100
<i>SOX9</i>	SRY-(sex determining region Y)-box 9	NM_000346	F 5'-ATCTGAAGAAGGAGAGCGAG-3' R 5'-TCAGAAGTCTCCAGAGCTTG-3'	59	262
<i>VNN1</i>	vanin 1	NM_004666	F 5'-GCGGCTGTTTATGAGCATGC-3' R 5'-AAGTGCATCTTCTGGAGTCAC-3'	58	160
<i>DMRT1</i>	doublesex and mab-3 related transcription factor 1	NM_021951	F 5'-CCAGTGGCAGATGAAGAACA-3' R 5'-TGACGGGAGTGACTGTGAAG-3'	55	250
<i>DHH</i>	desert hedgehog homolog	NM_021044	F 5'-CTACTACGAGTCCCGCAACC-3' R 5'-CGCCAAAACCCAGTCTCC-3'	55	159
<i>NR5A1 (SF-1)</i>	nuclear receptor subfamily 5, group A, member 1	NM_004959	F 5'-CCTCATCCGGTGTGAGAGC-3' R 5'-GGTGCACGTGTAGTGCTGT-3'	56	197
<i>GATA4</i>	GATA binding protein 4	NM_002052	F 5'-CTTCCCTCTTCCCTCCTCAA-3' R 5'-GAGATGCAGTGTGCTCGTGC-3'	55	242
<i>FGF9</i>	fibroblast growth factor 9 (glia-activating factor)	NM_002010	F 5'-GCAGCTATACTGCAGGACTG-3' R 5'-CAGTTCAGGTACTTTGTCTCG-3'	60	409
<i>AMH</i>	anti-Müllerian hormone	NM_000479	F 5'-CGGTGACAGGCAGGCTGCCCT-3' R 5'-GGCTCCTGGAACCTCAGCGA-3'	60	138
<i>CST9</i>	cystatin 9-like (mouse)	NM_080610	F 5'-GACTGGGGCACATCTTGAAT-3' R 5'-GGATAACAAACAAGTCCAAAGC-3'	57	232
<i>SOX8</i>	SRY (sex determining region Y)-box 8	NM_014587	F 5'-AAGAAGGACCACCCGACTAC-3' R 5'-TGCTGAAGTCGATGTTCTGG-3'	58	300
<i>KIAA0800</i>	Vpr-binding protein	NM_014703	F 5'-ACTACAGTAGTGGTACATGTGGAC-3' R 5'-TGGATCAGCTCGACCAGGATGTCG-3'	58	197
<i>NR0B1 (DAX1)</i>	nuclear receptor subfamily 0, group B, member 1	NM_000475	F 5'-CCGCTTGCACTGCGAGACTGTG-3' R 5'-CTCATGGTGAAGTGCCTACTG-3'	68	521
<i>WNT4</i>	wingless-type MMTV integration site family, member 4	NM_030761	F 5'-GCTGGAAGTGTCCACACTCG-3' R 5'-CCCGCATGTGTGTCAGGATGG-3'	58	360
<i>FOXL2</i>	forkhead box L2	NM_023067	F 5'-CGCTGTCCGTCTACCAG-3' R 5'-CCGGGTCCAGCGTCCAGTAG-3'	62	367
<i>ATRX</i>	alpha thalassemia/mental retardation syndrome X-linked	NM_000489	F 5'-GTGCCAGTGCTAATGCTTCA-3' R 5'-AGCGCATAGAAGCCAGTGAT-3'	58	254
<i>FOG2</i>	friend of GATA 2	NM_012082.2	F 5'-GTGGCATCTGGTATCGGAGT-3' R 5'-GGGCAGGAATTCTTCCATTT-3'	55	227
<i>NT3</i>	neurotrophin 3	NM_002527	F 5'-GATCAAAAACGGGCAACTCTC-3' R 5'-CCAGCCCACGAGTTTATTGT-3'	63	178
<i>ALDH1A1</i>	aldehyde dehydrogenase 1 family, member A1	NM_000689	F 5'-TGTTAGCTGATGCCGACTTG-3' R 5'-TTCTTAGCCCGCTCAAACT-3'	57	154
<i>PTGDS</i>	prostaglandin D2 synthase	NM_000954	F 5'-GACAAGTTCCTGGGGCGCTGGT-3' R 5'-GCTGTAGAGGGTGGCCATGC-3'	54	342
<i>NACA</i>	nascent-polypeptide-associated complex alpha polypeptide	NM_005594	F 5'-TTCAAGGTGAAGCTGTCTCAA-3' R 5'-GCCTTTGCTCTCGACACATT-3'	57	151
<i>CD164</i>	CD164 antigen, sialomucin	NM_006016	F 5'-TGCCAACAGCCAATTCTACA-3' R 5'-ACACCCAAGACCAGGACAAT-3'	57	178
<i>PRKCSH</i>	protein kinase C substrate 80K-H	NM_002743	F 5'-AACGGGGAGTTTGCTTACCT-3' R 5'-CGTGCCTTGCTCATACTTCA-3'	60	195
<i>MAGED1</i>	melanoma antigen, family D, 1	NM_006986	F 5'-TGGAGATGAGGCTGTGTCTG-3' R 5'-AGAAACCAATGGCACCAAAG-3'	60	236
<i>YWHAZ</i>	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	NM_003406	F 5'-TGAAGCCATTGCTGAAGT-3' R 5'-GGCAGACAAAAGTTGGAAGG-3'	62	172
<i>UGALT2</i>	UDP-galactose translocator 2	D87989	F 5'-TTACCAAACAGGCTCCAACC-3' R 5'-GCCCCAAAGAGCAGGATGTTA-3'	59	155
<i>PSMC2</i>	proteasome (prosome, macropain) 26S subunit, ATPase, 2	NM_002803	F 5'-CGCTCGTTCAATGAGTGTG-3' R 5'-GAAAGCCTTCAGGGTTCAGTT-3'	60	244
<i>USP19</i>	ubiquitin specific protease 19	XM_114325	F 5'-CTGGCGTGACAAGATCAATG-3' R 5'-GGCTCTCGTCTACCGTTGTC-3'	60	251

Table 1 (continued)

Gene symbol	Gene name	Accession No.	RT-PCR primers	Annealing temp., °C	Product size, bp
<i>HBPI</i>	HMG box containing protein 1	NM_012257	F 5'-AATGCCTTCATGCTTTTTC-3' R 5'-TTGTTGTGAGCCTGAATTGG-3'	59	225
<i>SMAP</i>	Homo sapiens golgi membrane protein SB140	NM_030799	F 5'-GCCCCATGATCCTACTTTCCA-3' R 5'-TTGTACATCTGGCCCACTGA-3'	57	250
<i>SYNE2</i>	synaptic nuclei expressed gene 2	NM_015180	F 5'-CTCCTCTCACGAAGAGGACGAGG-3' R 5'-CTGTGGGTGGCCATTCAGGACTCG-3'	62	300
<i>TDRD5</i>	Tudor domain containing protein 5	XM_088994.5	F 5'-CTGGCACAACAGGACTCAA-3' R 5'-GCACGGCTGCTAGAAAAATC-3'	59	286
<i>CRR9</i>	cisplatin resistance related protein 9	NM_030783	F 5'-AGCACCGTGGTCACTTTCT-3' R 5'-CGCTGTAAGTGCCAACTGA-3'	57	169
<i>EEF1A1</i>	eukaryotic translation elongation factor 1 alpha 1	NM_001402	F 5'-GATCGCCGTTCTGGTAAAAA-3' R 5'-TCCACTGCTTTGATGACACC-3'	56	188
<i>LHX9</i>	LIM homeobox 9	AY273889	F 5'-CTTGCCAGAAAAACAGGTCT-3' R 5'-CTGCAGATGCTACACACCAAGCTG-3'	55	451
<i>PTCH</i>	patched homolog	NM_000264	F 5'-ACATGTACAACAGGCAGTGG-3' R 5'-GCAAAGGAGGTTTACCTAGG-3'	55	191
<i>CYP11A1</i>	cytochrome P450, family 11, subfamily A, polypeptide 1	NM_000781	F 5'-CCTGCAGTGGCACTTGTATG-3' R 5'-GGTCATCTCTAGCTCAGCGA-3'	55	417
<i>INSL3</i>	insulin-like 3	NM_005543	F 5'-CCCAGAGATGCGTGAGAAGT-3' R 5'-GTAGGGACAGAGGGTCAGCA-3'	60	315
<i>TPM1</i>	tropomyosin 1	NM_001018020	F 5'-AAGTCCGACAGCTGGAAGAA-3' R 5'-TCATTGGCACTTTGAATGGA-3'	54	598
<i>CALD1</i>	caldesmon 1 transcriptional variant 4	NM_033139	F 5'-GTCACCAAGTCCTACCAGAAGA-3'	55	820
	caldesmon 1 transcriptional variant 5	NM_033140	R 5'-GCTGCTTGATGGGTGATTTGA-3'		742
<i>TPH2</i>	tryptophan hydroxylase 2	NM_173353	F 5'-GCCTTTCCTCTGTGTTTATTAG-3' R 5'-TCATTCCAACTGCTGTGTTACC-3'	55	164
<i>TGM3</i>	transglutaminase 3	NM_003245	F 5'-GACCCAGGACTCTCCAAAC-3' R 5'-GTGGTGGCCTGAGAGAGAAG-3'	55	103
<i>IRX3</i>	iroquois homeobox protein 3	NM_024336	F 5'-GCGGAACAGATCGCTGTAGT-3' R 5'-CACACAAAGGCAGACACGTT-3'	58	411
<i>SPRR2D</i>	small proline-rich protein 2D	NM_006945	F 5'-GGAGCTAAGAAAAGGAAGTCCTCA-3' R 5'-TTATTCAGGGAGTGAACGATAAA-3'	55	179
<i>FST</i>	follistatin	BC004107	F 5'-GTTTTCTGTCCAGGCAGCTCCAC-3' R 5'-GCAAGATCCGGAGTGCTTTACT-3'	55	437
<i>NES</i>	nestin	NM_006617	F 5'-CACTCCCCTGGGCTTCTACC-3' R 5'-AGGGGACGCTGACACTTACA-3'	58	369
<i>PDGFRA</i>	platelet-derived growth factor α -receptor	L25829	F 5'-TGCAGAAAGCTGAAGGAGGCTCTGG-3' R 5'-CACGGGCAGAAAGGTACTGCCT-3'	55	850
<i>B2M</i>	beta-2-microglobulin	AF072097	F 5'-TGAATTGCTATGTGTCTGGGT-3' R 5'-CCTCCATGATGCTGCTTACAT-3'	58	234

Fibroblast growth factor 9 (*FGF9*) is required for migration, differentiation and proliferation of Sertoli cells [Colvin et al., 2001; Willerton et al., 2004], a mechanism conducted through the *FGFR2* receptor [Schmahl et al., 2004]. The functions of this molecule have placed it downstream of *SRY* in the sex-determining pathway. Likewise, the role of the prostaglandin D2 signalling pathway and its converting enzyme prostaglandin D2 synthase (*PTGDS*) has been placed in a role downstream of *SRY*. Ectopic prostaglandin D2 can masculinize XX gonads by acting to stimulate Sertoli cell differentiation as a paracrine factor to activate *SOX9* expression and an

autocrine factor inducing *SOX9* nuclear localisation [Adams and McClaren, 2002; Malki et al., 2005; Wilhelm et al., 2005]. The neurotrophin factor, neurotrophin 3 (*NT3*), is expressed by Sertoli cells and induces the migration of cells from the nearby mesonephros [Cupp et al., 2003].

Mice mutant for the desert hedgehog (*Dhh*) signalling molecule have impaired Leydig cell development [Yao et al., 2002]. As this gene is initially expressed in Sertoli cells at 11.5 dpc, this molecule is thought to be important for signalling to developing germ cells [Bitgood et al., 1996]. The importance of this factor is highlighted by the un-

Fig. 2. Expression profile of genital ridge-specific genes in NT2/D1 cells. Semi-quantitative RT-PCR on genes expressed in the developing gonad. RNA isolated from NT2/D1 cells (N) was used for RT-PCR. As positive controls, human testis RNA (T) or human ovary RNA (O) were used. As a negative control, no nucleic acid template (-). Relatively low cycle numbers of PCR (30) were used throughout. The house-keeping gene β_2 -microglobulin (*B2M*) was amplified as an RNA loading control.

derlying clinical phenotypes, including XY male-to-female sex reversal, which occur when the gene is mutated [Umehara et al., 2000; Canto et al., 2004, 2005]. The Vanin-1 (*VNN1*) molecule isolated from a differential screen [Bowles et al., 2000; Grimmond et al., 2000] has recently been shown to be regulated by both SF-1 and SOX9 and is also thought to play a role in providing an appropriate environment for male germ cell development [Wilson et al., 2005]. Cystatin 9 (*CST9*, also known as *Testatin*), a member of the family 2 cystatins, was also isolated from a differential screen [Tohonen et al., 1998]. *CST9* is up-regulated strongly after the onset of SRY and has therefore been implicated as playing a role in the sex-determining pathway. However, recent results have shown that no abnormal gonadal phenotype is observed in *Cst9* knockout mice [Tohonen et al., 2005].

RT-PCR showed that all of these signalling molecules are expressed in NT2/D1 cells (fig. 2). This result suggests that the cellular environment of NT2/D1 cells is somewhat reflective of the morphological state seen during testis formation. As NT2/D1 cells are pluripotent,

these signalling molecules may be acting on cells to maintain a Sertoli-like environment.

Genes That Present Clinical Phenotypes when Dysfunctional

Some genes already discussed and found to be expressed in NT2/D1 can be characterised by their clinical phenotypes such as *WT1*, *SF-1*, *SRY*, *SOX9* and *DHH*. There are other genes that have also been elucidated from cases of individuals showing sexual ambiguities.

DMRT1 (doublesex- and mab-3-related transcription factor 1) is the most conserved sex-determining gene in metazoans [Raymond et al., 1998]. *DMRT1* is expressed in both Sertoli and germ cells of the 12.5 dpc gonad. However, by 15.5 dpc *DMRT1* expression is detected only in ovaries [Raymond et al., 1999]. The role of *DMRT1* in sex determination is unclear, however. Deletions of *DMRT1* loci on chromosome 9 result in XY male-to-female sex reversal [Veitia et al., 1997]. This region also contains at least two other DM genes. RT-PCR detected the expression of *DMRT1* in NT2/D1 cells (fig. 2). The expression

observed may be a direct consequence of the presence of GATA4 as GATA4 has recently been found to directly regulate the expression of *Dmrt1* [Lei and Heckert, 2004]. Lei and Heckert [2004] also demonstrated the binding of an unknown factor to the *Dmrt1* promoter by DNase I footprinting, a factor that is also possibly expressed in NT2/D1 cells and required for *DMRT1* regulation.

Mutations in the *ATRX* (α -thalassemia and mental retardation associated with the X chromosome) gene result in various phenotypes including severe mental retardation and α -thalassemia [Gibbons et al., 1995]. *ATRX* mutations also cause variable genital ambiguities with some cases leading to complete XY male-to-female sex reversal [Ion et al., 1998]. The exact role that *ATRX* plays in sex determination is not known, however, it is thought to be downstream of *SRY* as sex-reversed individuals lack Müllerian ducts, indicating that *AMH* is functional [Lee and Donahoe, 1993]. The expression of *ATRX* was also observed by RT-PCR in NT2/D1 cells (fig. 2). Regulation of *ATRX* in the gonad is not known, but *ATRX* expression indicates that in NT2/D1 cells these factors may also be present.

Dosage sensitive sex-reversal-adrenal hypoplasia congenital-critical region of the X chromosome, gene 1 (*DAX1*) is an X-linked gene in humans, which when duplicated results in XY male-to-female sex reversal [Bardoni et al., 1994]. These results suggest that *DAX1* is an antagonist of the testis-determining pathway. Subsequent studies in mice showed that *DAX1* is required for testis determination including Leydig cell development, somatic cell differentiation and *Sox9* up-regulation [Meeks et al., 2003a, b; Bouma et al., 2005; Park et al., 2005]. The expression of *DAX1* was, however, not observed in NT2/D1 cells (fig. 2), despite the expression of *SF-1* which is known to regulate its transcription [Kawabe et al., 1999].

Candidate Genes Required for Gonad Formation

A range of high-throughput screens have been employed to identify new genes that may play roles in sex determination/differentiation. These studies have produced a number of candidate genes that are expressed specifically in the developing male gonad at the time of sex determination. In an effort to ascertain which of these genes are expressed in NT2/D1 cells, RNA isolated from NT2/D1 cells was screened for the expression of these genes. In a suppression subtractive hybridization experiment genes were subtracted from 12–12.5 dpc testes and ovaries to identify genes that potentially lie within the male sex-determining pathway [McClive et al.,

2003]. Likewise, a similar screen of the differential expression pattern from cDNA libraries isolated from 12.5–13.5 dpc testes and ovaries has produced a large array of genes [Bowles et al., 2000]. Fifteen genes were selected for screening in NT2/D1 cells obtained from these two studies.

Two of these genes were found to be male-specifically expressed in testis cords, aldehyde dehydrogenase 1 family, member A1 (*ALDH1A1*) and UDP-galactose translocator 2 (*UGALT2*) [McClive et al., 2003]. Retinoic acid (RA) is derived from vitamin A and plays a critical role in embryonic development [Chytil, 1996]. *ALDH1A1* is required for the oxidation of retinal to retinoic acid [Lee et al., 1991]. As RA receptors are expressed in different cell types in the testis, including Sertoli cells [Cupp et al., 1999], the male-specific expression of *ALDH1A1* may indicate that RA plays a role during gonad formation. Indeed, recent experiments have demonstrated that retinoid signalling causes germ cells in the ovary to enter meiosis and initiate oogenesis, a process that is retarded by the male-specific retinoid-degrading enzyme CYP26B1 [Bowles et al., 2006]. Furthermore, it has also been reported that Androgen Insensitive Syndrome (AIS) patients who show testicular feminization also lack the expression of *ALDH1A1* in genital cells [Yoshida et al., 1993]. RT-PCR showed that this gene is expressed in NT2/D1 cells (fig. 2). The *UGALT2* gene was also expressed in NT2/D1 cells.

The *TDRD5* gene and the *HBPI* gene have both been shown to be expressed during the development of the male germ line in mice [Smith et al., 2004a, b]. Both these genes are expressed in NT2/D1 cells (fig. 2). Eight other selected genes (*NACA*, *EEF1A1*, *CD164*, *PRKCSH*, *MAGED1*, *YWHAZ*, *PSMC2* and *USP19*) were found not to be exclusively expressed in the male [McClive et al., 2003]. The three remaining genes (*CRR9*, *SYNE2*, and *SMAP*) have not been fully characterised for their expression pattern in the developing gonad. Expression profiling of NT2/D1 cells revealed that all 15 of these genes are expressed in NT2/D1 cells (fig. 2). The 15 genes screened in NT2/D1 represent only a small sample of potential new candidate genes arising from genome screens.

Genes Up-Regulated in the Developing Ovary

Genes required for ovary determination are yet to be fully classified. In the present study, 7 candidate ovary-determining genes were analysed for their expression in NT2/D1 cells, including *FOXL2* and *WNT4*, which previously have been implicated in the subsequent formation and maintenance of the ovary. Mutations in *FOXL2*, a

forkhead domain/winged-helix (WH) transcription factor, have been detected in patients with the Blepharophimosis/Ptosis/Epicanthus inversus Syndrome (BPES) [Crisponi et al., 2001; De Baere et al., 2001]. *FOXL2* is essential for granulosa cell differentiation and ovary maintenance [Schmidt et al., 2004]. Furthermore, XX gonads of mice null for *Foxl2* develop somatic cells with testicular properties [Ottolenghi et al., 2005]. *WNT4*, a member of the WNT signalling molecule family, is required for a number of cellular processes [Huelsken and Behrens, 2002]. *Wnt4* knockout mice have failure in Müllerian duct formation, differentiation of the Wolffian duct into the male ductile system and loss of oocytes [Vainio et al., 1999]. Recent experiments have also shown that *WNT4* prevents both the formation of the male-specific coelomic blood vessel and ectopic steroid production by repressing endothelial and steroidogenic cell migration into the developing XX gonad [Jeays-Ward et al., 2004; Heikkila et al., 2005].

Neither *FOXL2* nor *WNT4* were expressed in NT2/D1 cells or the testis but were detected in the ovary (fig. 2). The absence of expression of these ovary-determining genes is significant as it demonstrates that NT2/D1 cells have maintained their male-specific properties. This correlates with the observation that XY male-to-female sex-reversed *Sox9* knockout mice continue to express *FOXL2* and *WNT4* [Barrionuevo et al., 2006]. As *SOX9* is expressed in NT2/D1 cells, it can be hypothesized that the absence of these two ovary-determining genes is a result of repression by *SOX9*.

Recent differential screens have yielded a greater insight into genes up-regulated during the sex determination period in the XX gonad that may be required for proper formation of the ovary. We investigated the expression profile of five candidate ovary-determining genes that are significantly elevated in the XX gonad compared to the XY gonad at 11.5 dpc in two independent differential screens – follistatin (*FST*), transglutaminase 3 (*TGM3*), tryptophan hydroxylase 2 (*TPH2*), iroquois homeobox protein 3 (*IRX3*) and small proline-rich protein 2D (*SPRR2D*) [Nef et al., 2005; Beverdam and Koopman, 2006]. Expression of these genes was detected in the ovary, but also in the testis and NT2/D1 cells (fig. 2). The detection of these transcripts in the testis and NT2/D1 cells suggests that the initial sexual dimorphic expression pattern observed in the ovary is not maintained. Indeed, expression of *Tgm3* has been detected in the adult mouse testis [Hitomi et al., 2001] and expression of *Irx3* mRNA was reported to be low during embryonic testis development [Jorgensen and Gao, 2005]. No information on the

testicular expression of *TPH2* or *SPRR2D* in humans has been reported.

The expression of *FST* is lost in *Wnt4* null ovaries suggesting a regulatory link, furthermore, similar phenotypic defects arise in *Fst* null ovaries compared to those of *Wnt4* null ovaries [Yao et al., 2004]. Interestingly, *FST* expression is maintained in NT2/D1 cells despite the absence of *WNT4* expression. Expression of *FST* has previously been described in the human testis [Tortoriello et al., 2001], therefore, parallel regulatory pathways must exist to maintain expression of *FST* in both the testis and NT2/D1 cells.

Cell Types Represented in NT2/D1

The screening of genital ridge specific genes has revealed that NT2/D1 cells represent a number of cell types found in the developing gonad including Sertoli cells (*SOX9*) and germ cells (*HBPI*). To define other cell types represented we screened additional cell markers. Expression of the steroidogenic enzyme *CYP11A1* is confined to Leydig cells and its expression is regulated by SF-1 [Ikeda et al., 1994]. *CYP11A1* was detected in NT2/D1 cells and may correlate with the persistence of SF-1 expression (fig. 2). The receptor Patched (*PTCH*) is expressed in developing male gonads from 11.5 dpc in endothelial cells and also Leydig cells [Clark et al., 2000]. *PTCH* is the receptor of DHH signalling [Oldak et al., 2001] and along with *DHH*, its expression was also detected in NT2/D1 cells.

Peritubular myoid cells surround testis cords with a flattened morphology that phenotypically distinguishes them from other cell types in the interstitium. The similarities of origin of peritubular myoid cells and other cell types within the interstitium have made finding cell-specific markers difficult. NT2/D1 cells were screened for the expression of caldesmon 1 (*CALD1*) and tropomyosin 1 (*TPM1*) genes that were recently identified as being expressed by peritubular myoid cells, although not strictly specific [Jeanes et al., 2005]. Both these genes were detected in NT2/D1 cells (fig. 2). Interestingly, a second transcriptional variant of *CALD1*, variant 4, was detected in NT2/D1 whereas only variant 5 was detected in the testis. Transcriptional variant 4 may have other functional roles in NT2/D1 cells.

Targeted disruption of the LIM homeobox 9 gene (*Lhx9*) in mice results in XY male-to-female sex reversal in the absence of other anomalies [Birk et al., 2000]. *Lhx9* is considered as a marker of the mesothelial cell lineage known to give rise to Sertoli cells but also to some interstitial cells of the developing testis [Mazaud et al., 2002].

LHX9 expression was detected in the testis; however, we did not detect expression of *LHX9* in NT2/D1 cells. Mazaud and colleagues [2002] demonstrated that *LHX9* expression ceased in both sexes as cells began to differentiate into their respective lineages. Therefore, the absence of *LHX9* expression in NT2/D1 cells is reflective of cells that have already undergone the differentiation into their respective cell lineages, including those of Sertoli, Leydig and germ cells described in this study.

The screening of genital ridge-specific genes in NT2/D1 cells has focused on genes expressed during the sex determination period that may instigate the eventual differentiation of the gonad. To address the developmental stage which the NT2/D1 cells may reflect, we screened for the expression of the insulin-like 3 gene (*INSL3*) that is known to be expressed after differentiation of the gonad. Expression of *Insl3* is first detected at 14 dpc and is restricted to Leydig cells [Adham et al., 1993; Zimmermann et al., 1997]. RT-PCR showed that *INSL3* mRNA is detected in the testis but absent in NT2/D1 cells. This result correlates with the findings that NT2/D1 cells express genes that reflect the events of sex determination.

Concluding Remarks

In summary, the transcriptional profiling of NT2/D1 cells by RT-PCR has shown that these cells express 35 of 39 testis genes and lack the expression of 2 ovary-specific genes. The genes screened included a number of known sex-determining genes as well as numerous candidate genes possibly required for proper gonad formation. The four genes which were not expressed in NT2/D1 cells were *WT1*, *DAX1*, *WNT4* and *FOXL2*. The absence of *WT1* expression was surprising given the role of *WT1* in

SRY regulation. The relatively low levels of *SRY* expression could be explained in part by the absence of *WT1*. *DAX1*, with a newly identified testis-determining function, was also expected to be expressed – however, the absence of *DAX1* could also be a consequence of the lack of *WT1* expression. In contrast, the absence of expression of the ovary-specific genes *WNT4* and *FOXL2* was expected due to the derivation of NT2/D1 from testicular cells. However, the detection of five other genes up-regulated in the ovary at 11.5 dpc in NT2/D1 suggests that their initial sexually dimorphic expression patterns may vary as they are also detected in the adult testis. The cell types represented in NT2/D1 cells including Sertoli, Leydig, germ and peritubular myoid cells are reflective of the pluripotent nature of these embryonal carcinoma cells. The absence of the expression of *LHX9* also indicates that these cells have differentiated along these respective lineages. The absence of *INSL3* also suggests that these cells are indicative of cell types reflecting the early events of mammalian sex determination. Furthermore, given the high majority of the genes present in NT2/D1, future studies directed towards examining their regulation will be beneficial since many of the regulatory mechanisms are likely to be intact. This study extends known properties of the NT2/D1 cell line, further supporting its use in the study of sex-determining genes, for example, using promoter studies and repression/knock-down studies, to identify regulatory links between genes.

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