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Regulation of the gonadal transcriptome during sex determination and testis morphogenesis: comparative candidate genes

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Abstract

Gene expression profiles during sex determination and gonadal differentiation were investigated to identify new potential regulatory factors. Embryonic day 13 (E13), E14, and E16 rat testes and ovaries were used for microarray analysis, as well as E13 testis organ cultures that undergo testis morphogenesis and develop seminiferous cords *in vitro*. A list of 109 genes resulted from a selective analysis for genes present in male gonadal development and with a 1.5-fold change in expression between E13 and E16. Characterization of these 109 genes potentially important for testis development revealed that cytoskeletal-associated proteins, extracellular matrix factors, and signaling factors were highly represented. Throughout the developmental period (E13–E16), sex-enriched transcripts were more prevalent in the male with 34 of the 109 genes having testis-enriched expression during sex determination. In ovaries, the total number of transcripts with a 1.5-fold change in expression between E13 and E16 was similar to the testis, but none of those genes were both ovary enriched and regulated during the developmental period. Genes conserved in sex determination were identified by comparing changing transcripts in the rat analysis herein, to transcripts altered in previously published mouse studies of gonadal sex determination. A comparison of changing mouse and rat transcripts identified 43 genes with species conservation in sex determination and testis development. Profiles of gene expression during E13–E16 rat testis and ovary development are presented and candidate genes for involvement in sex determination and testis differentiation are identified. Analysis of cellular pathways did not reveal any specific pathways involving multiple candidate genes. However, the genes and gene network identified influence numerous cellular processes with cellular differentiation, proliferation, focal contact, RNA localization, and development being predominant.

Reproduction (2007) **134** 455–472

Introduction

Prior to gonadal sex determination, primordial germ cells migrate from extra-embryonic sites in the yolk sac through the mesentery of the gut and the mesonephros to colonize the genital ridge at embryonic day 10 (E10)–E11 in the rat (Jost *et al.* 1981, Ginsburg *et al.* 1990). A bipotential gonad is formed at E12 in the rat and has the potential to develop into either a testis or an ovary (Jost *et al.* 1981). Sex determination begins at E12.5 in the rat when the sex determining region of the Y chromosome (*Sry*) gene is expressed in the Sertoli cells of males. *Sry* is a mammalian sex determining factor leading to male development (Koopman *et al.* 1990). In the male, Sertoli cells arise from proliferating supportive precursor cells between E12.5 and E13 in the rat and coincides with the onset of *Sry* expression. Sertoli cell precursors form aggregates with the primordial germ cells (Magre *et al.* 1980, Karl *et al.* 1998). Mesenchymal peritubular myoid precursor cells migrate from the mesonephros into the gonad in a male specific manner and surround Sertoli-germ cell aggregates to promote formation of seminiferous cords around E14 in the rat (Magre *et al.* 1980, Jost *et al.* 1981, Buehr *et al.* 1993, Martineau *et al.* 1997, Levine *et al.* 2000, Schmahl *et al.* 2000, Cupp *et al.* 2003). Once peritubular myoid cells and Sertoli cells are in contact, an extracellular matrix is secreted and separates the testis cords from the interstitial tissue (Tung *et al.* 1984, Kanai *et al.* 1992). This cord formation is the first morphological event in sex determination and is imperative for proper testis development.

Vasculature development and coelomic vessel formation is also characteristic in testis development (Yao *et al.* 2006). During this embryonic period Leydig cells, which

ISSN 1470–1626 (paper) 1741–7899 (online)
DOI: 10.1530/REP-06-0341
Online version via www.reproduction-online.org

are later responsible for testosterone production, arise from the mesenchymal interstitial cells (Merchant-Larios & Moreno-Mendoza 1998, Capel 2000, Nishino *et al.* 2001). After seminiferous cord formation, there is sex-specific growth in the male gonad that includes proliferation of Sertoli, germ, peritubular, and interstitial cells (Mittwoch *et al.* 1969, Chubb 1992, Levine *et al.* 2000).

A number of genes have previously been shown to be critical for sex determination and testis development (Morrish & Sinclair 2002, Yao *et al.* 2002, Jameson *et al.* 2003). *Sry* was determined to be an essential factor for sex determination in the early 1990s (Berta *et al.* 1990, Koopman *et al.* 1990, Sinclair *et al.* 1990), however, it is presently unknown how *Sry* directs sexual differentiation. *Sox9*, a high mobility group (HMG) domain transcription factor closely related to *Sry*, causes sex reversal when over-expressed in the female or when inactivated in the male (Jost *et al.* 1981, Ginsburg *et al.* 1990, Wagner *et al.* 1994, Vidal *et al.* 2001). Doublesex and mab3 related in testis 1 (*Dmrt1*) is turned off in the developing ovary and is expressed in the developing testis of a number of species. Male *Dmrt1* mutants are sex reversed or infertile, while females appear normal (Raymond *et al.* 1999, 2000). The dosage-sensitive sex reversal – adrenal hypoplasia congenita gene on the X chromosome gene 1 (*Dax1*) is an orphan nuclear receptor thought to be an SRY antagonist since *Dax1* over-expression masks the effects of *Sry* in sex reversal (Swain *et al.* 1998). *Dax1* is expressed prior to sex determination in the bipotential gonad, then is repressed in the male during sexual differentiation, while persisting in the ovary (Swain *et al.* 1996). Anti-Müllerian hormone (AMH) is a secreted factor produced by Sertoli cells responsible for the regression of the Müllerian ducts in the mesonephros leaving the Wolffian ducts to develop a subsequent male reproductive tract (Munsterberg & Lovell-Badge 1991, De Santa Barbara *et al.* 1998). SOX9, in conjunction with steroidogenic factor 1, is thought to regulate the *Amh*/Müllerian inhibiting substance expression (De Santa Barbara *et al.* 1998, Jamin *et al.* 2002). Fibroblast growth factor 9 (*Fgf9*) is expressed in male gonads early in testis development and *Fgf9* null mutants show some sex reversal (Colvin *et al.* 2001). In addition, the neurotropic growth factor NT3 has been shown to be important for testis cord formation (Levine *et al.* 2000, Cupp *et al.* 2003). NT3 is a Sertoli cell product that acts as a chemotactic agent (Cupp *et al.* 2003) to promote cell migration of peritubular cell precursors from the mesonephros by binding its receptor TRKC (Levine *et al.* 2000, Cupp *et al.* 2002, Cupp *et al.* 2003). The insulin family of receptors is also involved in testis development. Male XY mice with all three insulin receptors knocked out are sex reversed (Nef *et al.* 2003). Although several growth factors and transcription factors have been shown to be involved in sex determination and testis cord formation, the present study uses a genomic approach to identify novel factors and signal transduction events. Further identification of factors involved in gonadal development will help elucidate the genomic control of sex determination and differentiation.

Sexual differentiation is required to produce the dimorphic sexes, essential for mammalian reproduction. A delay in sexual differentiation and testis cord formation can lead to sex reversal, infertility, or gonadal dysgenesis. A better understanding of embryonic testis development will help determine how these abnormalities arise. Numerous factors are anticipated to be involved in the key events of embryonic testis development including cellular proliferation, cell migration, cell associations, extracellular matrix remodeling, and vascularization that leads to testis morphogenesis. The present study was designed to identify new candidate genes involved in sex determination and testis development using a genomic approach involving a microarray analysis of gene expression during embryonic gonadal differentiation. The gene expression in E13, E14, and E16 male and female gonads were compared in order to identify sex differences during this developmental period. In addition, rat E13 testis organ cultures, which develop seminiferous cords *in vitro*, were used and compared with *in vivo* testis development (Martineau *et al.* 1997, Cupp *et al.* 2000, 2003, Levine *et al.* 2000, Uzumcu *et al.* 2002). Comparative microarray analysis was also used to narrow the candidate list of potential regulatory factors for testis development. The rat microarray data obtained herein were compared with data produced for gonadal differentiation in mouse (Nef *et al.* 2005, Small *et al.* 2005, Beverdam & Koopman 2006). Gene lists resulting from these comparative microarray analyses will assist in identifying potential candidate genes in gonadal sex determination.

Materials and Methods

Animals

Sprague–Dawley rats were kept in a temperature controlled environment and given food and water *ad libitum*. Estrous cycles of female rats were monitored by cellular morphology from vaginal smears (Uzumcu *et al.* 2002). Rats in early estrus were bred overnight and matings confirmed by sperm positive smears, denoted day 0 of pregnancy. Animals were euthanized at E13, E14, and E16 of pregnancy, and gonads were collected for RNA isolation and histology. Sex was determined by PCR using primers specific for *Sry* on genomic DNA isolated from embryo tails as previously described (Levine *et al.* 2000). All procedures were approved by the Washington State University Animal Care and Use Committee.

Organ cultures

Rat gonads from E13 embryos were dissected with mesonephros intact and cultured 3 days as previously described (Cupp *et al.* 2000). Briefly, gonads were placed in drops of medium on Millicell CM filters (Millipore, Bedford, MA, USA) floating on 0.4 ml of CMRL 1066 medium (Gibco BRL) supplemented with penicillin–streptomycin, insulin (10 μg/ml), L-glutamine (350 μM), transferrin (10 μg/ml), and BSA (0.01%). Media were changed on the second day of culture. Gonads were maintained in culture for 3 days at which time testis cords formed and testes were used for histological analysis or separated from mesonephros and used for RNA collection. Embryonic cultures undergo similar testis morphogenesis after 3 days of culture as that seen *in vivo* (Levine *et al.* 2000, Cupp *et al.* 2003). Analysis of E13 testis viability in culture demonstrated no abnormal histology, with similar morphology as observed *in vivo* (Levine *et al.* 2000, Cupp *et al.* 2003).

Histology

Tissue specimens were fixed in Bouin's solution for 1 h and embedded in paraffin using standard procedures. Serial sections of 5 μm were stained with hematoxylin and eosin (H&E) using standard procedures by the Histology Core Laboratory of the Center for Reproductive Biology, Washington State University. Sections were visualized by light microscopy.

RNA preparation

Gonads without mesonephros were collected from female and male Sprague–Dawley rat E13, E14, and E16 embryos. Stage of development was confirmed by counting tail somites of each embryo. Gonad samples were stored in TRIZOL at −20 °C (Invitrogen) until extraction following the manufacturer's protocol. Two separate gonadal sample sets were collected for each E13, E14, and E16 testis and ovary, and E13 cultured testis for replicate analysis. Generally 20–30 gonads were combined for an individual sample. Each separate RNA sample was used for a single microarray chip, such that two separate experiments involving two separate sets of animals and RNA isolations were performed.

Microarray analysis

High quality RNA samples were assessed with gel electrophoresis and required a minimum $OD_{260/280}$ ratio of 1.8. At least 5 μg RNA per sample was delivered to the Center for Reproductive Biology, Genomics Core Laboratory, Washington State University for processing as previously described (McLean *et al.* 2002, Small *et al.* 2005). RNA was transcribed into cDNA, and cDNA transcribed into biotin-labeled RNA. Biotin-labeled RNA was then hybridized to the rat RAE230A arrays containing ∼16 000 transcripts (Affymetrix, Santa Clara, CA, USA) and labeled with phycoerythrin-coupled avidin. Hybridized chips were visualized on an Affymetrix Scanner 3000 (Affymetrix). Once raw data were obtained, they were processed using GeneChip Operating Software (GCOS) version 1.1 (Affymetrix) and analyzed by Genespring version 7.2 (Silicon Genetics, Redwood City, CA, USA), and Pathway Assist software (Stratagene, La Jolla, CA, USA).

Bioinformatics and statistical analysis

Initial analysis of microarray data was completed as previously described (Small *et al.* 2005). Microarray hybridization data were examined for physical anomalies on the chip and background noise above a value of 3. Default GCOS statistical values were used for the analysis. All probe sets were scaled to a mean of 125, where signal correlates to the amount of transcript in the sample. An absolute analysis was performed with GCOS to assess the relative abundance of the transcripts on the RAE230A chip based on signal and detection calls (present, absent, or marginal). This information was imported into GeneSpring 7.2 (Silicon Genetics) and normalized using the recommended default normalization methods. This includes setting signal values below 0.01, total chip normalization to the 50th percentile, and normalization of each gene to the median, which allows visualization of data based on relative abundance for a given sample, rather than by comparison with a specific control value (Small *et al.* 2005). The reproducibility between replicate chips was determined and an $R^2 > 0.95$ was judged sufficient to allow two chips to be used per data point, with a $P < 0.05$ confidence. The criteria to consider chip number has been previously described (Chen *et al.* 2004).

Gene expression during sexual differentiation was determined using data restriction and analytical tools within the GeneSpring software. Transcripts with raw signal values above 75 were selected. Previously a raw signal of 50 has been determined to be near background for an absent/present call for expression of most genes. Therefore, a signal of 75 was selected to minimize the inclusion of false positive calls in the analysis. In contrast, a signal of >100 does not include low expressing genes and excludes positive signals. Therefore, a signal of 75 was selected as the optimal cut-off. Transcripts with an average fold change of 1.5 or greater in signal intensity between the developmental stages were also selected. Transcripts expressed differentially in a statistically significant manner were determined using a one-way ANOVA parametric test with variances not assumed equal and $P < 0.05$. Statistics were applied to all time points for both testis and ovary samples to determine when statistically relevant changes occurred.

Two replicates for each sample were prepared and this allowed a 2×2 factorial comparison in the experiment. Unsupervised cluster analysis within the set of transcripts expressed above a signal of 75 allowed for organization of samples by relatedness based on similarity of the expression profiles between different genes and samples (Eisen *et al.* 1998). Gene expression data from mouse embryonic gonads at the time of sex determination produced by Small *et al.* (2005) were obtained from the gene expression omnibus available through NCBI. This data came from E11.5, E12.5, E14.5, and E16.5 mouse embryonic gonadal RNA hybridized to MGU74v2 arrays (Small *et al.* 2005). The raw data were analyzed by GCOS and GeneSpring 7.2 in the same manner as the rat chips above. The finalized mouse list comprised transcripts with a signal above 75, and statistically significant change in expression of 1.5-fold or more. A comparison of mouse genes present with those in the analogous rat list was made.

The rat genome 9999 program included in the GeneSpring software was used to search available promoters in finalized gene lists up to 2500 bases (Chaudhary *et al.* 2005) for the putative SRY binding element (A/T)AACAA(A/T) which would be expected to appear at a frequency of 4096 bps at random. Pathway Assist software (Stratagene) was used to further analyze lists of genes produced in GeneSpring as described previously (Asirvatham *et al.* 2006). The software excludes redundant and non-annotated genes. The final list is then used to produce shortest pathways for identification of cell processes affected and connections between genes of interest. Each connection was then verified using the PubMed/Medline hyperlink given for each node.

Results

The Affymetrix RAE230A chip represents ~16 000 transcripts of the rat genome and allows for a large portion of the rat transcriptome to be evaluated. In the present study, male and female developmental periods E13, E14, and E16 were evaluated to examine gonadal development during sex determination and gonadal differentiation. E13 testes cultured for 3 days form testis cords and were then used to elucidate transcripts consistently involved in testis development and cord formation *in vivo* and *in vitro*. Histological analysis of the testis from E13, E14, E16, and cultured E13 testis verified the progress of testis cord formation at each stage of development (Fig. 1). No cords were observed at E13 in the male or in the female samples. No significant morphology was observed in the ovary except for the development of oocyte nests at E16 (Fig. 1C). Testis cords with aggregated germ cells and Sertoli cells were seen in both E14 and E16 testis and in cultured E13 testis (Fig. 1E–G).

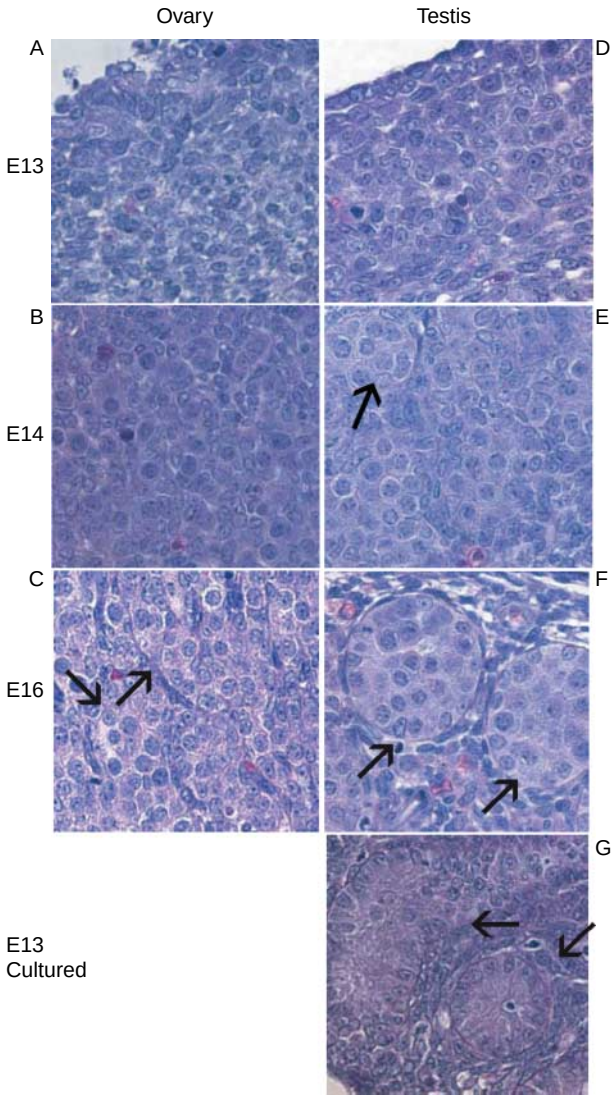

Figure 1 Histology of embryonic rat testis and ovary. Tissue sections from E13 (A), E14 (B), and E16 (C) ovary were analyzed. Tissue sections from E13 (D), E14 (E), and E16 (F) testis, as well as from E13 testis cultured for 3 days (G) were fixed and stained for morphological analysis. Serial sections were stained. Black arrows indicate testis cords in testes or oocyte nests in ovaries.

The transcripts expressed in the E13, E14, and E16 testis and ovary samples and the relationships of the transcriptomes were investigated. A comparison of the duplicate chips for each developmental time point had an $R^2 > 0.96$ demonstrating that the animal, sample, and microarray chip variability was negligible suggesting two chips are adequate. Expression profiles obtained for each sample were analyzed by determining the number of genes present above a raw signal value of 75 in at least one time point of the male or female developmental periods (i.e. E13, E14, or E16). A dendrogram and hierarchical clustering analysis of E13, E14, and E16 samples using genes with a signal above 75 separately in male and female gonadal development are shown for

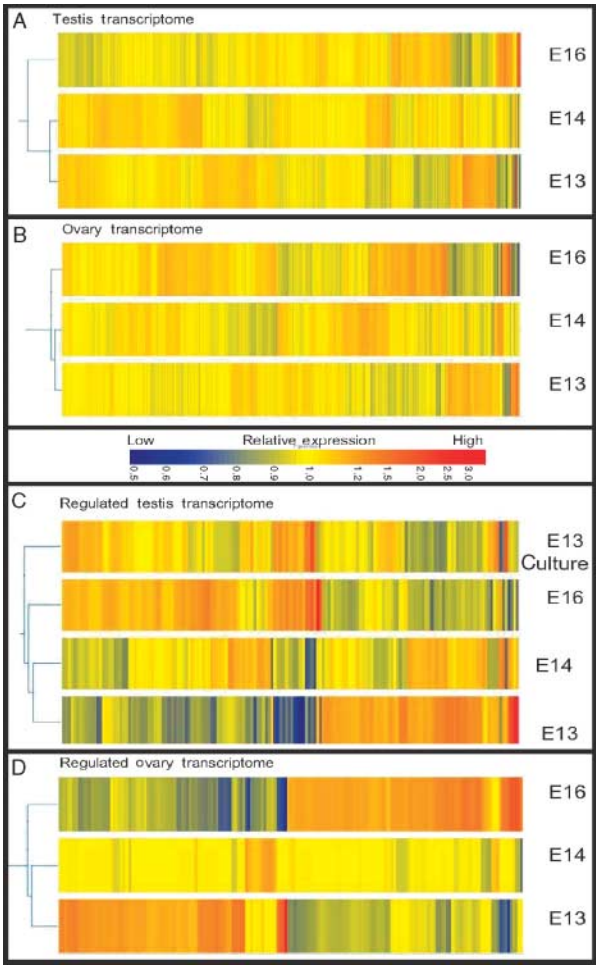

Figure 2 Dendrogram analysis of microarray data reveals the relative relatedness of gonadal transcriptomes. Dendrograms were produced in GeneSpring 7.2 using an unsupervised cluster analysis. Genes are clustered by pattern of expression. Sample sets or time points are clustered by relatedness of gene expression patterns as indicated by the left margin connective illustrations (i.e. links). (A) Dendrogram of male E13, E14, and E16 testis gene expression above a signal value of 75. (B) Dendrogram of female ovary gene expression above a signal of 75 at E13, E14, and E16. (C) Male gonadal genes for E13, E14, E16, and E13 cultured testis expressed above a signal of 75 and with a 1.5-fold significant change during the developmental period. (D) Female gonadal genes for E13, E14, and E16 expressed above a signal of 75 and with a 1.5-fold significant change.

male and female sample sets (Fig. 2). The cluster analysis using GeneSpring software (i.e. link relationships shown on the left side of each dendrogram set) revealed that expression was similar between the E13 and E14 testis (Fig. 2A). The E13 and E14 ovary were also similar (Fig. 2B). The E13 testis and E13 ovary were also found to be similar (data not shown). E16 ovary clustered distally to E13 and E14 ovary transcriptomes. E16 testis also clustered distally to E13 and E14 testis transcriptomes. This suggests that the later E16 time points are more divergent. A second set of dendrograms were produced for male and female transcriptomes using genes present above 75 with a minimum of a 1.5-fold significant change in the developmental period studied. This analysis was done separately for male (Fig. 2C) and female (Fig. 2D) gonadal development. The E13 cultured testis was included in analysis of the male samples. Although the E13 culture testis and E16 testis transcriptomes appear similar (Fig. 2C), the E13 cultured testis clustered distally to the freshly isolated E13, E14, and E16 testis samples (Fig. 2C). A comparative analysis of transcripts expressed in the E13 cultured testis that have formed testis cords to those expressed *in vivo* at E16 in the testis was performed. Regulated genes with similar expression changes *in vivo* and *in vitro* are more likely to be important candidates in testis development and cord formation, while those not similar may not be essential for these processes. Subtracting those transcripts not consistently regulated *in vivo* and *in vitro* allowed the candidate regulatory gene list for involvement in testis cord formation and development to be reduced. The pattern of expression changes was identified by selecting genes that changed over the entire developmental period (E13, E14, and E16). Genes increasing in expression are represented by a color change from blue to red, and genes decreasing in expression are represented by a color change from red to blue. Examples of genes with both increasing and decreasing expression are prevalent in the differentiating testis (Fig. 2C) and ovary (Fig. 2D).

The number of genes expressed above a raw signal of 75 was determined individually for male and female E13, E14, and E16 samples. Genes expressed at each time point were organized in a Venn diagram (Fig. 3). From the 16 000 genes on the chip, 7740 transcripts were expressed in the male gonad in at least one time point. There were 6560 genes with a signal above 75 in all the three (E13, E14, and E16) testis samples. The remaining 1180 genes were expressed in only one or two of the time points analyzed (Fig. 3A). In the female gonad, 7489 transcripts had a raw signal over 75 in at least one time point. Expression similar to the male was seen with 6472 genes above a signal of 75 in all the three

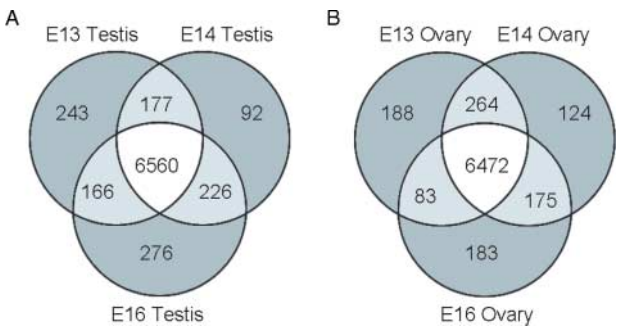

Figure 3 Expressed gene numbers in male and female gonadal development during sexual differentiation. Venn diagrams comparing numbers of genes expressed above a raw signal of 75 were produced in Genespring 7.2. Genes expressed over 75 in E13, E14, and E16 in the rat testis (A) and ovary (B) are compared.

E13, E14, and E16 ovary samples. The remaining 1017 ovary transcripts had a signal above 75 in only one or two time points (Fig. 3B). The similar numbers of expressed genes in the male and female developmental periods suggest that the male and female transcriptional controls of gonad development are equally active.

Genes known to be involved in sex determination and gonadal development; (*Sry, Dax1, Wnt4, Amh, Vanin, Fgf,* and *Wt1*) were used to determine whether expression in the microarray was comparable with that demonstrated in previous studies (Table 1). Expression trends throughout the developmental period matched previously published expression trends for these genes. The *Sry, Vanin, Fgf9,* and *Amh* genes all were expressed in the testis with negligible expression found in the ovary during this developmental period. *Dax1* was initially present in both sexes and then downregulated in the testis as previously described (Swain *et al.* 1996). As expected, the *Wnt4* gene was predominantly expressed in the ovary.The *Wt1* gene is required for gonadal development, hence is expressed in both sexes. Unfortunately, *Sox9* is not in the list as it is not on the RAE230A rat chip. This short list of sex determination genes was selected to validate the microarray and experimental approach.

To identify novel genes that were differentially regulated between E13, E14, and E16 testis or ovary samples, genes with a statistically significant 1.5-fold increase or decrease between time points were determined separately for male (Fig. 4A) and female samples (Fig. 4B). Male and female developmental periods had approximately the same number of statistically significant changes in gene expression between E13, E14, and E16. There were 160 genes with at least a 1.5-fold change in expression in the testis and 175 changing in the ovary between E13, E14, and E16 samples. In the male, the majority of changes occurred between E13 and E14 with 46 genes increasing and 36 genes decreasing in expression (Fig. 4A). Between E14 and E16, the male had 40 genes increase and 16 genes decrease. In the female (Fig. 4B), the majority of gene expression changes occurred between E14 and E16 where there were 43 genes with increased expression and 37 with decreased expression. Between E13 and E14 in the ovary, 36 genes had decreased expression and only 18 increased. The similar number of regulated genes during E13–E16 development in male and female suggests that both have active transcriptional regulation during this development period. Interestingly, the majority of changes in the female occurred later than in the male suggesting that there is a delay in transcriptional activation in gonadal differentiation of the female as compared with the male.

A comparative and subtractive analysis between genes in the *in vivo* E13, E14, and E16 testis developmental period and genes expressed in the E13-cultured testis was performed to narrow the list of potential candidate genes for involvement in testis cord formation and morphogenesis (Fig. 4C). This allowed elimination of genes not essential for seminiferous cord development, as required genes would need to be expressed both *in vivo* and *in vitro* for gonadal and cord development. The testis culture comparison reduced the number of genes regulated in the E13–E14 period to 29 increasing and 18 decreasing, and in the E13–E16 period to 46 increasing and 35 decreasing. Comparative and subtractive analyses lead to a reduction in the list of overall candidate genes from 160 (Fig. 4A) to 109 (Fig. 4C and Table 2). The 51 genes subtracted in this analysis are presented in Supplementary Table 1, which can be viewed online at www.reproduction-online.org/supplemental/. The 109 gene list represents genes regulated during embryonic E13–E16 testis development and contains potential candidate genes associated with male gonadal sex determination and testis morphogenesis. As discussed below, further subtraction of the female expressed genes provides a male enhanced and regulated list (Fig. 4D).

Functional categorization of the 109 gene list (Table 2) revealed that 23 of the genes are cytoskeletal and

Table 1 List of genes known to be involved in sex determination. The highest and lowest expression (Exp.) values detected in the microarray analysis for each of these genes are given. Signals denoted non-detectable (ND) were considered absent for the microarray analysis and generally have an expression <50 unless indicated.

Name	High exp.	Signal	Low exp.	Signal	GenBank	Description
Sry	E13 testis	174	E14 ovary	ND	AF275682	*Rattus norvegicus* sex-determining region Y protein
Vanin	E16 testis	169	E13 ovary	31	BI289085	Vanin 1
Fgf9	E14 testis	80	E13 ovary	ND	D14839	Fibroblast growth factor 9
Dax1	E13 testis	175	E16 testis	66.4	NM_053317	Nuclear receptor subfamily 0, group B, member 1
Amh	E16 testis	953	E16 ovary	ND	AI059285	UI-R-C1-lb-f-01-0-UI.s1 UI-R-C1 *Rattus norvegicus*
Wt1	E14 ovary	234	E16 ovary	125	NM_031534	Wilms tumor 1
Wnt4	E16 ovary	249	E16 testis	63	NM_053402	Wingless-type MMTV integration site family, member 4

extracellular matrix factors and 21 are signaling factors (Fig. 5). Other represented categories include 16 metabolism genes, 9 growth factors, 7 receptors, and 6 transcription/translation factors (Fig. 5). The categorizations were made based on the major cellular function of the gene. The 109 gene list was imported into Pathway Assist (Stratagene) to evaluate cell processes and signaling pathways affected by the 109 genes (Fig. 6). The number of arrows pointing to or from each cellular function box for this gene network indicates the connectivity (Asirvatham *et al.* 2006) and was used to determine major cell processes affected by the gene set. The resulting gene network indicated that proliferation, differentiation, and maturation (i.e. development) are all affected. Focal contact, RNA localization, and pathogenesis are also connected to the gene network (Fig. 6). A further analysis of 130 different known cellular and signaling pathways using GeneSpring KEGG pathways did not identify any specific pathways where multiple genes (>2) from the 109 list were involved (data not shown). Therefore, no specific pathways were identified to be influenced by multiple genes in the 109 list. In contrast, major cellular processes were influenced by the 109 list of genes, Fig. 6.

The list of 109 rat genes was compared with mouse genes regulated during E11.5, E12.5, and E14.5 of mouse gonadal development from a previously generated microarray database (Small *et al.* 2005; available at www.ncbi.nlm.nih.gov/geo). The 15 genes found in both the 109 rat list and identified in the mouse are noted with an 'S' in Table 2. The lack of some homologous transcripts on the mouse and rat chips limits the number of genes in both analyses. From the 109 rat gene list, 68 genes were present on the mouse microarray chip. A calculation of the random chance genes would overlap between the rat and mouse chips indicated that 0.75 transcripts may potentially randomly overlap. Therefore, the 15 genes identified significantly exceed any random overlap expected. Genes appearing in both the rat and mouse analyses can be considered good candidates for involvement in sex determination and testis development.

The 109 genes differentially regulated in the male throughout the developmental period were screened for potential SRY binding elements in promoters. The frequency of the SRY element to randomly appear is every 4096 bp. From the 109 gene list, 21 genes had searchable promoters. From these 21 genes, 13

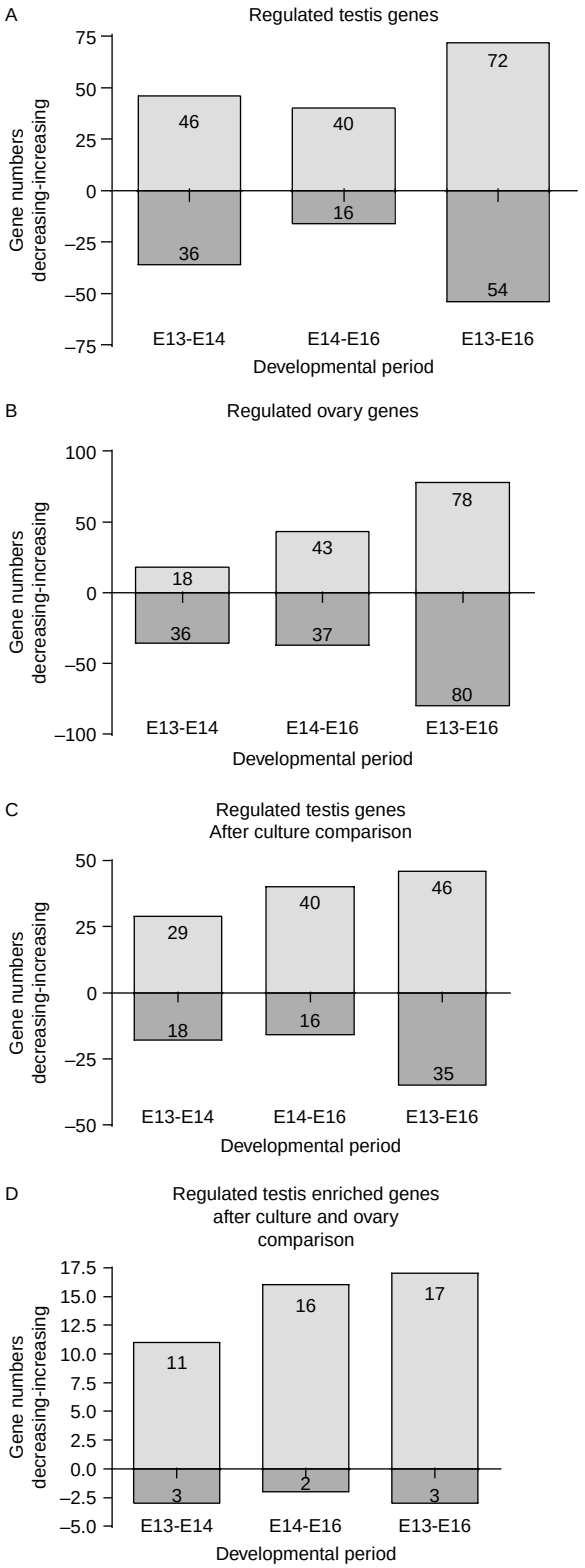

Figure 4 Numbers of regulated genes from testis and ovary developmental periods. (A) Numbers of genes expressed during testis development that have an expression signal of at least 75 and a statistically significant increase or decrease of 1.5-fold between each time interval are represented in a bar graph. (B) Numbers of ovary development genes with a signal of at least 75 and a statistically significant 1.5-fold increase or decrease. (C) The number of candidate genes for involvement in testis development and cord formation was reduced by comparison with E13 testis culture. Genes that are expressed above a raw signal of 75, have a 1.5-fold increase or decrease in the male time course and have similar patterns of expression changes from E13 to cultured testis as from E13 to E14 or E13 to E16. (D) Candidate testis development genes after subtraction of ovary expressed genes and comparison of testis organ cultures.

Table 2 List of 109 genes expressed in the rat testis from E13 to E16 with a signal of at least 75, a 1.5-fold or greater significant change, and with similar expression in E13 testis cultured for 3 days. Genes in bold are not in the ovary above a signal of 75 at E13, E14, or E16. Underlined genes were also differentially expressed between the sexes in at least one time point. An '*' denotes genes found to contain a potential SRY binding element in there promoter. An 'S' denotes genes identified in analysis of mouse data from Small *et al.* (2005).

Gene symbol	High exp.	Signal		GenBank	Description
Cytoskeletal and extracellular matrix					
Tgfbi	E13	1359		BG379319	Transforming growth factor β induced
Podxl	E13	468		AF109393	Podocalyxin-like
Tpm1	E13	223		AF370889	Tropomyosin-α
Epb4.1l3	E13	142		NM_053927	Erythrocyte protein band 4.1-like 3
Crtl1	E13	125		NM_019189	Cartilage link protein 1
Mmp16	E13	102	*	NM_080776	Matrix metalloproteinase 16
Sponf	E14	112	*	M88469	F-spondin
Col1a1	E16	1646		BBI285575	Collagen, type 1, α1
	E16	771		BI296340	Similar to cadherin 11
	E16	530		BM389149	Immunoglobulin superfamily/RNase inhibitor
	E16	505	S	AI177055	Similar to myosin, light polypeptide kinase
Bgn	E16	477		NM_017087	Biglycan
	E16	302	S	AI176126	Similar to procollagen, type VI, α 3
	E16	292		BM384071	Tubulin, *β2*
	E16	252		BG672591	Plastin 3 (T-isoform)
	E16	237		BM391858	Similar to dynein, axonemal, heavy polypeptide 11
	E16	203		BF406693	Similar to Laminin α-4 chain precursor
	E16	183		AI008689	Similar to transforming acidic coiled-coil containing protein
	E16	170		BE110691	Similar to EH-domain containing 2
Tagln	**E16**	**148**	**S**	**NM_031549**	**Transgelin**
	E16	123	S	BI296640	Similar to Epsin 2
	E16	108		BI287851	Similar to procollagen, type VI, α 2
Ril	**E16**	**103**		**NM_017062**	**Reversion-induced *LIM* gene**
Signaling					
Bambi	E13	512		AF387513	BMP and activin membrane-bound inhibitor
Gucy1b3	E13	237	*	NM_012769	Guanylate cyclase 1, β3
Gucy1b3	**E13**	**232**	*	**BF399387**	**Guanylate cyclase 1, soluble, β 3**
Ren1	E13	183	*	J02941	Renin 1
	E14	88		BG371889	Similar to phosphodiesterase 6G, cGMP-specific
	E16	502		AI639128	Similar to wingless-type MMTV integration site 5A
Sfrp4	**E16**	**241**	*	**AF140346**	**Secreted frizzled-related protein 4**
	E16	237		BF287964	Similar to annexin A11
Socs2	**E16**	**224**		**BM384088**	**Similar to suppressor of cytokine signaling 2**
	E16	151	S	AI408442	Similar to deltex 4 homolog
	E16	**142**		**BI296275**	**Similar to monocyte to macrophage differentiation-associated 2**
Pawr	**E16**	**136**	*	**U05989**	**PRKC, apoptosis, WT1, regulator**
Wnt5a	**E16**	**134**	*	**NM_022631**	**Wingless-type MMTV integration site 5A**
	E16	129		AI406490	Similar to tyrosine kinase, non-receptor, 2
	E16	126		BF283621	Similar to Ras GTPase-activating-like protein
Sh3kbp1	**E16**	**118**		**AF230520**	**SH3-domain kinase-binding protein 1**
	E16	**108**		**AI178741**	**Similar to PRA1 family 2 (mouse)**
	E16	**101**	**S**	**AI071649**	**Similar to adenylate cyclase 7**
	E16	**91**		**BE112895**	**Similar to phosphoprotein enriched in astrocytes 15**
Dusp6	**E16**	**85**		**AA957292**	**Dual specificity phosphatase 8**
	E16	**85**		**BI277482**	**Similar to MAP kinase interacting kinase**
Metabolism					
	E13	286		BI284270	Similar to glucan (1,4-α-), branching enzyme 1
	E13	228		AA799700	Similar to selenophosphate synthetase 2
	E13	221		BI289467	Similar to expressed in non-metastatic cells 4, protein
	E13	170		BF420664	Similar to ubiquitin-specific protease 29
Pppr2b2	E13	141	S	NM_022209	Protein phosphatase 2 (formerly 2A)
Slc2a3	E13	93		AA901341	Solute carrier family 2, member 2
Nedd4a	E14	873		BG379338	Similar to ribonucleotide reductase M2
	E16	468		AI411530	Similar to aminoacylase 1
	E16	414		AI227941	Similar to tumor-related protein
	E16	232		BG673187	Similar to four and a half LIM domains 1
	E16	**169**	**S**	**BI289085**	**Similar to vanin 1**
Enpep	**E16**	**157**		**AF214568**	**Aminopeptidase A**
Gatm	**E16**	**134**	**S**	**NM_031031**	**Glycine amidinotransferase**
	E16	128		BF406832	Similar to leprecan-like 2
Tm6p1	E16	119		NM_139107	Fasting-inducible integral membrane protein
	E16	100		AI412948	Similar to ataxin 7-like 3

Table 2 *(Continued).*

Gene symbol	High exp.	Signal		GenBank	Description
Growth factor					
Bmp4	E13	137	*	NM_012827	Bone morphogenetic protein 4
	E13	115	S	BG671943	Similar to ephrin A5
	E16	647		BG375362	Similar to latent transforming growth factor β-binding protein 4
	E16	**239**		**AI170324**	**Similar to C-fos-induced growth factor (Figf)**
Nppc	**E16**	**184**	*	**NM_053750**	**Natriuretic peptide precursor C**
Jag1	**E16**	**118**	*	**NM_019147**	**Jagged 1**
	E16	**117**	**S**	**BG664221**	**Similar to osteoglycin**
Tgfb3	**E16**	**102**	*	**NM_013174**	**Transforming growth factor, β 3**
Nrg1	**E13**	**78**		**U02319**	**Neuregulin 1**
Receptor					
Grinl1a	E13	300		BG664035	Glutamate receptor, ionotropic
Gpr48	E13	285	S	BI300274	G-protein-coupled receptor 48
	E13	196		AI072459	Similar to Eph receptor A4
Itpr1	E13	185	*S*	J05510	Similar to inositol 1,4,5-triphosphate receptor 1
Cxcr4	E13	128		AA945737	Chemokine receptor (LCR1)
Lrp4	E13	85		AI070976	Low-density lipoprotein receptor-related protein 4
	E14	123		BI275605	Similar to unc-5 homolog B
Transcription and translation					
Egr1	E13	171		NM_012551	Early growth response 1
Hoxa5	E13	163		BE107303	Similar to Homeo box A5
	E14	147		BE107296	Ribosomal protein S6 kinase polypeptide 6
	E16	319		AW253720	Nuclear factor I/B
	E16	135		AI409308	Max interacting protein 1-negative reg. of cell proliferation
Nupr1	**E16**	**91**	*	**NM_053611**	**Nuclear protein 1**
Ion transport					
Scn4b	E13	313		AI137995	Sodium channel, voltage-gated, type IV, β
Lgals9	E13	107		U72741	Lectin
Nme7	E16	345		AI232036	NME7
Other/unknown					
	E13	851		AI599621	EST251324 cDNA clone REMEH36
	E13	566		AA956417	*Rattus norvegicus* transcribed sequences
	E13	246		AI175861	*Rattus norvegicus* transcribed sequences
	E13	222	S	AA924756	Similar to ES neuronal differentiation 2
	E13	167		BI294768	Hypothetic protein–DNA-binding domain
	E13	143		AW529759	*Rattus norvegicus* transcribed sequences
	E13	**104**		**AA799470**	***Rattus norvegicus* transcribed sequences**
	E13	**88**		**AI236229**	**Similar to RNA binding motif protein 24**
	E13	**77**	**S**	**AI178384**	**Similar to kelch-like 6**
	E14	172		AA860014	Similar to hemoglobin: SUBUNIT=ζ
	E14	**92**		**AI410969**	**Hypothetical protein MGC27854**
	E14	**76**		**AI101385**	***Rattus norvegicus* transcribed sequences**
A5D3	E16	1514		AY007690	A5D3 protein
	E16	203		AI102758	Transcribed sequence-similarity to ref:NP_080909.1
	E16	**183**		**BE103235**	***Rattus norvegicus* transcribed sequences**
	E16	**178**		**AI009714**	**Similar to serum deprivation response**
	E16	157		AA891255	Hypothetic protein
	E16	147		BM388789	*Rattus norvegicus* transcribed sequence
	E16	147		BI281129	Unknown
	E16	**138**		**BI274243**	**Transcribed sequence-similarity**
	E16	112		AI410305	*Rattus norvegicus* transcribed sequences
	E16	111		AI716904	Predicted hypothetical protein
	E16	**79**		**BE099060**	**Hypothetical protein LOC311430**
Pak3	**E16**	**75**		**NM_019210**	**P21 (CDKN1A)-activated kinase 3**

promoters were found to contain putative SRY binding elements and are marked with an asterisk in Table 2. Although the number of genes identified with potential SRY elements is in part due to random appearance of the SRY element, the lack of an element likely precludes direct binding and regulation by an HMG box protein such as SRY. Therefore, the presence of an SRY element simply indicates the potential for regulation, but does not indicate functional relevance.

Genes with a raw signal above 75 in any female samples (E13, E14, or E16) were removed from the list of the 109 genes to reveal regulated transcripts with enriched expression in the testis versus the ovary. This resulted in 34 genes that were differentially regulated

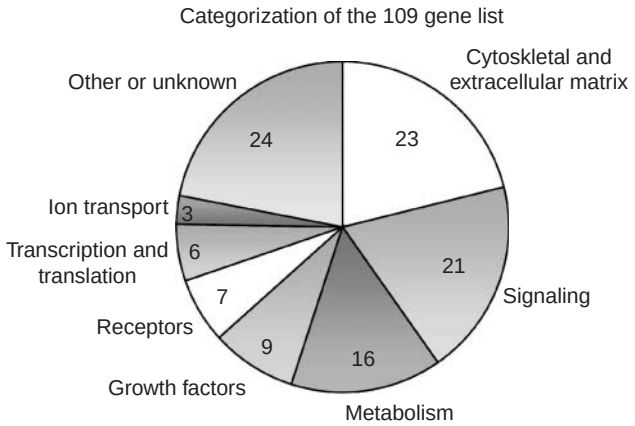

Figure 5 Functional categorization of the 109 gene list. The number of genes in each functional category of the 109 gene list of male-enhanced transcripts regulated in testis development.

between E13, E14, and E16 in the testis and enriched in the male rat gonad (Fig. 4D). These 34 genes are indicated in bold in Table 2. A similar reduction of the 175 genes expressed above a signal of 75 and with a 1.5-fold change in the ovary resulted in no gene candidates that were both differentially regulated between E13, E14, and E16 in the ovary and female enriched.

To determine whether any expressed genes were female enriched, genes expressed in male and female gonads above a given microarray signal at any time point were determined. This was done for raw microarray signal cut-off values of 75, 100, 150, 200, 250, and 500 (Fig. 7A). Genes expressed above a signal of 75 in the opposite sex were then subtracted to find the number of gender-enriched genes at each raw signal cut-off value (Fig. 7A). The numbers of genes that are female enriched at any cut-off value during this developmental period are fewer than those that are male enriched. Genes enriched above a signal value of 75 represent the portion of genes form the Venn diagrams in Fig. 3 that are gender-enriched. Genes enriched in the male and female above 100 are listed in Supplementary Table 2, which can be viewed online at www.reproduction-online.org/supplemental/. A cut-off of 100 was used for Table S2 due to the large number of genes present above 75, which would make a prohibitively lengthy list.

Genes that are differently regulated between the sexes at a single time point were identified (Fig. 7B) to allow comparison with previously reported mouse studies (Nef *et al.* 2005, Beverdam & Koopman 2006). Genes with a raw microarray signal of at least 75 and

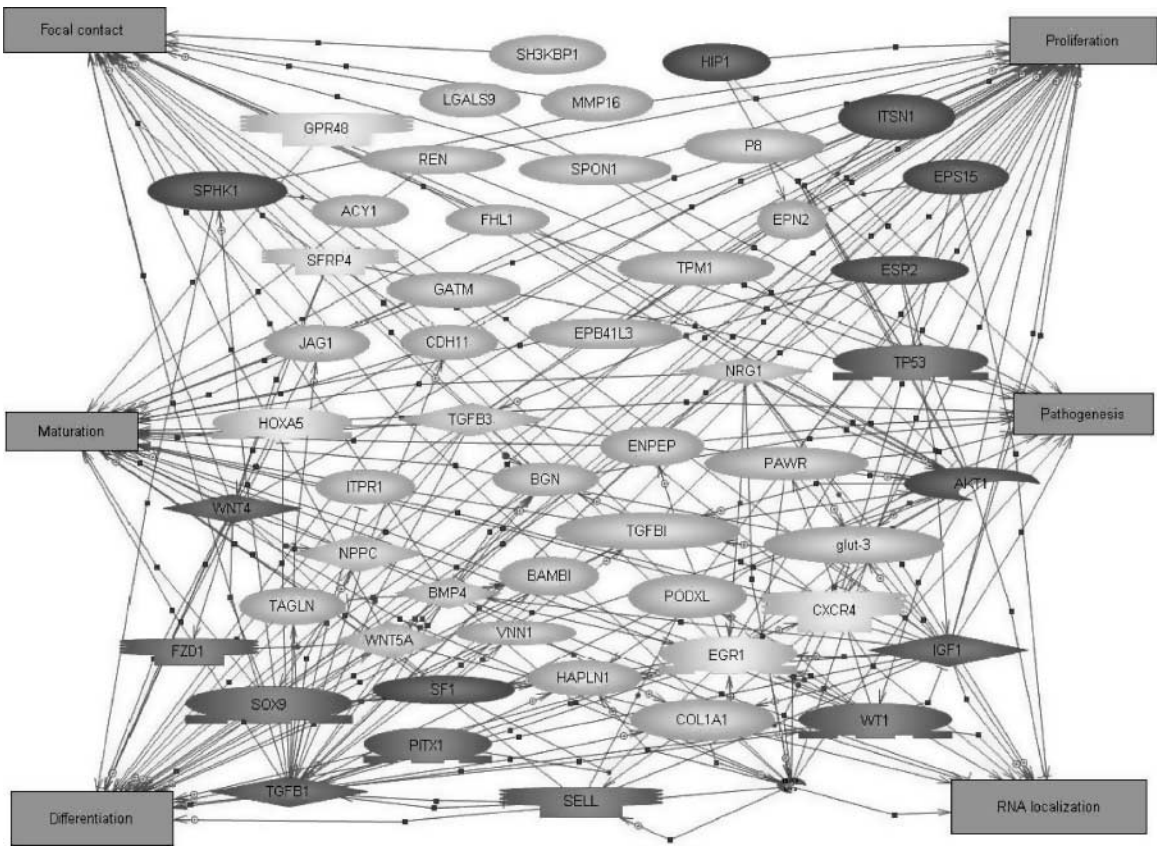

Figure 6 Functional gene network analysis of testis development genes. The 109 gene list was analyzed by Pathway Assist. Cell processes involved in testis development were determined based on the number of arrows connected to each box (connectivity). Rectangles are the cellular processes, light shaded shapes are a subset of the 109 list, and dark circles represent interconnecting proteins not on the 109 list.

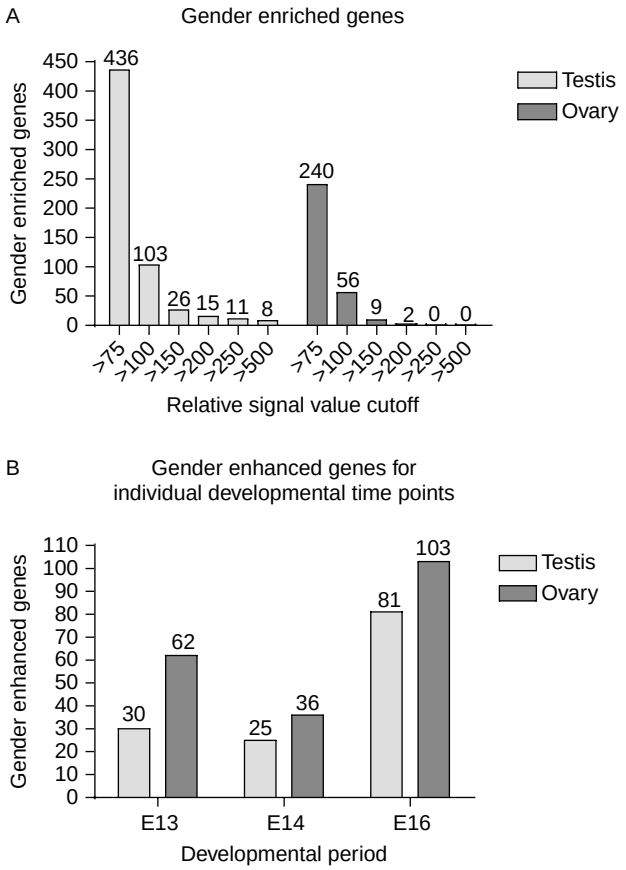

Figure 7 The number of gender-enriched (A) enhanced (B) transcripts between E13 and E16 in the testis and ovary. (A) All genes gender-enriched in the male and female time courses with given signal cut off values. Genes expressed above a signal of 75 in any time point of one sex were removed from lists of genes above a specified signal cut-off in at least one time point of the other sex to obtain the number of gender-enriched genes for a given signal cut off. (B) Numbers of gender-enhanced genes at a single given time point with at least a 1.5-fold change between the sexes were determined.

a statistically significant 1.5-fold increase between testis and ovary were identified at each time point individually (Fig. 7B). Genes identified in this analysis will be referred to as gender-enhanced to distinguish them in the discussion herein from the enriched genes represented in Fig. 7A. For each time point, more genes were over-expressed in the female than in the male. There were 62 transcripts found with an expression 1.5-fold higher in the female than in the male at E13, and 30 transcripts with 1.5-fold higher expression in the male at E13. At E14, 36 transcripts had enhanced expression in the female and 25 in the male. At E16, 103 transcripts had enhanced (> 1.5-fold increase) expression in the female and 81 had enhanced expression in the male. A combined total of 316 genes were differentially regulated between the sexes at a given time point (E13, E14, or E16; Supplementary Table 3, which can be viewed online at www.reproduction-online.org/supplemental/). The list of 109 genes regulated in the male between E13, E14, and E16 (Table 2) was then compared with the 316 genes differentially regulated between the sexes at a single time point (Supplementary Table 3). The 33 genes in both lists are underlined in Table 2 and indicate genes regulated between the sexes and regulated over the developmental period of E13, E14, and E16 in the testis.

Comparison of differentially regulated genes for individual developmental time points between the sexes identified 316 total genes (Fig. 7B and Supplementary Table 3). A direct comparison of genes differentially regulated between the sexes in the rat at E13, E14, and E16 was made to lists of genes differentially regulated between sexes in the mouse at E10.5, E11.5, and E12.5. A list of genes generated in a similar manner for the mouse at individual developmental time points (Nef *et al.* 2005) was used for comparison, revealing 19 genes differentially regulated between the sexes in both the mouse data and rat data marked with an 'N' (Table 3). A similar comparison was made between the rat and the mouse data generated by Beverdam & Koopman (2006). Genes found to be differentially expressed in the rat between the sexes, as shown in Fig. 7B, or changing throughout the developmental period for testis or ovary, as shown in Fig. 4C and B respectively, were compared with published lists of genes regulated in mouse gonadal sex determination (Beverdam & Koopman 2006). This revealed 17 conserved genes between the Beverdam & Koopman mouse data and the rat data from the present study marked with a 'B' (Table 3). Previous comparison of the rat 109 list to mouse data by Small *et al.* (2005) revealed a total of 15 genes conserved and marked with an 'S' in Table 2. Three of these genes from the Small *et al.* (2005) mouse comparison also appear in the comparisons with the Nef *et al.* (2005) or Beverdam & Koopman (2006) mouse data and are also marked with an 'S' in Table 3. Genes identified as being regulated in the mouse and rat during this developmental period of sex determination and gonadal development appear to be conserved in sex determination and are potential candidates for further consideration. A complete list of candidate genes was compiled and is shown in Table 4. Genes included in the list had to appear in at least two of the comparative analysis lists, one of which had to be the novel rat list from the present study. The rat and mouse lists in which each gene appeared are indicated in Table 4. The candidate regulatory gene list contains 36 candidate genes including 25 potentially conserved between rat and mouse, and 23 genes in three or more lists (Table 4). In addition, 11 novel genes were identified in the present study and are also listed. The present study presents this list of candidate genes in gonadal sex determination for both testis and ovary development that are conserved between rodent species.

Table 3 List of genes conserved in the rat and mouse. Genes present in the Nef *et al.* 2005 (N) or Beverdam & Koopman (2006) (B) or Small *et al.* (2005) (S) studies are indicated. The time in the mouse and rat that the gene is expressed (Exp) differently between the sexes is indicated for mouse and rat. Genes in bold also appear in the 109 list.

Study pools	Mouse exp.	Rat exp.	Name	GenBank	Description
Testis					
N, B	**E11.5**	**E16**	***A5D3***	**AY007690**	**A5D3 protein**
N, B	**E11.5**	**E16**		**BI296275**	**Monocyte-macrophage differentiation**
N	**E12.5**	**E16**	*Tcf21*	BE113336	Transcription factor 21
N	**E12.5**	**E16**		AI227742	Bcl-2-related ovarian killer protein
N	**E12.5**	**E16**		AI179988	Ectodermal-neural cortex 1
N, B	**E12.5/11.5**	**E14, E16**	*Gatm*	NM_031031	Glycine amidinotransferase
N	E12.5	E16, E13	*Ednra*	NM_012550	Endothelin receptor type A
N, S	**E12.5**	**E16**	***Jag1***	**NM_019147**	**Jagged 1**
N, S	**E12.5**	**E14**	***Gatm***	**NM_031031**	**Glycine amidinotransferase**
N	E12.5	E13		AI233246	Insulin-like growth factor binding protein 7
B	E10.5	E13, E16		AI410924	*Rattus norvegicus* transcribed sequences
B	E11.5	E14		**B1296340**	***Rattus norvegicus* transcribed sequences**
B	E11.5	E13–E16		**BG664221**	**Transcribed sequence similar to osteoinductive factor**
B	E11.5	E13–E16		AI103641	*Rattus norvegicus* transcribed sequences
B	E11.5	E13–E16		**BM384088**	**Transcribed sequence similar to** β
Ovary					
NSB	E11.5	E13	*Fst*	NM_012561	Follistatin
N	E12.5	E16	*Amhr2*	NM_030998	Anti-Müllerian hormone type 2 receptor
N	E12.5	E16	*Axin2*	BF398114	Axin2
N	E12.5	E16		AI172116	Zinc finger protein 672
N	E12.5	E14	*Igfbp2*	NM_013122	Insulin-like growth factor binding protein 2
N	E12.5	E14	*Enpp2*	NM_057104	Ectonucleotide pyrophosphatase/phosphodiesterase 2
N	E12.5	E14		BI291872	Solute carrier family 37 member 2
N	E12.5	E13		AI412658	Fibroblast growth factor receptor 2
N	E12.5	E13	*Wnt4*	NM_053402	Wingless-type MMTV integration site family, member 4
B	E11.5	E13, E14	*Klk1*	NM_012593	Kallikrein 1
B	**E11.5**	**E14, E16**	***Cxcr4***	**AA945737**	**Chemokine receptor (LCR1)**
B	E11.5	E16		AI102517	*Rattus norvegicus* transcribed sequences
B	E11.5	E16	*Msx1*	NM_031059	Homeo box, msh-like 1
B	E11.5	E16		BG374285	*Rattus norvegicus* transcribed sequences
B	E11.5	E13–E16	*Ifi27l*	NM_130743	Interferon, α-inducible protein 27-like
B	E11.5	E13–E16		AI172218	Simlar to RIKEN Cdna 2810002N01
B	E11.5	E13–E15	*Bzrp*	AI008680	Benzodiazepin receptor

Discussion

The present study used a microarray analysis to identify new potential candidate genes for rat sex determination and differentiation. Whole gonads were used such that all the different somatic cells and germ cells are present. Clearly, changes in the individual cell type transcriptomes will contribute to the total gonadal transcriptome and, as such, correlations of the present data to individual cell types must be made with caution. The present analysis used two different samples and two different microarray chips to obtain the individual data points. Criteria to assess chip number has been previously described (Chen *et al.* 2004). Previous studies have demonstrated two chips for each data point, assuming an $R^2 > 0.95$ and confident $P < 0.05$, provide a critical and statistically relevant analysis (McLean *et al.* 2002, Kezele *et al.* 2005, Small *et al.* 2005). The reproducibility of the different microarrray chips used in this study was assessed as variance between samples and had an $R^2 > 0.96$ for each dataset.

Genes known to be involved in sex determination had patterns of expression that match what is known from previous literature (Table 1). Some of these genes were eliminated from the candidate male development lists due to the stringency of the analyses. For example, FGF9 and WT1 do not exhibit the 1.5-fold statistically significant change in expression parameter used in the study. The elimination of some potentially important genes allowed for a stringent selection of candidate genes. Analysis of genes expressed with a raw signal of at least 75 revealed that approximately half the genes on the rat RAE230A chip were considered present in the embryonic testis and ovary. Approximately 2% had a 1.5-fold statistically significant change in the male and female E13, E14, and E16 developmental periods. The list of 160 genes with a 1.5-fold change in the male was reduced to 109 genes by comparison with the gene expression levels from the 3-day cultured embryonic testis. This narrowed the list of genes to be considered as candidates critical for testis development and cord formation by ∼32%. The list of subtracted genes is

Table 4 Final compiled candidate list of genes for involvement in sex determination and gonadal development. Genes were selected based on the number of rat analysis (rat lists) and mouse lists they appeared in and potential function. 109 and 316 refer to gene lists in Table 2 and Supplementary Table 2 respectively.

Name	High exp.	Signal	Rat lists	Mouse lists	GenBank	Description
Testis development candidates						
A5D3	E16	1514	109,316	NB	AY007690	A5D3 protein
	E16	840	316	N	AI233246	Insulin-like growth factor binding protein 7
Sfrp4	E16	241	109,316,M,*	B	AF140346	Secreted frizzled-related protein 4
Socs2	E16	224	109, M	N	BM384088	Similar to suppressor of cytokine signaling 2
	E13	222	109,316	S	AA924756	Similar to ES neuronal differentiation 2
Itpr1	E13	185	109,316	S	J05510	Inositol 1,4,5-triphosphate receptor 1
Cxcr4	E16	178	109,316	B	AA945737	Similar to chemokine receptor (LCR1)
	E16	169	109,M	S	BI289085	Similar to vanin 1
	E16	151	109,316	S	AI408442	Similar to deltex 4 homolog
	E16	142	109,316,M	NB	BI296275	Similar to monocyte to macrophage differentiation-
Ednra	E16	126	316	NS	NM_012550	Endothelin receptor type A
Jag1	E16	118	109,316,M,*	N	NM_019147	Jagged 1
	E16	117	109,M	SB	BG664221	Similar to osteoglycin
	E16	106	316	B	AI410924	*Rattus norvegicus* transcribed sequences
	E16	101	109,316,M	NS	AI071649	Similar to adenylate cyclase 7
	E16	239	109,316,M		AI170324	Similar to C-fos induced growth factor (Figf)
Nppc	E16	184	109,316,M,*		NM_053750	Natriuretic peptide precursor C
Pawr	E16	136	109,316,M,*		U05989	PRKC, apoptosis, WT1, regulator
Wnt5a	E16	134	109,316,M,*		NM_022631	Wingless-type MMTV integration site 5A
	E14	123	109,316		BI275605	Similar to unc-5 homolog B
Tgfb3	E16	120	109,M,*		NM_013174	Transforming growth factor, β 3
	E16	91	109,316,M		BE112895	Similar to phosphoprotein enriched in astrocytes 15
Nupr1	E16	91	109,M,*		NM_053611	Nuclear protein 1
Dusp6	E16	85	109,316,M		AA957292	Dual specificity phosphatase 8
Nrg1	E13	78	109,316,M		U02319	Similar to neuregulin 1
Pak3	E16	75	109,M		NM_019210	p21 (CDKN1A)-activated kinase 3
Ovary development candidates						
Igfbp2	E16	1478	316	N	NM_013122	Insulin-like growth factor binding protein 2
Amhr2	E14	964	316	N	NM_030998	Anti-Müllerian hormone type 2 receptor
Fst	E16	406	316	NSB	NM_012561	Follistatin
	E14	332	316	N	AI412658	Similar to fibroblast growth factor receptor 2
Wnt4	E16	249	316	N	NM_053402	Wingless-type MMTV integration site family, member 4
Enpp2	E16	233	316	N	NM_057104	Ectonucleotide pyrophosphatase/phosphodiesterase 2
Klk1	E13	219	316	B	NM_012593	Kallikrein 1
	E14	135	316	N	BI291872	Similar to solute carrier family 37 member 2
Msx1	E16	130	316	B	NM_031059	Homeo box, msh-like 1
	E16	128	316	B	BG374285	*Rattus norvegicus* transcribed sequences

M indicates male-enhanced genes from Table 2. An asterisk indicates genes with an SRY element in the promoter. S, Small *et al.* (2005) data; N, Nef *et al.* (2005) data; B, Beverdam & Koopman 2006 data.

presented in Supplementary Table 1. Since the cultured embryonic E13 testis undergoes cord formation and testis differentiation similar to *in vivo*, those genes regulated both *in vitro* and *in vivo* are assumed to be important. Although potential subtraction of important genes is a limitation, selection of regulated genes present from the *in vivo* and *in vitro* studies is likely to identify candidate genes for cord formation. The subtraction of genes expressed in the ovary above a raw signal of 75 from the 109 gene list revealed 34 male-enriched genes. In contrast, the subtraction of genes expressed above a signal of 75 in the male from the 175 ovary gene list yields no female-enriched genes. Therefore, the stringent subtraction analysis used revealed no ovary-enriched

genes that were regulated during the E13–E16 developmental period.

Increased transcriptional changes occur in the male between E13 and E14, while major changes in the female were delayed and occur between E14 and E16. When considering genes enriched in each sex for a given signal cut off, Fig. 7A, more genes appear to be enriched in the testis. The increased number of male-enriched genes suggests that there is an active female developmental process, but a reduced number of unique transcripts at this point in female development. These observations support the concept that it is the male process of development which requires expression of unique transcripts for testis determination. In contrast, when looking at the number of transcripts differentially regulated between the sexes at a single time point, as was done in a previous mouse analysis (Nef *et al.* 2005) and shown in Fig. 7B, there appear to be more female-enhanced genes than male. At only a single time point, a sex-enhanced gene may be present due to up- or downregulation in that sex. Therefore, it is not until gene expression is considered in the context of the developmental time course that it can be determined in which sex it is regulated and enhanced. The 33 genes identified in the 109 list of genes and in the 316 list of genes gender enhanced are particularly interesting candidates for further consideration in male sex differentiation and development. The 33 gene list includes 10 out of the 21 signaling factors, 4 out of the 9 growth factors, and 3 out of the 7 receptors in Table 2. These genes are interesting because they are potential regulatory gene candidates and warrant further investigation. The 33 gene list does not include many cytoskeletal and extracellular matrix factors despite the fact that this is the largest category in the 109 list. Signaling is the second largest represented functional gene category in the 109 gene list and the largest category represented by genes also testis-enriched or -enhanced. Genes appearing in this category such as *Tgfb3, Sfrp4*, and *Jag1* suggest involvement of specific signaling pathways in testis development. There appears a high level of connectivity of these genes with other genes of interest in a gene network built from the 109 list (Fig. 6). These pathways have the potential to influence gonadal differentiation. A thorough pathway analysis of over 130 different cellular and signaling pathways revealed no specific pathways where multiple (>2) genes within the 109 list are present. Clearly, a large number of critical cellular processes are influenced (Fig. 6); however, subsets of grouped genes involved in specific pathways were not identified. The assumption that defined specific pathways involving multiple-regulated genes are needed for male sex determination is questioned and instead observations suggest that a larger number of cellular processes influenced by a smaller number of different genes are likely. The concept that a smaller group of genes that influence larger gene networks is critical for development and disease etiology has been suggested (Schadt *et al.* 2005).

Recently, three studies have investigated genome wide transcriptional regulation of sex determination in the mouse using multiple time points (Nef *et al.* 2005, Small *et al.* 2005, Beverdam & Koopman 2006). Several others have also been done for selected genes and developmental periods (Koopman & Koopman 2002, Smith *et al.* 2003). The study by Small *et al.* (2005) focused on transcriptional changes throughout the gonadal developmental period, while the study by Nef *et al.* (2005) focused on changes between the sexes at E10.5, 11.5, 12.5, and 13.5 separately. The Beverdam study (Beverdam & Koopman 2006) examined changes between the sexes and between E10.5 and E11.5 in mouse. These studies were able to identify genes that were regulated early in gonadal differentiation in a sex-enhanced manner. The gonadal development and transcriptional processes in mouse and rat are similar. Investigation of the gonadal transcription during sex determination in the rat and comparison with the mouse allowed identification of genes conserved in mammalian gonadal sex determination.

The list of candidate genes involved in sex determination and gonadal development was compiled from genes identified in the present rat study (Table 4). The majority of the genes in this list also appeared in one of the mouse studies used for comparison. There was a subset of 11 genes that appear in several of the analyses performed on the rat data and did not appear in the mouse analyses. These genes may be novel in rat gonadal development, but the absence of some on the mouse microarray chips must be considered. The 36 genes in this candidate list (Table 4) had 15 genes conserved in mouse and rat testis development. To confirm the validity of the approach used in the present study, several genes known to be essential for testis development were also identified. *VANIN1*, *A5D3*, and *IGFBP7* were all identified as significant candidate genes using this genomic approach. VANIN1 is a membrane-linked protein that has been shown to be expressed in a sex-specific manner in the developing testis (Wilson *et al.* 2005). A5D3 is highly expressed in the testis and contains potential leucine zipper and phosphorylation sites (Blomberg *et al.* 2002). A5D3 has also been found in a study of vitamin A deficient synchronized testis where it was called VAD4 (Luk *et al.* 2003). Insulin-like growth factor binding protein 7 (IGFBP7) belongs to a family of proteins that regulates IGF function. Interestingly, it has been shown that IGF receptors are required for testis development (Zhou & Bondy 1993, Nef *et al.* 2003).

A developmental signaling pathway suggested to have a role in sex determination is the WNT signaling pathway (Kim *et al.* 2006). WNTs are secreted proteins implicated in cell growth, migration, and differentiation. Secreted frizzled related protein 4 (SFRP4) is a secreted lipoprotein receptor complex similar to the frizzled

receptors in the WNT signaling cascade, but without the transmembrane activation domain (Hewitt *et al.* 2006). SFRP4 is thought to antagonize cell survival and inhibit WNT signaling by binding WNT without activating its signaling cascade. SFRP4 has been implicated in placental growth and ovulation (Drake *et al.* 2003, Hewitt *et al.* 2006). *Wnt5a* also appears on the candidate gene list. WNT5A has been shown to activate signaling cascades in a manner dependant on the receptor to which it binds (Mikels & Nusse 2006). A potential role for WNT5A and SFRP4 in gonadal development is yet to be determined and the present observations support a role of the WNT signaling pathway in sex determination.

Another developmental signaling pathway potentially involved in sex determination involves the notch pathway. JAGGED1 is a notch signaling ligand involved in cell fate decisions (de La Coste & Freitas, 2006), and has been found in mouse testis where it may function in cell fate decisions during spermatogenesis (Dirami *et al.* 2001). DELTEX4 homolog belongs to a family of proteins originally identified in *Drosophila* able to interact with the ankrin repeats in notch to mediate notch signaling (Ordentlich *et al.* 1998, Kishi *et al.* 2001). Identification of these transcripts in the present study suggests that the notch pathway may be involved in male sex determination.

A number of other signal transduction-related genes were present in the candidate list, but did not group to specific pathways. Suppressor of cytokine signaling 2 is an inhibitor of JAK/STAT signaling (Leung *et al.* 2003, Leroith & Nissley 2005). Inositol 1,4,5-triphosphate receptor 1 has an intrinsic calcium ion channel opened in response to $InsP_3$ (Mignery *et al.* 1990). CXCR4 is a G-protein-coupled chemokine receptor with a c-x-c motif (Habasque *et al.* 2002, Khan *et al.* 2005, Smith *et al.* 2005). Endothelin receptor type A is also a G-protein-coupled receptor expressed in Sertoli and peritubular myoid cells (Ergun *et al.* 1999). Osteoglycin is a keratan sulfate proteoglycan (Iozzo & Murdoch 1996, Osawa *et al.* 2006). Adenylate cyclase 7 catalyzes conversion of ATP to cAMP (Suzuki *et al.* 1998). These genes affect a number of different signal transduction pathways and are likely important for sex determination.

There are 11 genes proposed as testis development candidates that were not found in the mouse studies. Although these 11 genes did not cross rodent species analysis, they display a high probability of significant impact on sex determination in the rat, and hence require further scrutiny. This includes the c-fos-induced growth factor, a secreted factor involved in cell growth and morphogenesis (Orlandini *et al.* 1996). NPPC is the precursor protein for CNP, which activates a Gi signal cascade (Hobbs *et al.* 2004, Anand-Srivastava 2005). Unc-5 homolog B is a netrin receptor that plays a role in morphogenesis of the vascular system (Lu *et al.* 2004) and oligodendrocyte precursor motility (Jarjour *et al.* 2003). PAWR is a pro-apoptotic protein containing a leucine zipper and death domain, and is important for cell sensitization to apoptotic stimuli (Boosen *et al.* 2005). Transforming growth factor (TGF)β3 is a growth factor that is a growth inhibitor and present in the testis and involved in spermatogenesis (Jarjour *et al.* 2003, Lui *et al.* 2003). Nuclear protein 1 (NUPR1 or p8) is an HMG-I/Y like protein that functions as a transcriptional regulator (Hoffmeister *et al.* 2002). Neuregulin 1 is a critical signaling factor in cell–cell interactions (Falls 2003). The p21 activated kinase (PAK3) is a serine–threonine kinase involved in apoptosis (McPhie *et al.* 2003, Boda *et al.* 2004). A phosphoprotein enriched in astrocytes and a dual specificity phosphatase are also included in the testis development candidate list. Although these 11 candidate rat genes did not appear on the mouse lists, individually they are viable candidates for roles in sex determination due to the correlations observed in the rat data provided.

There are ten candidate genes listed for ovarian development in Table 4. Follistatin and WNT4, known to be involved in ovarian development (Schneyer *et al.* 2004, Yao *et al.* 2004, Yao 2005), appear on the ovary development candidate list. These observations help validate the experimental approach and this ovary list. IGFBP2 is involved in growth inhibition in fetal development, and is abundant in Leydig cells (Wang *et al.* 1994, Schneyer *et al.* 2004, Terrien *et al.* 2005, Yao 2005). AMH receptor 2 (AMHR2) is known to bind AMH to promote Müllerian duct regression in the developing male, and to negatively regulate postnatal Leydig cell differentiation (Jamin *et al.* 2002, Mendis-Handagama *et al.* 2006). A role in the fetal ovary has not been identified. FGF receptor 2 is a receptor tyrosine kinase. A role for FGFR2 in male sex determination has been identified (Schmahl *et al.* 2004), however, a role in female development has not. ENPP2 (also known as autotaxin) promotes cell motility, angiogenesis, and myelination (Moolenaar 2002). Kallikrein 1 is a secretory serine protease (Clements *et al.* 1994). Solute carrier family 37 member 2 is homologous to the GlpT antiport sn-glycerol 3-phosphate transporter family in bacteria (Bartoloni *et al.* 2000). *Msx1* is a homeobox gene (Blin-Wakkach *et al.* 2001, Ramos & Robert 2005). The genes identified in Table 4 are candidates for involvement in female sex determination and early gonadal development and should be considered for further investigation.

This study characterizes transcriptional regulation of sex determination in the rat on a genomic scale and compares regulated genes in the mouse and rat. It has provided a resource for identifying candidate genes in mammalian sex determination. Profiles of expression of over 8000 genes present between E13 and E16 in the rat testis and ovary and transcripts expressed in cultured E13 testis are available (www.skinner.wsu.edu). These expression profiles provide information on genes regulated during the time of gonadal sex determination

and testis development in the rat and can be used in comparative studies with the mouse and other organisms to gain insight into potentially conserved regulatory mechanisms. A functional analysis of the identified candidate genes is now required to help elucidate their potential significance in gonadal sex determination and differentiation process. Observations from the present study suggest that a smaller group of genes regulated during sex determination may have a role in influencing a large number of different cellular processes (i.e. gene networks), rather than larger groups of genes specific to selected pathways. This genomic analysis of the gonadal transcriptome during sex determination has provided a global assessment of genes and pathways potentially involved in sex determination and gonad development.

Acknowledgements

We acknowledge the expert technical assistance of Mrs Michelle Schmidt and Dr Marina Savenkova, as well as the assistance of the Center for Reproductive Biology Genomics and Bioinformatics Core Laboratories and the expert assistance of Mr Derek Pouchnik. We thank Ms Jill Griffin and Ms Rochelle Pedersen for the assistance in preparation of the manuscript. The present address of Dr Mehmet Uzumcu, Department of Animal Science, Rutgers University, 84 Lipman Drive, New Brunswick, NJ 08901. This research was supported by a grant from NIH, NICHD to MKS. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received 22 November 2006
First decision 16 January 2007
Revised manuscript received 22 May 2007
Accepted 4 June 2007