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Using real time RT-PCR analysis to determine multiple gene expression patterns during XX and XY mouse fetal gonad development

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Abstract

New techniques are being applied to identify all the genes involved in mammalian gonad development and differentiation. As this list of genes increases, understanding the potential interactions between these genes will become increasingly difficult. We used a real time reverse transcription PCR (real time RTPCR) protocol to examine and compare the relative expression levels of 55 genes in individual mouse fetal gonads. Real time PCR analysis demonstrated that except for *Sry*, no differences in relative gene expression were detectable between XX and XY gonad/mesonephroi complexes at embryonic day (E)11.5. Following *Sry* peak expression at E11.5, a number of genes were expressed at significantly higher relative levels in E12–14 XY than XX gonads. Of six genes expressed at higher levels in E12.5–14 XX than XY gonads, three, *Bmp2*, *Emx2*, and *Fgfr2*, had not been reported previously. Our results caution that differential localization patterns observed with whole mount in situ hybridization techniques may not accurately reflect changes in transcript levels. We conclude that real time PCR is an efficient and powerful tool for studying multiple gene expression patterns during gonad development and differentiation, and can provide insight into gene interactions.

Keywords: Embryo; Testis; Ovary; Multi-gene platform; Gonad differentiation; Transcript; GPR

The discovery of the testis determining gene *Sry* in 1990 was an important first step towards elucidating the mechanism of gonadal sex determination and differentiation in mammals (Berta et al., 1990; Gubbay et al., 1990). Since then, additional genes have been identified that play important roles in establishment of the genital ridge and differentiation of normal testes and ovaries (reviewed in Scherer, 2002; and primary references therein). The discovery rate for new gonadal genes, however, has been limited because most genes were identified by chance. Humans or mice carrying a null mutation were found either lacking gonads or their sex chromosome complement did not match their gonadal phenotype (i.e. XY individual with ovaries) (reviewed Scherer, 2002; Sarafoglou and Ostrer, 2000).

New molecular techniques are being applied to more efficiently identify all the genes involved in gonad differentiation (McClive et al., 2003; Menke and Page, 2002; Nordqvist and Tohonen, 1997; Wertz and Herrmann, 2000). These techniques, including differential display, subtractive hybridization and high-throughput gene expression analysis, are generating a growing list of genes expressed preferentially in XX or XY fetal gonads. As of 2004 very little is known about the molecular pathways regulating gonad development for the limited number of genes that have been identified. As the list of genes increases, studying their interactions will become even more daunting.

One approach to investigate gene interactions in genital ridge development and/or gonadal sex differentiation is to compare gene expression levels during normal and impaired fetal gonad development. Real-time reverse transcription polymerase chain reaction (real time RTPCR) is a powerful tool for studying gene expression levels because it requires minimal amounts of RNA, is highly sensitive, allows multiple genes to be analyzed simultaneously, and can compare relative gene expression levels between different experimental groups (Schmittgen, 2001; and other references in this special topics issue). Real time PCR is widely used for quantification of transcript levels, but to date its

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application in monitoring gene expression levels during fetal gonad development and differentiation has been limited to single gene measurements (i.e. Sarraj et al., 2003). The aim of this study was to utilize a real time PCR protocol to quantitatively examine and compare the relative expression levels of multiple genes during female and male fetal gonad development. The DNA binding fluorescent dye SYBR Green was chosen for detection chemistry because it yields reproducible results and allows multiple genes to be analyzed simultaneously. Here we report the relative expression level of 55 genes (Table 1) during fetal gonad development in XX and XY mice.

1. Results and discussion

1.1. Primer validation

Because SYBR Green binding is not sequence specific, careful validation of the primer pairs was undertaken to ensure that only the target gene sequence was amplified. To verify the specificity of each primer pair, three requirements were fulfilled. First, a single gel band of the expected size was amplified for each primer pair when tested on a cDNA pool generated from embryonic and neonate XX and XY gonads. Second, the amplified PCR products corresponded to the target gene as determined by bidirectional DNA sequencing. Finally, a single PCR product and no primer dimers were generated by each primer pair as confirmed by dissociation curve analysis (data not shown).

1.2. Real time PCR results

Significant differences in relative gene expression level between XX and XY gonads were determined using two methods: (1) Student's *t*-test on 18s ribosomal RNA (18s rRNA) normalized data, and (2) the software algorithm global pattern recognition (GPR) program (Akilesh et al., 2003). The results are shown in Tables 2–4, and Fig. 1.

1.2.1. Gene expression in E10.5 and E11.5 samples

Midday a vaginal plug was detected and noted as embryonic day (E)0 for the developing embryos. More accurate individual embryonic age was determined by counting tail somites, and fore and hind limb morphology (Theiler, 1989). A list of genes expressed in XX and XY samples on E10.5 and E11.5 is presented in Table 2. Before E12, XY and XX gonads are morphologically indistinguishable and said to be 'undifferentiated' (Tilmann and Capel, 2002). *Emx2*, *Lhx9*, *Nr5a1* (*Sf1*), and *Wt1* transcripts were detected in E10.5 XX and XY samples. Previous studies have shown the importance of these genes in early gonad formation (Birk et al., 2000; Kreidberg et al., 1993; Luo et al., 1994; Miyamoto et al., 1997). No differences in relative gene expression levels were observed between XX and XY samples at E10.5 or E11.5, with the exception of *Sry* (Table 3). *Sry* expression in the XY fetal gonad was observed from E11.5 until E13, after which transcript levels fell below the detection limit. In a separate study, we detected *Sry* expression in E11 XY samples (data not shown), in agreement with previously published work by Hacker et al. (1995).

1.2.2. Gene expression in E12–14 XY gonads

From E12 through E14, when gonads and mesonephroi could be cleanly separated, a number of genes were expressed in XY gonads that were not expressed in XX gonads (Table 3). One example is *Amh*, a gene encoding a protein that induces regression of the internal female reproductive tract derivatives (Mullerian ducts) in male embryos. Munsterberg and Lovell-Badge (1991) detected *Amh* transcripts in XY gonads at E12.5, but not E11.5 using radioactive in situ hybridization. Our results indicate that *Amh* is expressed at E12.

As noted in Table 3, the genes encoding the steroidogenic enzymes *Cyp11a1* and *Cyp17a1* were expressed only in XY gonads. *Cyp11a1*, and *Cyp17a1* were first detected at E12.5. This is in agreement with published work by O'Shaughnessy et al. (1998) and moves the previous reported expression for these genes from E13 to E12.5 (Table 3). Both enzymes are part of the steroidogenic pathway leading to testosterone synthesis and suggest that fetal Leydig cell differentiation immediately follows *Sry* expression in the developing testis (Baker et al., 1999; Jeays-Ward et al., 2003; O'Shaughnessy et al., 1998). Early testosterone production by fetal Leydig cells is important for the stabilization of the Wolffian ducts and their differentiation into male internal genital ducts (reviewed in Hughes, 2001).

1.2.3. Comparison between genes expressed in E12–14 XX and XY gonads

At E12, several genes were identified that had significantly different relative expression levels between XX and XY gonads (Table 4A). These genes are of particular interest because they are the first genes differentially expressed immediately after peak *Sry* expression in XY gonads at E11.5, and before obvious morphological differentiation occurs at E12.5.

In agreement with previous studies, *Fgf9*, *Sf1*, and *Sox9* were expressed at higher levels in XY than XX gonads (Table 4A) (Colvin et al., 2001; Ikeda et al., 1996; da Silva et al., 1996). These genes are known to be critical for male gonadal sex differentiation.

A number of genes were expressed at similar transcript levels in E12–14 XX and XY gonads (data not shown). These genes include *Wt1* (*Wt1* + *KTS*, *Wt1* − *KTS*), *Dmrt1*, *Cdh11*, and *Wnt5a*. The developmental expression patterns observed for *Wt1* and *Dmrt1* confirm results reported by others (Armstrong et al., 1993; De Grandi et al., 2000). Similar gonad developmental expression data are unavailable for *Wnt5a*.

Table 1
Primer sequences for the 55 genes examined

Gene symbol	Name, synonym	GenBank accession number	Primer sequence (5′ to 3′ direction)	
			Forward	Reverse
18s rRNA	18s ribosomal RNA	X56974	GAGGCCCTGTAATTGGAATGAG	GCAGCAACTTTAATATACGCTATTGG
Aard	Alanine and arginine rich domain containing protein	NM_175503	CATGAAAATGCAGCAGCTGAA	GCCTCGGACTCTCCACTATGC
Adamts19	A disintegrin-like and metalloprotease with thrombospondin type 1 motif, 19	NM_175506	GGTCACAACATGGGCATCAAC	CTGGACCATGAAACATCACCAA
Amh	Anti-mullerian hormone	NM_007445	CTATTTGGTGCTAACCGTGGACTT	AAGGCTTGCAGCTGATCGAT
Bmp 2	Bone morphogenetic protein 2	NM_007553	ACACAGGGACACACCAACCAT	TGTGACCAGCTGTGTTCATCTTG
Cbln1	Cerebellin 1 precursor protein	NM_019626	TGTTTGTTCTCTGATGCTTGTCAAT	AAAATTTGGCTATTCACACGTATGTT
Cbln4	Cerebellin 4 precursor protein	NM_175631	GGCACCGAGGAAAGGAATCTAT	TCACCAGCAAATGCAGAGATG
Cdh11	Cadherin 11	AK028271	TGAAGATAGAGGCCGCCAAT	CCAAGAACATGGGAGGCTCAT
Col2a1	Procollagen type II alpha 1	NM_031163	TTCCTCCGTCTACTGTCCACTGA	CTACATCATTGGAGCCCTGGAT
Col9a3	Procollagen, type IX, alpha 3	NM_009936	CAAGATTTATGGCAGCCCAATAC	TCTCTTGAGTGTTTTTATCTCATGGAA
Cst9	Cystatin 9	NM_009979	AAGAAAGCTCTGCCTCTCACCA	TCCACTGTGGGAATGAAATGAAC
Cyp11a1	P450scc, cholesterol side chain cleavage	NM_019779	ACATGGCCAAGATGGTACAGTTG	ACGAAGCACCAGGTCATTCAC
Cyp17a1	Cytochrome P450, family 17, subfamily a, polypeptide 1	NM_007809	CTCCAGCCTGACAGACATTCTG	TCTCCCACCGTGACAAGGAT
Cyp26b1	Cytochrome P450, family 26, subfamily b, polypeptide 1	NM_175475	GAGAATGTGCGCAAGATCCTACT	TGGATGTCGCCAATGGAATT
Dhh	Desert hedgehog	NM_007857	ACCCCGACATAATCTTCAAGGAT	GTACTCCGGGCCACATGTTC
Dmrt1	Doublesex and mab-3 related transcription factor 1	NM_015826	GTGCCTGCTCAGACTGGAAAC	GATCTGGGACATGCTCTGGC
Dtna	Dystrobrevin alpha	NM_010087	CCTGGTATGGCTGCCTCTTC	TGTTGACATCGGTATCGAAATCC
Emx2	Empty spiracles homolog 2	NM_010132	TCCAAGGGAACGACACAAGTC	CAAAAGCGTGCTCTAGCCTTAAA
Etd	Embryonic testis differentiation	NM_175147	TTCTGGAACGCTGAGGTTTGTT	CTGGTATTATCCAATGGCCTCAA
Fgf9	Fibroblast growth factor 9	NM_013518	TACTATCCAGGGAACCAGGAAAGA	TCGTTCATGCCGAGGTAGAGT
Fgfr2	Fibroblast growth factor receptor 2	NM_010207	GCCGTGATCAGTTGGACTAAGG	CAAGCATAGAGGCCGGAGTCT
Fst	Follistatin	NM_008046	CCAGGCAGCTCCACTTGTGT	AGTCACTCCATCATTTCCACAAAG
Gapd	Glyceraldehyde-3-phosphate dehydrogenase	NM_008084	GGGAAGCCCATCACCATCTT	GCCTTCTCCATGGTGGTGAA
Gata2	GATA binding protein 2	NM_008090	CATGAAGAAGGAAGGGATCCAG	GGCGGTGACTTCTCTTGCAT
Gata4	GATA binding protein 4	NM_008092	CCTGGAAGACACCCCAATCTC	AGGTAGTGTCCCGTCCCATCT
Gpi1	Glucose phosphate isomerase 1	NM_008155	TCCGTGTCCCTTCTCACCAT	TGGCAGTTCCAGACCAGCTT
Gpr73l1	G protein-coupled receptor 73-like 1	NM_144944	AAGATGGGAGGCTCCTGACA	CTGGAAACCTCGGCTCTTTCT
Hhip	Hedgehog-interacting protein	NM_020259	TGCCACCAACAACTCAGAATG	CTAGGTCCCCATCCAGGACA
Hprt	Hypoxanthine guanine phosphoribosyl transferase	NM_013556	CATTATGCCGAGGATTTGGAA	CACACAGAGGGCCACAATGT
Hsd3b1	Hydroxysteroid dehydrogenase-1 delta5-3-beta	NM_008293	ACATGGCTCTGGGAGTTATAAGGT	TTAGTGACTGGCAAGGCTTCTG
Lhx9	LIM homeobox protein 9	NM_010714	TGGGAGTGGACATCGTGAATT	GAAAGAAGTTCGCATCCGTTTG
Mc2R	Melanocortin 2 receptor	NM_008560	CCTTCTGCCCAAATAACCCTTA	TGGGCTCCGAAAGGCATATA
Mro	Maestro	AK017067	CAAGATTCGCTCTTAGGCCATTT	TGGAAACTCGGCACCTTTCT
Ngfr	Nerve growth factor receptor	NM_033217	GTAGCCTGCCCCTGACCAA	GCCTCGTGGGTAAAGGAGTCT
Nr0b1	Nuclear receptor subfamily 0 group B member1, Dax1	NM_007430	GCCCTTTTCCTGCTGAGATTC	TCACAGCTTTGCACAGAGCAT
Nr5a1	Nuclear receptor subfamily 5 group A member1, steroidogenic factor 1 (Sf1)	NM_139051	CGCAACAACCTTCTCATTGAGA	TGGATCCCTAATGCAAGGAGTCT
Pdgfa	Platelet derived growth factor alpha	NM_008808	CAGTGTCAAGGTGGCCAAAGT	TGGTCTGGGTTCAGGTTGGA
Pdgfra	Platelet derived growth factor receptor alpha polypeptide	NM_011058	TCTGTGACCTTTAAGGATGCTTCA	GATGCCCACATAGCCTTCATTC

(*continued on next page*)

Table 1 (*continued*)

Gene symbol	Name, synonym	GenBank accession number	Primer sequence (5′ to 3′ direction)	
			Forward	Reverse
Ptgds	Prostaglandin D2 synthase (brain)	AB006361	GCTCTTCGCATGCTGTGGAT	GCCCCAGGAACTTGTCTTGTT
qk	Quaking	NM_021881	GAAATGGAAACGAAGGAGAAGC	CGCTCGAGGTGGTTGAAGAT
Ren1	Renin 1 structural	NM_031192	CACCCCAGACCTTCAAAGTCA	TCAGAGGACTCATAGAGGCTGTGA
Scarb1	Scavenger receptor class B member 1	NM_016741	CTCCCAGACATGCTTCCCATA	CAGATGGATCCTGCTGAAATTCT
Serpine2	Serine (or cysteine) proteinase inhibitor, clade E, member 2	NM_009255	ACGGTGATGCGATATAATGTAAACG	CATTCCTGAGAAACACAGCATTG
Sostdc1	Sclerostin domain containing 1	NM_025312	TACACCCGTCAGCACAACGA	CTCAGACTGTGCTTGCTGGATT
Sox9	SRY-box containing gene 9	NM_011448	AAGAAAGACCACCCCGATTACA	CAGCGCCTTGAAGATAGCATT
Sry	Sex determining region of chromosome Y	NM_011564	TTATGGTGTGGTCCCGTGGT	GGCCTTTTTTCGGCTTCTGT
StAR	Steroidogenic acute regulatory protein	NM_011485	TCTCTAGTGTCTCCCACTGCATAGC	TTAGCATCCCCTGTTCGTAGCT
Tcf21	Transcription factor 21, capsulin, podocyte-expressed 1	NM_011545	GACCTTCAAGAGGTGGAGATGCT	CCTTCTGTGGAGACCCGTTCT
Tdl	Testis specific beta defensin-like	NM_145157	AACAGGCCTACTTCTACTGCAGAAC	TGGCCAGTGCTTCTCGTAAGA
Vnn1	Vanin 1	NM_011704	TGGCCAAGAACAACTCCATCT	CACCACATCAGTGTTGTACTGGAAT
Wnt5a	Wingless-realted MMTV integration site 5a	NM_009524	CGCTAGAGAAAGGGAACGAATC	TTACAGGCTACATCTGCCAGGTT
Wt1	Wilms tumor homolog	NM_144783	CGGTCCGACCATCTGAAGAC	GTTGTGATGGCGGACCAATT
Wt1 + KTS		NM_144783	AAAAGACACCAAAGGAGACACACA	AGGGCTTTTCACTTGTTTTACCTGTA
Wt1 − KTS		NM_144783	AAAAGACACCAAAGGAGACACACA	GCTGAAGGGCTTTTCACCTGTAT
Zfpm2	Zinc finger protein multitype 2, friend of Gata 2 (Fog2)	NM_011766	GAGTAAACCCCGGCAGATCAA	GCTTCCCTCCAGTGGAAAGTC

Table 2
Genes expressed before E12 in XX and XY tissues, as defined by having an average Ct ≤ 37.5

First detected in E10.5 samples		First detected in E11.5 gonad/mesonephroic complexes	
Aard	*Hhip*	*Cyp11a1*	
Adamts19	*Lhx9*	*Dmrt1*	
Bmp2	*Mc2R*	*Sry*	
Cbln1	*Mro*		
Cbln4	*Ngfr*		
Cdh11	*Pdgfa*		
Col2a1	*Pdgfra*		
Col9a3	*Qk*		
Cyp26b1	*Scarb1*		
Dax1	*Serpine2*		
Dma	*Sf1*		
Emx2	*Sostdc1*		
Fgf9	*Sox9*		
Fgfr2	*StAR*		
Fog2	*Tcf21*		
Fst	*Vnn1*		
Gata2	*Wnt5a*		
Gata4	*Wt1*(+ and − KTS)[a]		
Gpr7311			

[a] Lee and Haber, 2001.

Menke and Page (2002) reported that *Cdh11* is expressed at higher levels in E13.5 XY than XX gonads. Their observations are based on wholemount in situ hybridization (WISH), which revealed that *Cdh11* was localized in E13.5 ovaries along the gonad/mesonephros boundary, whereas *Cdh11* transcripts were localized throughout the E13.5 testis. Our results indicate that *Cdh11* transcript levels in E13.5 XX and XY gonads are similar. The apparent discrepancy between our results and the results of Menke and Page (2002) is an example of comparing total transcript levels to cellular localization patterns obtained with WISH. These seemingly conflicting results, in fact, may not be different if fewer cells in XX gonads express higher *Cdh11* transcripts levels whereas a greater number of cells in XY gonads express lower transcript levels. Thus, while gene localization patterns obtained with WISH may not accurately reflect gene expression levels, WISH combined with

Table 3
Genes expressed in XY but not XX gonads

Gene	Expression period
Amh[a]	E12–14
Cbln1[a]	E12–14
Col9a3	E12–14
Csrp[a]	E12–14
Cyp11a1	E12.5–14
Cyp17a1[a]	E12.5–14
Cyp26b1[a]	E12–14
Mc2R	E12–14
Ptgds	E12–14
Ren1	E12–14
Sry[a]	E11.5–12.5

[a] Not expressed in XX and XY mesonephroi.

Table 4A
Genes expressed at significantly higher relative levels (GPR score ≥0.4 and $P < 0.05$) in XY compared to XX fetal gonads

Gene	Developmental time period				
	E12	E12.5	E13	E13.5	E14
Aard	0.656	0.829	0.829	ST	0.767
Cbln4	0.594	0.743	0.743	ST	0.633
Col2a1	0.406	0.400	0.486	0.639	–
Dhh[a]	0.406	0.813	0.857	0.972	0.800
Dtna	–	0.714	ST	0.611	ST
Etd[a]	–	0.571	0.857	0.806	0.567
Fgf9	0.531	0.514	–	–	–
Fog2	–	–	0.400	–	–
Gata2	–	–	0.743	0.778	–
Gata4	–	–	0.486	–	GPR (0.400)
Gpr73l1	0.406	0.800	0.800	–	0.767
Hhip	–	0.743	0.714	0.778	0.600
Hsd3b1	–	0.600	0.857	0.750	0.633
Lhx9	–	–	–	0.639	GPR (0.433)
Mro	0.406	0.600	0.486	–	0.633
Ngfr	–	0.571	0.543	0.583	0.433
Pdgfa	–	GPR (0.457)	0.457	GPR (0.472)	GPR (0.433)
Pdgfra	–	0.429	0.543	0.556	GPR (0.467)
Qk	–	–	0.571	–	–
Scarb1	–	GPR (0.400)	0.657	ST	0.633
Serpine2	0.563	0.486	0.829	0.778	0.667
Sf1	–	–	0.457	0.417	0.633
Sostdc1	–	–	–	0.750	0.967
Sox9	–	0.657	0.743	0.667	0.667
StAR	–	–	0.686	0.778	0.833
Tcf21	–	0.457	0.571	0.611	–
Tdl	0.563	0.743	0.829	0.944	–
Vnn1	ST	0.629	0.571	0.611	–

GPR scores indicate the fraction of normalizer genes to which the gene was found to be significantly different. ST, Student's *t*-test. Significantly different ($P < 0.05$) between XY and XX using Student's t-test on normalized data using 18s rRNA. GPR, Global pattern recognition. Significantly different between XX and XY using GPR software algorithm. –, Not significantly different between XX and XY using either GPR software algorithm or Student's *t*-test on normalized data using 18s rRNA.

[a] Not expressed in XX and XY mesonephroi.

Table 4B
Genes expressed at significantly higher relative levels (GPR score ≥0.4 and $P < 0.05$) in XX compared to XY fetal gonads

Gene	Developmental time period			
	E12.5	E13	E13.5	E14
Adamts19	0.914 (18.59)	0.886 (8.71)	0.917 (12.83)	0.700 (7.16)
Dax1	–	–	–	0.533 (1.69)
Fst	0.943 (59.95)	1.000 (194.24)	1.000 (100.12)	0.967 (179.77)

GPR scores indicate the fraction of normalizer genes to which the gene was found to be significantly different. Fold change difference between XX and XY calculated with 18s rRNA as the normalizer is given between the parenthesis. –, Not significantly different between XX and XY, using either GPR software algorithm or Student's *t*-test on normalized data using 18s rRNA.

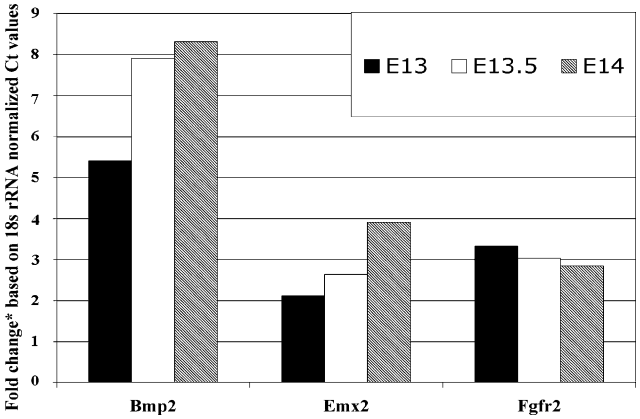

Fig. 1. Significant fold difference ($P < 0.05$; GPR score ≥0.4) in relative gene expression levels for three genes expressed at higher levels in XX compared to XY. *Fold changes were calculated as 2^(ΔCt XY − ΔCt XX).

real time PCR can provide better insight into gene expression patterns during gonad development.

Wertz and Herrmann (2000) reported that *Cdh11* was not detected in E9.5 and E11.5 urogenital ridges, but it was present in E14.5 testes. Our results detected *Cdh11* in E10.5 tissue containing genital ridges (Table 2). The discrepancy between these two conclusions is likely due to the more sensitive real time RT-PCR assay used in this study compared to the cDNA library screen combined with a high-throughput whole-mount in situ hybridization (HI-WISH) assay used by Wertz and Herrmann.

Several other genes were expressed at higher levels in XY compared to XX gonads (see Table 4A). Most of these genes were included in our analysis because of previously published sexual dimorphic expression (Wertz and Herrmann, 2000; Menke and Page, 2002; McClive et al., 2003 and references therein). Our results confirm these previous findings, and further validate the use of real time PCR as a powerful tool to study relative gene expression levels during gonadal sex differentiation.

1.2.4. Genes expressed preferentially in XX gonads

Six genes were identified that had significantly higher relative expression levels in XX than XY gonads (Table 4B, Fig. 1). These include *Fst* and *Adamts19*, two genes reported to be preferentially expressed in E12.5–14 XX gonads (Menke and Page, 2002), and *Bmp2*, *Emx2*, *Fgfr2*, and *Nr0b1* (*Dax1*). *Bmp2*, *Emx2*, and *Fgfr2* were expressed at higher levels in E13–14 XX than XY gonads (Fig. 1), a finding not previously reported. The higher expression of these three genes in XX fetal gonads suggests that they play an important role in ovarian development. *Fst* encodes a binding protein that inhibits bone morphogenic protein (BMP) function, including BMP2 (Findlay et al., 2002). Although the role of *Fst* and *Bmp* genes in XX fetal gonad development is unknown, these genes play critical roles in folliculogenesis and fertility in the adult female (Erickson and Shimasaki, 2003; Jorgez et al., 2004). Mice lacking

Emx2 fail to develop a urogenital system, indicating that this gene is important in kidney and genital ridge formation (Miyamoto et al., 1997). However, specific functions for *Emx2* and *Fgfr2* in the developing XX gonad remain to be determined.

The other gene expressed at higher levels in XX than XY gonads is the orphan nuclear receptor, *Dax1* (Table 4B). *Dax1* was expressed equally in XX and XY samples at E10.5–13.5. At E14, however, *Dax1* relative transcript levels were significantly higher in XX than XY gonads, in general agreement with previous studies (Ikeda et al., 1996; Ikeda et al., 2001).

1.2.5. Gene expression in E12–14 XX and XY mesonephroi

Relative gene expression levels were analyzed in E12–14 XX and XY mesonephroi. No significant differences in relative gene expression level were apparent between XY and XX mesonephroi for any genes analyzed, nor were any of these genes expressed exclusively in mesonephroi (data not shown). Genes expressed in gonads but not in mesonephroi include *Amh*, *Cbln1*, *Cyp17a1*, *Cyp26b1*, *Cst9*, *Dhh*, *Dmrt1*, *Etd*, and *Sry*.

1.3. Points of consideration and conclusion

Because the day a mating plug is detected provides an inexact age of the developing fetuses, it is crucial to perform careful embryonic staging to determine the age of each individual fetus in a litter (i.e. counting tail somites and assessing paw morphology, see Section 2). This is especially important because developmental variation exists between individuals within a litter and differentiation occurs at a rapid rate. Apparent discrepancies between data from different laboratories in terms of presence or absence of gene expression at a developmental time point may simply reflect the method used to assess embryonic age. In addition, variation in gene expression data may reflect genetic background differences, another critical consideration when comparing data sets.

The high sensitivity of real time PCR to procedural errors, such as pipetting errors and poor RNA and/or cDNA quality, can lead to substantial differences between individual samples within experimental groups. This is especially the case when sample sizes are small and data is presented as fold differences. In these cases, minor variations in normalized Ct values (number of PCR amplification cycles to reach fluorescent intensity above threshold) can suggest large changes between experimental groups when no significant difference exists. It is crucial, therefore, to assess RNA quality before conducting real time PCR experiments. In addition, because variation can occur over time due to RNA/cDNA degradation, it is important to avoid long-term storage of RNA and cDNA, and to store cDNA samples at −20 °C.

The real time PCR results were analyzed using the statistical software algorithm GPR (Akilesh et al., 2003), and the Student's *t*-test on normalized Ct values using 18s rRNA as the normalizer gene. Owing to careful RNA preparation/validation, both methods identified the same genes that were significantly different in the majority of cases. However, although preliminary results indicated 18s rRNA to be the most stable expressing housekeeping gene compared to *Gpi*, *Gapd*, and *Hprt*, its use as normalizer gene has limitations (Bustin, 2000). For example, the higher expression of 18s rRNA relative to all the other genes examined can lead to errors in setting the baseline for the real time PCR analysis. Furthermore, 18s rRNA cannot be used when quantitating mRNA due to the lack of a poly(A) tail. Finally, in a few instances even 18s rRNA appeared to vary in expression compared to other genes. Because GPR does not require a single invariant housekeeping gene to normalize the data, it is less sensitive to small changes in Ct values caused by RNA and/or cDNA degradation, pipetting errors, or variability in expression levels of the selected normalizer gene. In addition, because each gene is considered individually, GPR is better able to handle PCR failures (Akilesh et al., 2003). For these reasons, we recommend GPR as the preferred method for analyzing multiple gene expression real time PCR data.

This study demonstrates that real time PCR is a powerful tool for determining multiple gene expression level profiles during gonad differentiation. In addition, it is an efficient method for determining if the expression profile of a gene suggests that it plays a role in gonad development and differentiation. For example, by simply adding to the assay gene specific primer pairs for a number of potential gonad genes, we found that *Bmp2*, *Emx2*, and *Fgfr2* are expressed at higher relative transcript levels in the XX than XY gonads, a previously unreported finding. We conclude that real time PCR together with other techniques, such as subtractive hybridization and differential displays, will be instrumental in elucidating the complex network of gene interactions regulating gonadal development and differentiation.

2. Material and methods

2.1. Animal tissue and genotyping

Mice from the C57BL/6JEi inbred strain were used in this study. Timed matings were performed and midday a vaginal plug was detected in the female was noted as embryonic day (E)0 for the developing embryos. Because of individual developmental variation within a litter, more precise embryonic staging was performed: Fetuses younger than E13 were staged by counting tail somites distal to the hindlimbs (~8ts corresponds to E10.5, ~18ts to E11.5, and ~28ts to E12.5) (Hacker et al., 1995), whereas fetuses E13 to E14 were staged according to fore and hind limb morphology (Theiler, 1989). A section containing the genital ridge/mesonephros complex was isolated from

E10.5 fetuses, whereas paired gonad/mesonephros complexes were dissected from E11.5 and older fetuses. At E12 and beyond, gonads and mesonephroi were separated before processing. Tissues from each fetus were immediately homogenized individually in lysis buffer containing β-mercaptoethanol (Qiagen RNAeasy kit, Qiagen Inc., Valencia, CA), and then stored at −80 °C until RNA isolation. Dissection instruments were cleaned with RNaseZap wipes (Ambion Inc., Austin, TX) between fetuses.

XX and XY mice were distinguished by the absence or presence, respectively, of the Y chromosome using a tail sample digested overnight in lysis buffer (0.05 M KCl, 0.05 M Tris at pH 8.3, 0.1 mg/ml gelatin, 0.45% Nonident P-40, 0.45% Tween, and 60 μg/ml Proteinase K) at 55 °C. Multiplex PCR was performed according to Capel et al. (1999) using primers for the YMT2/B locus on the Y chromosome (5′-CTGGAGCTCTACAGTGATGA-3′ and 5′-CAGTTACCAATCAACACATCAC-3′) and primers for the autosomal myogenin gene (5′-TTACGTCCATCGTG GACAGCAT-3′ and 5′-TGGGCTGGGTGTTAGTCTTAT-3′) as a control. The PCR amplification cycle was as follows: 95 °C (2 min), followed by 34 cycles at 96 °C (10 s), 60 °C (30 s), 72 °C (30 s), and one cycle at 72 °C (5 min).

2.2. RNA isolation and reverse transcription

Tissue lysate from each individual fetus was applied to QIAshredder spin columns (Qiagen Inc.). RNA was isolated from the flow through using the RNeasy Mini Kit (Qiagen Inc.) according to the manufacturer's description, with the exception that total RNA was eluted with 40 μl of RNAse free water (Ambion Inc.) directly into 1 μl SUPERase-IN™ (Ambion Inc.) to inhibit RNAse activity. To prevent DNA contamination, RNA was treated with 1 μl DNase 1 and 4 μl 10 × DNase 1 Reaction Buffer (DNA-free, Ambion Inc.) at 37 °C for 30 min. DNase was inactivated by addition of 10 μl DNase inactivation reagent (Ambion Inc.).

Total RNA quantity and quality was assessed using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Rockland, DE) and the Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA), respectively. Only samples with ~10–40 ng/μl total RNA, a 28s to 18s rRNA ratio of 1–2, and absence of genomic DNA were used in experiments.

RNA was reverse transcribed into cDNA using the MessageSensor™ RT kit (Ambion Inc.) with addition of random decamer primers and SUPERase-IN™ (Ambion Inc.). Briefly, nuclease-free PCR tubes (Robbins Scientific Corporation, Sunnydale, CA) containing 10 × RT-PCR buffer, 2.5 mM dNTP mix, 10 μM random decamer primers, 1 μl SUPERase-IN, 1 μl MessageSensor reverse transcriptase, and 10 μl total RNA were incubated for 30 min at 45 °C followed by 7.5 min at 95 °C. cDNA was stored overnight at 4 °C or used immediately for real time RTPCR.

2.3. Primer design and validation

Genes were selected for a real time PCR multi-gene platform expression profile analysis based on published literature indicating their importance in urogenital development, sex determination/differentiation, or expression in the developing gonad. Initially, 116 genes were selected, including gene family members for a number of genes (e.g. *Gata*, *Bmp*, and *Sox*). Based on their expression level and pattern obtained in preliminary experiments, the gene list was shortened to a final set of 55 genes. To allow similar amplification profiles, all primer pairs were designed to have a melting temperature of 58–60 °C and produce an amplicon of ~120 bp in length. This short amplicon length maximized amplification efficiency and allowed sequencing of the PCR product by the ABI 3700 sequencer (Applied Biosystems, Foster City, CA). To increase specificity, the last five nucleotides at the 3′ end of each primer were designed to contain at least three A's or T's. Primer pairs were obtained from MWG Biotech (High Point, NC). Table 1 contains a list of the 55 primer pairs used in this study.

To determine the target sequence specificity of each primer pair, PCR analysis was performed on a cDNA pool obtained from E10.5 XY and XX samples, E11, E12.5, and E13 XX and XY gonad/mesonephros complexes, and a neonate ovary and testis. PCR was conducted using AmpliTaq Gold PCR Master Mix (Applied Biosystems, Foster City, CA) containing AmpliTaq Gold DNA polymerase, Gold Buffer, dNTPs, and $MgCl_2$. The PCR conditions were: 95 °C for 5 min (one cycle), 95 °C for 15 s and 60 °C for 1 min (40 cycles). Approximate PCR product sizes were determined via gel electrophoresis using 2% SeaKEM LE agarose (BMA Inc., Rockland, ME).

PCR products with the expected band size of ~120 bp were further validated by DNA sequencing. Unwanted dNTPs and excess primers were removed by treating PCR products with Exo SAP-IT (USB Corp., Cleveland, OH) according to the manufacturer's instructions. Each PCR product was sequenced bi-directionally on an ABI 3700 sequencer (Applied Biosystems) using 3.2 μM of the gene specific forward and reverse primer. The resulting sequences were BLAST searched to verify the correct gene target sequence.

Final validation of primer specificity was performed by conducting dissociation curve analysis using real time PCR analysis for each gene primer pair on the cDNA pool according to the manufacturer's instructions (Applied Biosystems). This analysis is important because it detects decreases in SYBR Green fluorescence resulting from dissociation of the PCR products, and is routinely used to detect non-specific double stranded DNA and/or presence of primer dimmers (Ririe et al., 1997; Applied Biosystems).

One primer pair passed the primer validation process but did not produce a signal during the real time PCR analysis. This failure was likely due to the fact that this primer pair

was incompatible with the SYBR Green master mix, or the amount of cDNA template and/or primers used in the real time PCR analysis was to dilute. Redesigning the primers to a different region of the gene eliminated the problem.

2.4. Real time RTPCR

Real time PCR was used to determine expression profiles of 51 genes involved in urogenital development and gonadal sex determination/differentiation. Each analysis was performed in a total volume of 10 μl reaction mixture containing 1 μl cDNA sample (diluted 1:5), 5 μl 2 × SYBR Green PCR Master Mix (Applied Biosystems), 2.5 μl RNase free water, and 1.5 μl gene specific forward and reverse primer (0.75 nM each). For high throughput analysis, a 96-well primer pair plate was prepared with a forward and reverse primer pair in each well. Primers from this plate were added to 384 well plates so that 55 genes could be analyzed on six cDNA samples, each cDNA sample was obtained from a single fetus. For each cDNA sample analyzed, four housekeeping genes (18s rRNA, *Gpi1*, *Hprt*, and *Gapd*) were included to normalize the data. Real time PCR was performed using an ABI Prism 7900HT Sequence Detection System, with SDS version 2.1 software (Applied Biosystems). The default threshold of 0.2 was maintained, and the baseline was set from 3 to 12 to distinguish background fluorescence from fluorescence generated by SYBR Green binding to the PCR product. The PCR cycle conditions were: 50 °C for 2 min (one cycle), 95 °C for 10 min (one cycle), 95 °C for 15 s and 60 °C for 1 min (40 cycles).

2.5. Analysis

For each time point, a minimum of 10 cDNA samples (three XY and three XX gonads, three XY and three XX mesonephroi) were analyzed with the exception of E12, where only two XY gonad samples were available. The Ct number, defined as the number of PCR amplification cycles required to reach fluorescent intensity above threshold, was determined for each gene and each developmental time point analyzed.

Data were analyzed in two different ways. First, because 18s rRNA was the most constant expressing housekeeping gene, 18s rRNA was used to normalize the data by subtracting its Ct value from the Ct value obtained for each reaction. Fold changes between XX and XY fetal gonads then were calculated as 2^(ΔCt XX − ΔCt XY), with ΔCt indicating normalized values. Significant changes are determined at $P < 0.05$ using an unpaired, two-tailed Student's *t*-test on normalized Ct values. Second, because 18s rRNA expression can vary among cDNA preparations (Bustin, 2000; Akilesh et al., 2003) we also analyzed relative changes in gene expression between XX and XY samples using the recently developed statistical algorithm GPR (Akilesh et al., 2003). GPR uses the raw Ct values from each reaction and determines which genes qualify as normalizers. Significant relative changes in gene expression then are determined by comparing each gene with every other gene in the data set. Because GPR does not require a single invariable normalizer, it evaluates changes in gene expression with greater reliability (Akilesh et al., 2003). GPR determines significant changes based on repeated consistent changes compared to the multiple normalizers using a Student's *t*-test. Each gene is assigned a GPR score that indicates the fraction of normalizer genes to which the gene is found to be significantly different. The greater the number of normalizer genes identifying a candidate gene as significantly different, the higher the GPR score. A GPR score of ≥ 0.4 is considered a reliable significant difference between two experimental groups (Akilesh et al., 2003).

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