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Comparative Expression Analysis of Genes Associated with Gonadal Development in the Mouse

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Abstract

Mammalian sex determination involves differentiation of a bipotential gonad into either testis or ovary which is governed by a complex interplay of various genes that are spatio-temporally expressed. The changes in the expression levels of these genes coincide with the differentiation of various cells in the gonads and hence the mRNA levels of these genes are used as surrogate markers to understand the fate of the bipotential gonads during the window of sex determination. In this study, we performed a comprehensive analysis of the expression profiles of twenty genes known to be involved in some processes of sex determination and three housekeeping genes in mouse XX and the XY gonads between E11.5 to E14.5. We performed Real-time RT-PCR on RNA isolated from gonads that were collected after timed mating and free from the underlying mesonephric tissue. The results revealed that amongst the housekeeping genes studied, the expression of *18S rRNA* is most stable and not sexually dimorphic during window of sex determination while *Gapdh* was found to be highly variable and was higher in the XX gonads as compared to XY. Our results suggest that *Amh* and *Dmrt1* are exclusively expressed in the XY gonads; their expression appears only at E12.5 and 13.5 respectively indicating that they can be the best markers to determine terminal testicular differentiation. *Sox9* appeared as the best marker that could be used for studying testicular fate as early as E11.5 until E14.5. In case of ovarian soma, *Wnt4* and *Lhx9* were consistently XX dominant from E11.5 to E14.5 and had minimal variability. *Foxl2* is XX exclusive but appears only at E13.5 and hence could be used as terminal marker for ovarian fate. To the best of our knowledge, this is a first comprehensive analysis of important factors involved in mouse gonadal determination. We believe that our results will aid researchers in elucidating the complex network of gene regulations occurring during the gonadal development and differentiation

Keywords: gonads, sex determination, mouse, gene expression.

Introduction

During vertebrate sex determination, the bipotential gonad differentiates into either testis or ovary which is triggered by genes or environmental influence. The process of sex determination and further differentiation of gonads involves a complex interplay of several genes. Studies in various species with either genetic or environmental sex determination have shown that sex determination in the gonads involves a set of pro-testis genes, anti-testis genes and pro-ovary genes and the relative levels of these genes during the window of sex determination are critical determinants of the fate of bipotential gonads (Sekido and Lovell-Badge, 2013).

In the mouse at E10.5, the gonads are bipotential; the testis is morphologically defined by E12.5 and the ovary by E13.5. With the advent of high throughput gene expression technologies, it is evident that even before the morphological definition; there are several genes which are expressed in sexually dimorphic manner. Based on microarray profiling of early XX and XY gonads, it is evident that distinct changes

occur in expression of several genes that precede morphologic differentiation. Some genes are sexually dimorphic at the onset of sex determination and continue to remain so; whereas some genes are dimorphic only after sex determination. Conversely, there are several genes which are sexually dimorphic only during the critical window of sex determination and their levels become identical in both sexes thereafter (Jameson et al., 2012; Bouma et al., 2010). The changes in the expression levels of these genes coincide with the differentiation of various cells in the gonads. Therefore, the mRNA levels of these genes in the XX and XY gonads are used as surrogate markers to understand the fate of the bipotential gonads during the window of sex determination. Any alteration in levels of the transcripts of these genes is viewed as a defect in the process of gonad development.

During the process of sex determination, *Sry*, *Sox9* and *Dmrt1* are well established markers of Sertoli cells and are reportedly testis specific (Eggers et al., 2014). Thus their expression is used as indicator for Sertoli cell differentiationalbeit*Sox9* is expressed at lower levels in the developing ovary. However, unlike the testis, with the exception of *Foxl2* there are no unique genes to the ovary(Eggers et al., 2014; Ottolenghi et al., 2005; She and Yang, 2014). Based on knockout experiments, *Wnt4*, *Rspo1* and *B-Catenin* have been reported to be associated with granulosa cell differentiation(Liu et al., 2009; Chassot et al., 2012)and thus could be surrogate markers of ovarian development; however it is important to note that all these genes are also expressed in the developing testis(Chassot et al., 2012). Furthermore, like the somatic cells, there are differential expression of genes associated with germ cell differentiation and interstitial cell (steroidogenic cell and vascular cell) differentiation. While the functions of many of the genes associated with testicular or ovarian development are known, the information on patterns of changes in the expression of candidate marker genes (mainly associated with ovarian development) during the entire period of sex determination is limited. Also most reports have used whole gonad-mesonephric complexes to evaluate gene expression and it is recognized that several genes are expressed by both, the mesonephros and the gonad, however their expression vary in both these structures during the period of sex determination. For example,Bouma,*et al*(2004) observed that 46/ 55 genes studied by them were expressed in both, mesonephros and the gonads and the levels of the mRNA in the gonads, but not mesonephros was sexually dimorphic. This observation suggest that data derived from gonad-mesonephric complexes may not accurately reflect the changes occurring in the gonad. Secondly, in absence of a baseline data on the differential gene expression patterns in the gonads, for most researchers the choice of genes to be used as surrogate markers for somatic, germ and interstitial cell differentiation is rather empirical. For a rational use of gene expression as surrogate markers to asses different cell types during stages of gonadal development, it will be valuable to have a prior knowledge of the detailed expression profiles in the XX and XY gonads during the window of sex determination.

In the present study we have performed a comprehensive analysis of the expression profiles of twenty genes in mouse XX and the XY gonads between E11.5 to E14.5. These genes were chosen based on their known functions and are known to be reportedly involved in various processes of gonad development.

Materials and Methods

Mouse strains and tissue collection

The use of animals for this work was approved by animal ethics committee of NIRRH (IAEC number: 08/08, 13/12). C57BL6 mice were housed in the experimental animal facility at NIRRH with 12h day and night cycles at 25ºC. Mature 8 week old female mice were housed 1:1 with males of same strain and the day of the vaginal plug was taken as E0.5. Pregnant mice were euthanized in the afternoon of E11.5, E12.5, E13.5 and E14.5 and their gonads were dissected. The genital ridge was separated from the mesonephri and stored at -80ºC in Trizol (Gibco).

Embryo sexing

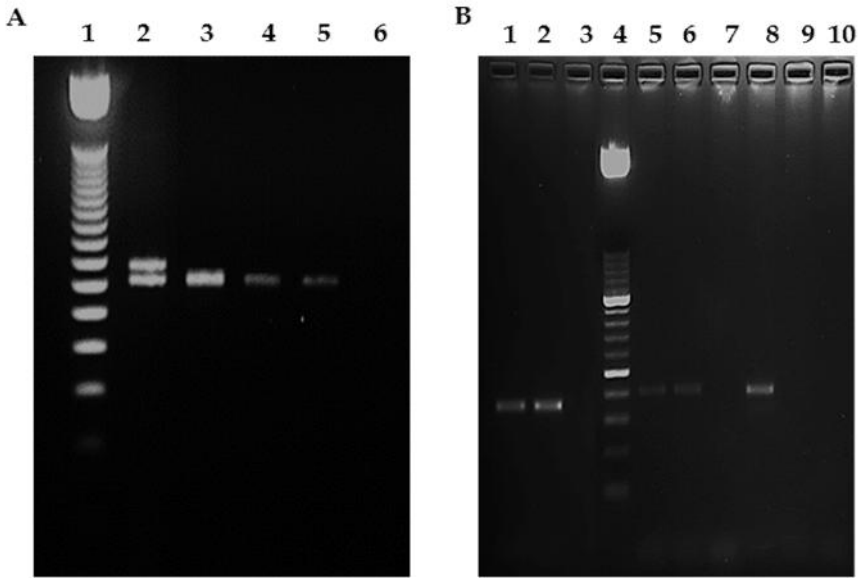
Sex of the embryo was determined by PCR for *Jarid1* (Clapcote and Roder, 2005). *Jarid1c* is X-chromosome-specific while *Jarid1d* is Y-chromosome-specific gene. The intron between the two exons 9-10 is 114 bp long in *Jarid1c* but 85 bp long in *Jarid1d* resulting in a difference of 29 bp.The primer pairs designed for this region (Clapcote and Roder, 2005) simultaneously amplify DNA fragments of 331 bp from the X chromosome homologue (*Jarid1c*) and 302 bp from Y chromosome (*Jarid1d*,there by permitting resolution on standard agarose gels (Supplementary Fig 1A). The primer sequences along with annealing temperature and expected amplicon size are mentioned in Table-1.

Table 1: Primer sequences with their respective annealing temperatures temperatures and expected size of PCR product. NA- single exon gene

Gene	Accession Number	Primer sequence	Exon No. spanned by For. and Rev. primer	Expected product size (bp)	Optimized annealing tempera ture (°C)
18S rRNA	NR_003278.3	F:5'-ACCCGGTGAGCTCCCTCCC-3' R: 5'-TCGAATGGGTCGTCGCCGC-3'	NA	114	68
Amh	NM_007445.2	F: 5'-GCGGGGCACACAGAACCTCTG-3' R: 5'-TCCGCGTGAAACAGCGGGAA-3'	Exon 2 - Exon 4	191	70
B-Catenin	NM_001165902.1	F: 5'-GCCTGAGGGTACCTGAAGCTC-3' R: 5'-GAAGGAGCTGTGGTGGTGGCA-3'	Exon 1 - Exon 3	189	66
Cyp17	NM_007809.3	R: 5'-CATGGGATCCGGGACGTTAG-3' F: 5'-GATCGGTTTATGCCTGAGCG-3'	Exon 7 - Exon 8	329	62
Cyp26b1	NM_175475.3	F: 5'- GCCTCCCCAGGCAATCTTTTT-3' R: 5'- GGCAGCTTGCAGCTCTTGTC-3'	Exon 1 - Exon 2	198	64
Dax1	NM_007430.5	R: 5'-AGGGCACTGTTCAGTTCAGCGG-3' F: 5'-TTAACCCAGACCTGCCTGGCCT-3'	Exon 1 - Exon 2	140	70
Dazl	NM_001277863.1	F: 5'-GTGTGTCGAAGGGCTATGGAT-3' R: 5'-ACAGGCAGCTGATATCCAGTG-3'	Exon 3 - Exon 7	328	68
Dmrt1	NM_015826.5	F: 5'-TGGAAACCAGTGGCAGATGAA-3' R: 5'-TTCGAGCTCTCGTTGCTCAT-3'	Exon 3 - Exon 5	240	64
Fgf9	NM_013518.4	F: 5'-TGGGTCAGTCCGAAGCAGGGG-3' R: 5'-TCCAGAATGCCGAAGCGGCTG-3'	Exon 1 - Exon 2	178	64

Figla	NM_012013.1	F: 5'-GAGCCCTTCCTGGTCACTCC-3' R: 5'-TTGTTTCTCTGAGACCTTCGCTT-3'	Exon 1 - Exon 2	349	62
Foxl2	NM_012020.2	F: 5'-GGCTCTTCGGGAGCGGAGGA-3' R: 5'-TGGCAGGAGGCGTAGGGCAT-3'	NA	163	64
Gata4	NM_008092.4	F: 5'-CCCTGGAAGACACCCCAATC-3' R: 5'-GGAAGCGGACAGGCGG-3'	Exon 2 - Exon 4	199	64
Jagged1	NM_013822.5	F: 5'-GCACCCGCGACGAGTGTGAT-3' R: 5'-TGTAGGACCTCGGCCAGGCG-3'	Exon 2 - Exon 3	201	68
Jarid-1c/d (Kdm5c/ d)	-	F: 5'- CTGAAGCTTTTGGCTTTGAG -3' R: 5'- CCACTGCCAAATTCTTTGG -3'	Exon 9 - Exon 10	302/331	61
Lhx9	NM_001025565.2	F: 5'-GGACCTCAAACAGCTTGCTC-3' R: 5'-AATTTTCAAACGTCGGGATG-3'	Exon 4 - Exon 5	103	60
Oct4	NM_013633.3	F: 5'-AGCTGCTGAAGCAGAAGAGG-3' R: 5'-GGTTCTCATTGTTGTCGGCT-3'	Exon 2 - Exon 3	198	64
Pdgfr	NM_001146268.1	F: 5'-CTGAGCACCCTCTCCATTCC-3' R: 5'- ATGGTGTCCGGGCTACAGAT-3'	Exon 1 - Exon 2	169	68
Rspo1	NM_138683.2	F: 5'-GCCGCTGCGCCAGGTCTATC-3' R: 5'-AGAGCCAGGCCCGGATCCAC-3'	Exon 1 - Exon 2	175	64
Sdha	NM_023281.1	F: 5'-TTGGCGTTAACTGGGGCGTGGC-3' R: 5'-CCAAATGCAGCTCGCAAGCCTG-3'	Exon 1 - Exon 3	191	68
Sf1	NM_001110791.1	F: 5'-GCTTGAATGGCACTCTACGG-3' R:5'-AGTTGTAGACATGAGAGACGGTG-3'	Exon 7 - Exon 9	341	64
Sox9	NM_011448.4	F:5'-ACTTCTGTGGGAGCGACAACTTTAC-3' R: 5'-GGTCTCAGCTGCCGGCTCTAAA-3'	Exon 1	157	68

Stra8	NM_009292.1	R: 5'-ATATCACAGCCTCAAAGTGGCA -3' F: 5'-TCTGGCATATTCTTCCTTGACC -3'	Exon 2 - Exon 4	158	66
Sycp3	NM_011517.2	R: 5'-TCCTCAGATGCTTCGAGGGTG-3' F: 5'-GGAGCCTTTTCATCAGCAACAT-3'	Exon 1 - Exon 3	207	66
Wnt4	NM_009523.2	F: 5'-TGGACTCCCTCCCTGTCTTTGGGA-3' R: 5'-TCCTGACCACTGGAAGCCCTGTG-3'	Exon 2 - Exon 4	188	65



Supplementary Fig. 1: PCR for detection of the sex of embryo and purity of cDNA preparations.
A) PCR using Jarrid primers for embryo sexing. Lane1: 50 bp DNA ladder, Lane 2 XY, Lane 3-5: XX, Lane 6: No template control.
B) PCR amplified products with cDNA from mesonephros and gonad using *Sdha*, *Jagged1* and *Oct4* primers. Lane 1, 2: *Sdha*; Lane 5, 6: *Jagged1*; Lane 8, 9: *Oct4*; Lane 4: 50 bp DNA ladder; Lane 1, 5 & 8: cDNA from gonad; Lane 2, 6 & 9: cDNA from mesonephros Lane 3, 7 & 10: Negative controls for *Sdha*, *Jagged1* and *Oct4*

For sexing, a portion of the somatic tissue (lower limb or tail) was solubilized in the solubilisation buffer (50mM NaOH) and incubated at 95°C for 10 mins. The extract was neutralised with 1M Tris-HCl (pH: 8.0) and 5µl was used to perform PCR reaction using Clontech Terra PCR kit (Clontech). PCR was performed on a standard thermal cycler (Veriti, Applied Biosystems) where initial denaturation was 95°C for 5 min, followed by 30 cycles of 95°C for 15 sec, 61°C for 15 sec, 72°C for 15 secs. The samples were run on 2.5% agarose gels and visualized under UV transilluminator.

RNA isolation and Reverse Transcription

Gonads were separated from the mesonephros, homogenised in Trizol and total RNA was extracted using the standard protocol. The RNA was treated with *DNase1* (Roche Diagnostics, USA) and the quantity of RNA was estimated using a NanoPhotometer P-360 (Implen). The samples having 260/280 ratio of ~2 were considered for Reverse Transcription. One microgram of total RNA was reverse transcribed using MMLV reverse transcriptase and random hexamer primers (BD-Clontech) as per kit instructions.

Quantitative Reverse Transcriptase PCR (qRT-PCR) analysis

To determine the expression of various genes, primers were designed using NCBI Primer-Blast software. The sequences and the standardised annealing temperatures of the primers are as mentioned in Table 1.

qRT-PCR was performed using SYBR green super mix (BioRad). Each reaction was carried out in triplicates of 25 µl each on a BioRad CFX96 Real-Time System (BioRad, US). Briefly, the master mix containing the cDNA and appropriate primer pair (Table 1), was subjected to initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 30 sec, appropriate annealing for 30 sec and 72°C for 30 sec. The negative controls included wells without cDNA. All PCR amplifications were followed by melt curve analysis from 55°C to 95°C at the ramp rate of 3.3ºC/sec with 0.5ºC increments, 5 sec dwell time and a read at each temperature.

The homogeneity of the PCR amplicons was verified by electrophoresis on 2.5% agarose gels and also by analysing the melt curves. For normalization, the expression of three genes (*18SrRNA*, *Gapdh* and *Sdha*) was initially screened during the course of gonadal development. Based on the data (shown in results) *18S rRNA* values were chosen for normalization. The fold change in the expression of each gene was calculated using the formula 2^ (-ΔCt) (Schmittgen and Livak, 2008). Data reported are the mean fold change ±SEM for five biological replicates.

Statistical analysis

Statistical significance between each samples was determined at $p < 0.05$ using an unpaired, two-tailed Student's T-test.

Results

Purity of the RNA preparations from the gonads

The primary aim of the study was to determine the changes in gene expression in isolated gonads, free from the underlying mesonephros. A first requisite for such a study would be to assess the purity of the RNA isolated from the gonadsor from the mesonephros. As evident from the supplementary figure 1B, the *Sdha* and *Jagged1* genes were found to be amplified when the cDNA was derived from both gonad and mesonephros. However, *Oct4* which is gonad specific could only be amplified in the cDNA isolated from gonad but not mesonephros. This suggests that the preparation was appropriate.

Determining an appropriate housekeeping gene

We first analysed the expression profiles of three commonly used housekeeping genes, viz*18s rRNA*, *Sdha* and *Gapdh* in the XX and XY gonads from E10.5 to E14.5 (Fig 1). Amongst these, the levels of *18S rRNA* were found to be most stable during the course of gonad development and the levels were not significantly different in XX and XY gonads. *Sdha* mRNA, although not sexually dimorphic, was least abundant and its expression varied (although not statistically significant) between E11.5 toE14.5. *Gapdh* mRNA levels had maximum variability and was significantly higher in the XX gonads in comparison to XY gonads from E12.5 onwards. Based on these observations, we chose *18S rRNA* for normalization in all further experiments.

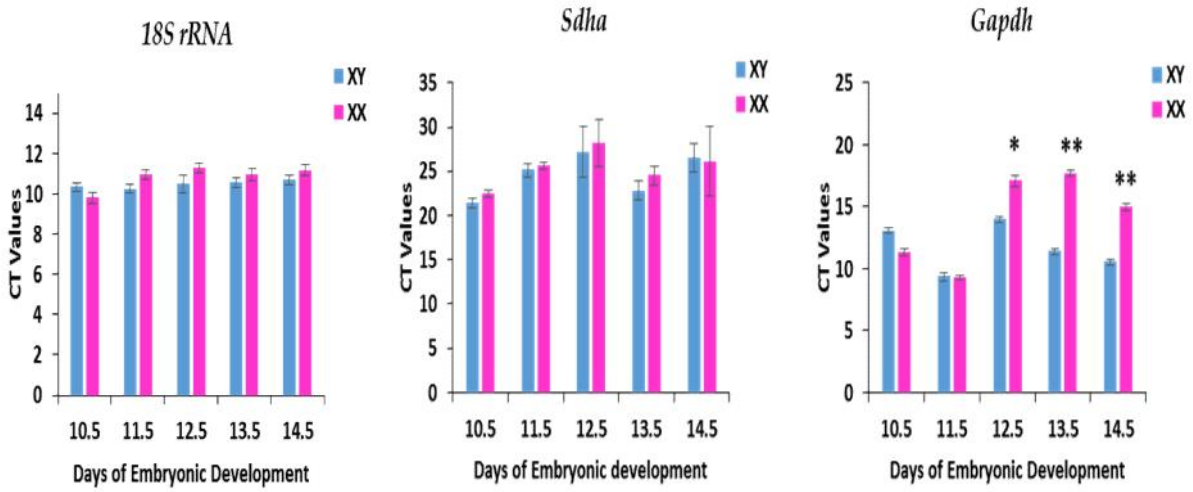


Fig. 1: RT-qPCR analysis of house keeping genes *18S rRNA*, *Sdha*and *Gapdh*in the murine XY and XX gonads from E10.5-E14.5. The threshold cycle (Ct) values for each time point were plotted. Values are gives as Mean ± SD. Student's T-test was used to show the statistical significance, * indicates $p< 0.05$ and ** indicates $p< 0.0005$.

Expression profiling of genes associated with gonadal development

Fig 2 gives the representative image of the PCR products for the various genes included in this study. A single band of expected size was observed in all the cases. The lanes with negative controls for each primer pair did not show any band suggesting that the amplification was not a result of any contamination or nonspecific primer-dimer interactions. The dissociation curve analysis (55°C to 95°C) for each primer pair revealed a single peak (Supplementary Fig 2).

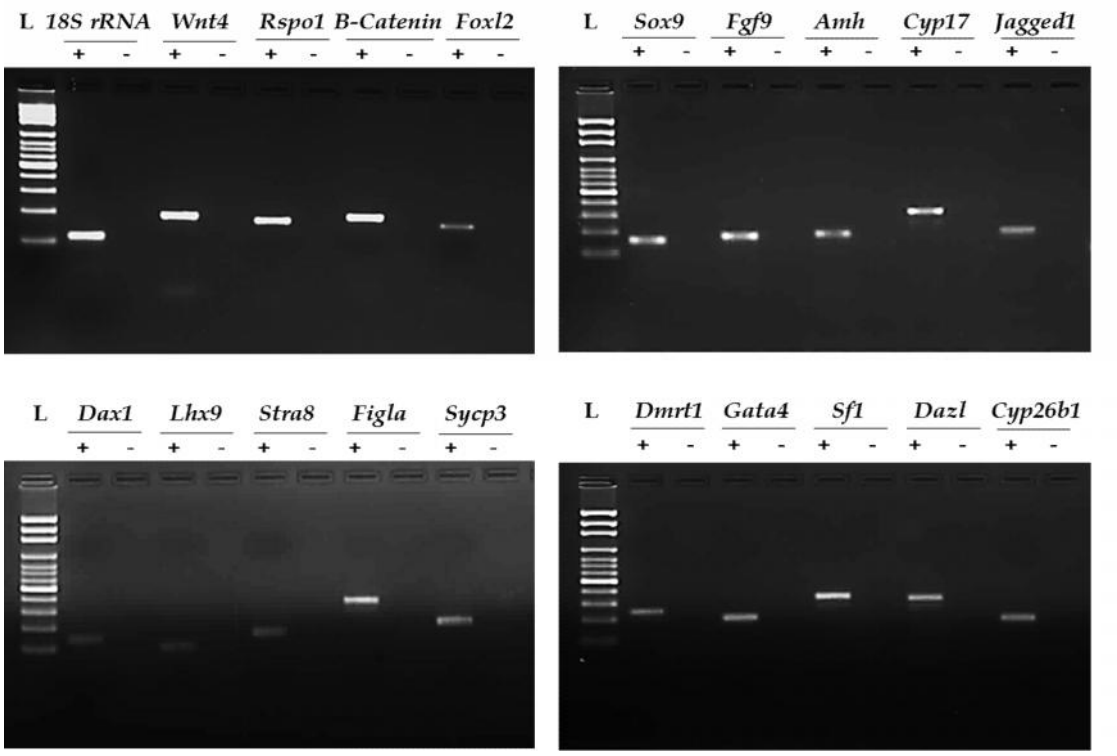
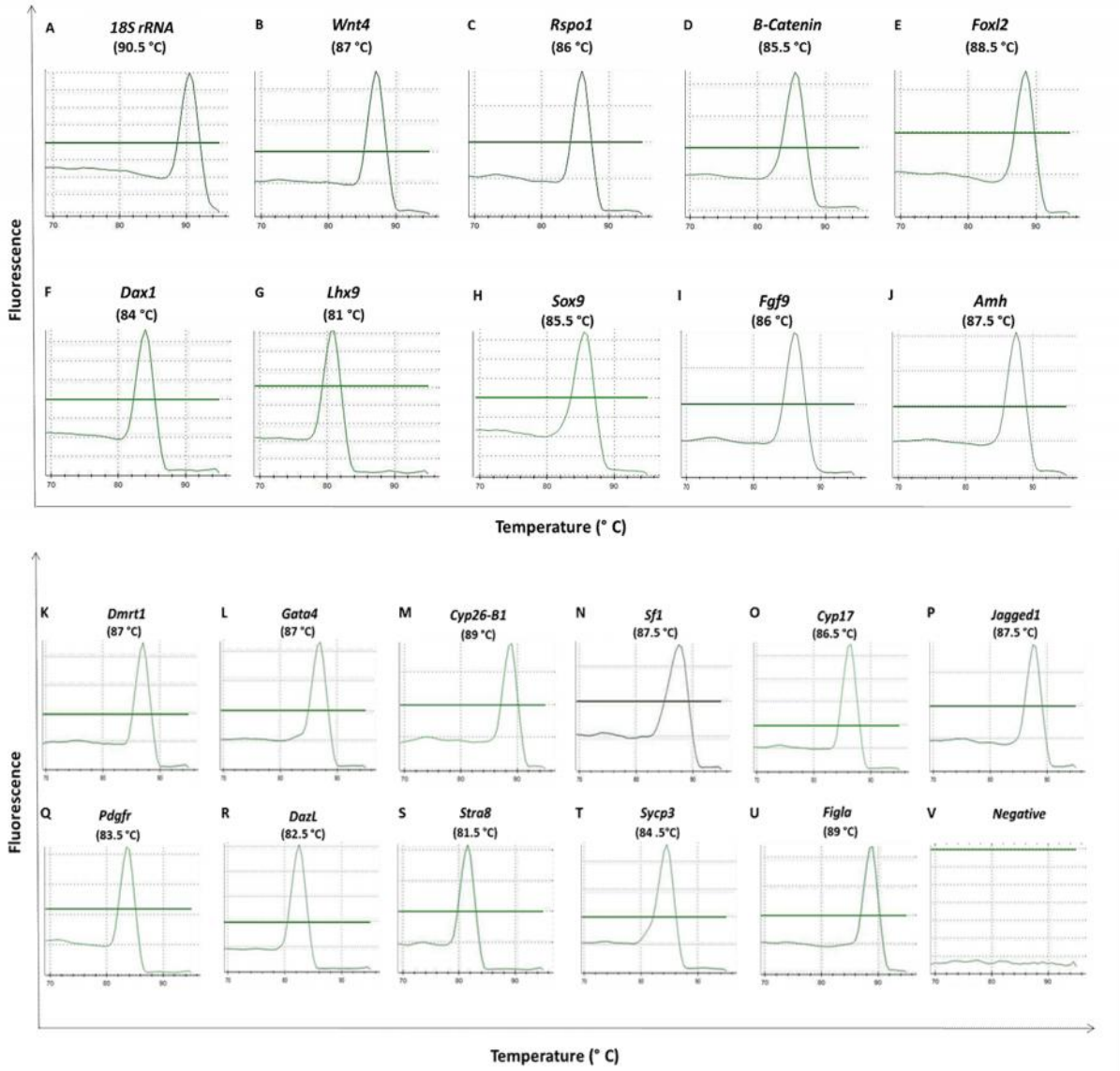


Fig. 2: Representative gel images of the RT-PCR products for various genes in the XX and XY gonads. '–' are no reverse transcriptase controls, '+' are wells with reverse transcriptase. L: 100 bp DNA ladder. The respective base pair sizes have been mentioned in Table 1.



Supplementary Fig. 2 Dissociation curve analysis of primer-specific amplification. Dissociation analysis was performed by constant monitoring of fluorescence signal from 55 to 95°C. The background cutoff for each curve is set to 100 RFU. All primer pairs (A-V) amplified single products with melting temperature between 75°C - 95°C

Genes involved in somatic cell differentiation

Wnt4

Wnt4 expression was found to be sexually dimorphic from E11.5 to E14.5, with higher mRNA levels in the XX gonads as compared to the XY gonads. At E11.5, there was 11 fold higher expression in the XX gonads as compared to the XY gonads. After E11.5, the expression was detected to be increased in the XX gonads, peaking by E13.5 where the XX gonads show almost 45 fold higher expression as compared to in the XY gonads. The expression of *Wnt4* dropped marginally by E14.5. In the XY gonads, *Wnt4* expression did not alter from E11.5 to E14.5 (Fig 3)

Rspo1

Rspo1 mRNA levels were identical in the XY and XX gonads at E11.5. At E12.5, there was almost 10 fold higher expression in the XX gonads as compared to the XY gonads. The expression of *Rspo1* in the XX gonads was highest at E13.5, with almost 12 fold higher expression as compared to in the XY gonads. As compared to E13.5, the expression of *Rspo1* marginally decreased on E14.5. In the XY gonads there was a marginal increase in expression of *Rspo1* from E11.5 to E12.5, after which the levels remained identical till E14.5 (Fig 3).

B-Catenin

B-Catenin mRNA levels were not sexually dimorphic in the gonads at E11.5. At E12.5 and onwards there was an increase in the expression of *B-catenin* in XX gonads and the levels were significantly higher as compared to XY gonads. The expression of *B-Catenin* in the XX gonads was highest at E13.5 with the levels almost 7 fold higher than the XY counterparts. As compared to E13.5, the levels of*B-Catenin* in XX gonads dropped marginally on E14.5. In the XY gonads the mRNA levels of *B-Catenin* did not alter significantly between E11.5 to E14.5 (Fig 3).

Foxl2

Foxl2 mRNA was detected in the XX gonads at E13.5 and E14.5; no expression was detected at E11.5 or E12.5. In the XY gonads, *Foxl2* mRNA was not detectable on any of the days during development (Fig 3).

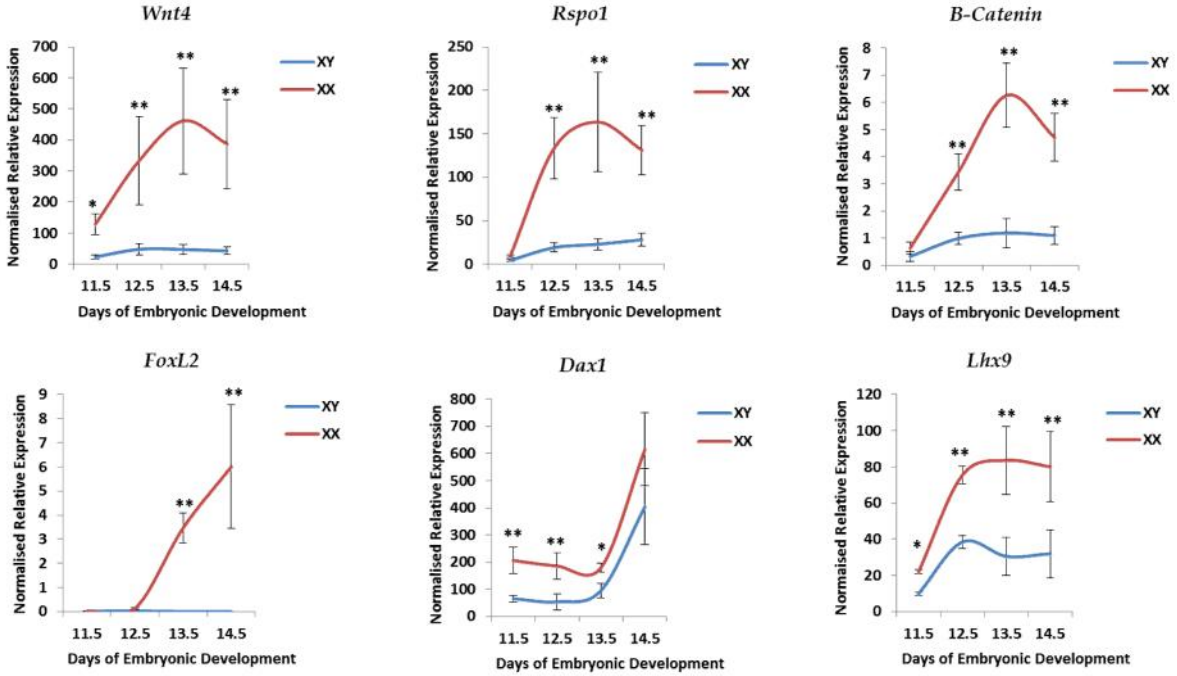


Fig. 3: Relative expression of genes associated with somatic cell differentiation in the murine XX gonads at E11.5 - E14.5. The expression levels were normalized to *18S rRNA*. Values are given as Mean ± SD. Student's T-test was used to show statistical significance, * indicates p< 0.05 and ** indicates p< 0.0005.

Dax1

*Dax1*was expressed in a sexually dimorphic pattern in the gonads from E11.5 to E14.5 with a 1.5-2 fold higher expression in the XX gonads as compared to XY gonads irrespective of the stage of development. Both, in the XX and the XY gonads, the expression of *Dax1* mRNA remained constant from E11.5 to E13.5. At E14.5, there was almost 3-4 fold increase in the expression as compared to its levels on E13.5 in both the sexes (Fig 3).

Lhx9

The levels of *Lhx*9 were 3-4 fold higher in the XX gonads as compared to XY gonads from E11.5 to E15.5. In both the sexes, the *Lhx9* mRNA expression increased at E12.5 as compared to E11.5, this increase was almost by 4 folds. Thereafter, no change in the levels of *Lhx9* was detected both in XX and XY gonads (Fig 3).

Sox9

Irrespective of the gestation age, the levels of *Sox9* were sexually dimorphic with higher expression in the XY gonads as compared to XX gonads. In the XY gonads at E11.5, the levels of *Sox9* mRNA were 40 fold higher as compared to the XX gonads, at E12.5 the levels were almost 70 folds higher. As compared to E11.5, the expression of *Sox9* increased in the XY gonads at E12.5, however the expression declined at E13.5 and E14.5 (Fig 4).

Fgf9

Fgf9 expression was higher in the XY gonads as compared to XX gonads from E11.5 to E14.5. At E11.5, the level of expression of *Fgf9* in the XY gonads was almost 2.5 fold higher than in the XX gonads. In the XY gonads, at E12.5 the expression of *Fgf9* was maximum with almost 4 fold higher in the XY gonads as compared to XX gonads. As compared to E12.5, the level of *Fgf9* mRNA were found to be decreased at E13.5 and E14.5. XX gonads had constant levels of *Fgf9* expression from E11.5 to E14.5 (Fig 4).

Amh

Amh mRNA was not detected at E11.5 in the gonads of either sexes. At E12.5 and onwards *Amh* was testis specific and only detected in the XY gonads. Its levels did not change in the XY gonads thereafter across development (Fig 4).

Dmrt1

Expression of *Dmrt*1 at E11.5 was similar in the XY and XX gonads. At E12.5 the levels were 40 fold higher in the XY gonads as compared to XX gonads. At E13.5 *Dmrt1* mRNA levels were 100 fold higher in the XY gonads as compared to XX gonads. A robust increase in *Dmrt1* mRNA was observed at E14.5 in the XY gonads and the expression was almost 500 folds higher as compared to XX gonads. In the XX gonads, no major change in expression of *Dmrt1* was observed across development (Fig 4).

Gata4

The mRNA levels of *Gata4* in XY and XX gonads were similar at E11.5. At E12.5, there was marginal increase in the expression of *Gata4* in the XY gonads as compared to E11.5. As compared to E12.5, the expression of *Gata4* increased 2 fold in the XY gonads at E13.5 and 4 fold at E14.5. The levels of *Gata4* were 4-5 fold higher in the XY gonads as compared to XX gonads both at E13.5 and E14.5. In the XX gonads the levels of *Gata4* did not change across development (Fig 4).

Cyp26b1

The levels of *Cyp26b1* in the XX and XY gonads were low and observed to be identical at E11.5. In the XY gonads at E12.5there was almost 400 fold increase in the expression of *Cyp26b1* mRNA as compared to at E11.5.*Cyp26b1* expression, in comparison to at E12.5, increased almost 10 fold at E13.5 and further 15 fold at E14.5 in the XY gonads. In the XX gonads at E12.5, *Cyp*26b1 expression was almost 17 fold lower than XY gonads and the levels continuedto be identical across development(Fig 4).

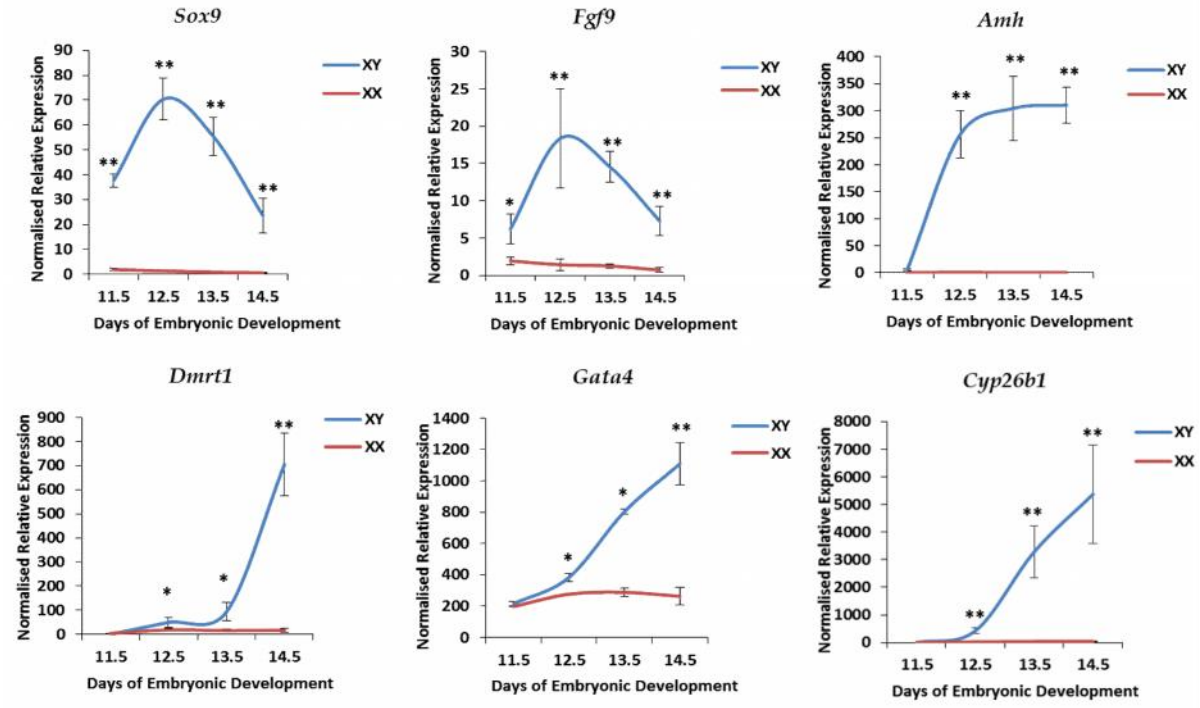


Fig. 4: Relative expression of genes associated with somatic cell differentiation in the murine XY gonads at E11.5 - E14.5. The expression levels were normalized to *18S rRNA*. Values are given as Mean ± SD. Student's T-test was used to show statistical significance, * indicates p< 0.05 and ** indicates p< 0.0005.

Genes involved in interstitial cell differentiation

In the developing gonads, *Sf1* and *Cyp17* are markers of the steroidogenic precursors, whereas*Jagged1* and *Pdgfr* labels the endothelial cells. Fig 5 gives the expression profiles of these genes in the XX and XY gonads on different stages of development.

Sf1

The expression of *Sf1* was identical in the XY and XX gonads at E11.5. In the XX gonads, the levels of *Sf1* mRNA steadily decreased and were not detectable on E14.5. In contrast, in the XY gonads, as compared to E11.5 gonads, *Sf1* mRNA levels increased on E12.5 and onwards. Highest *Sf1* mRNA levels were detected on E13.5 and E14.5. As compared to XX gonads, *Sf1* mRNA was 2.5 fold higher in the XY gonads at E12.5; the levels were more than 20 folds higher on E13.5 and E14.5 (Fig 5).

Cyp17

Cyp17 mRNA was not detected in the XX gonads at any stages of development. In the XY gonads *Cyp17* mRNA was first detectable on E13.5 which increased dramatically on E14.5 (Fig 5).

Jagged1

Jagged1 mRNA was found to be expressed in the XY dominant manner in the developing gonads. *Jagged1* mRNA was not detected in the gonads of either sexes at E11.5, but its expression was detectable in the XY gonads E12.5 onwards. In the XX gonads *Jagged1* mRNA was detectable on E13.5 and 14.5. As compared to XX gonads, the levels of *Jagged1* mRNA were 400 fold higher in the XY gonads at E13.5 the difference was further elevated at 14.5 (Fig 5).

Pdgfr

*Pdgfr*mRNA was not detectable in the XX and XY gonads at E11.5, after which it was found to be expressed in the XY dominant pattern. *Pdgfr* mRNA was first detected in the XY gonads at E12.5 and its expression was found to be increased by 3 fold at E13.5 and by 3.5 fold by E14.5. In the XX gonads, *Pdgfr* was observed at E13.5 and its expression was almost 20 fold lower as compared to XY gonad at same time point. This minimal level of expression was also detected at E14.5 (Fig 5).

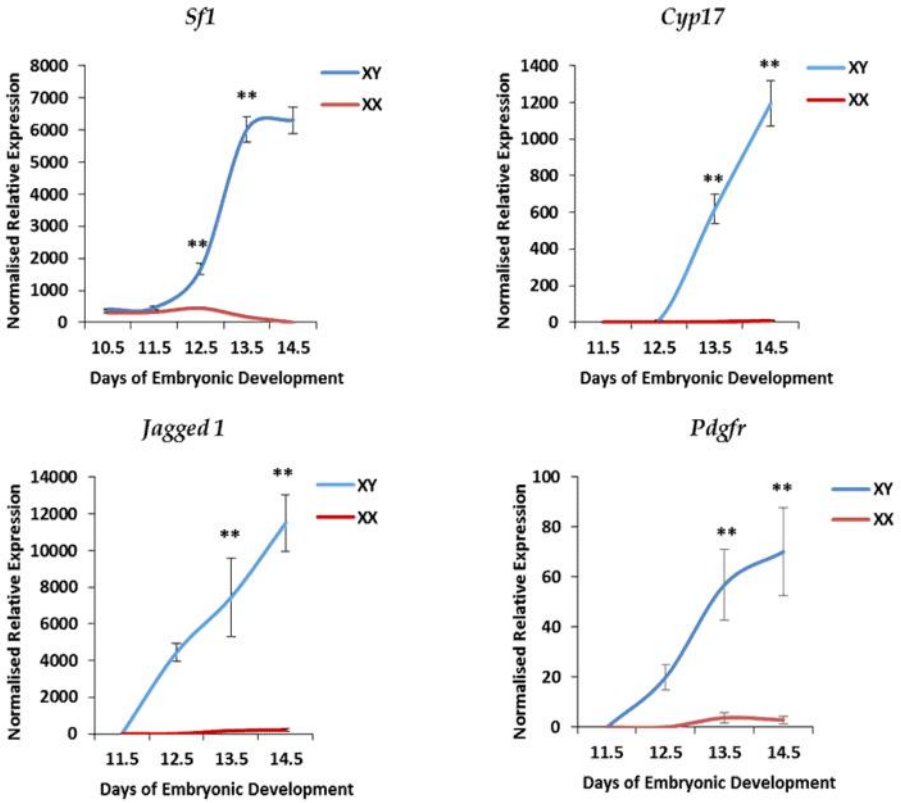


Fig. 5: Relative expression of genes involved in interstitial cell differentiation in the murine XY and XX gonads at E11.5 - E14.5. The expression levels were normalized to *18S rRNA*. Values are given as Mean ± SD. Student's T-test was used to show statistical significance, * indicates $p< 0.05$ and ** indicates $p< 0.0005$.

Expression of genes associated with germ cell development and differentiation

Dazl is known to be expressed by the PGCs, *Stra8* and *Sycp3* are reportedly expressed by the meiotic cells. *Figla* is expressed by the oocytes and is a terminal marker of germ cell differentiation in the XX gonads. Fig 6 gives the expression profiles of these genes in the XX and the XY gonads from E11.5 to E14.5.

Dazl

Expression of *Dazl* at E11.5 was identical in the XY and XX gonads (Fig 6). In both the sexes, as compared to E11.5, the expression of *Dazl* increased at E12.5 and E13.5 after which the levels reduced in the XY gonads on E14.5. The expression of *Dazl* in the XY gonads was higher as compared to XX gonads; and from E12.5 the differences were maintained until E14.5. Irrespective of the day of gestation the levels of *Dazl* were 2-3 folds higher in the XY gonads as compared to XX gonads.

Stra8

Stra8 expression was detected only in the developing XX gonads. No *Stra8* mRNA was detected in the gonads of either sex at E11.5. mRNA expression of *Stra8*was initiated in the XX gonads at E12.5 and their levels continue to increase until E14.5 only the XX gonads (Fig 6).

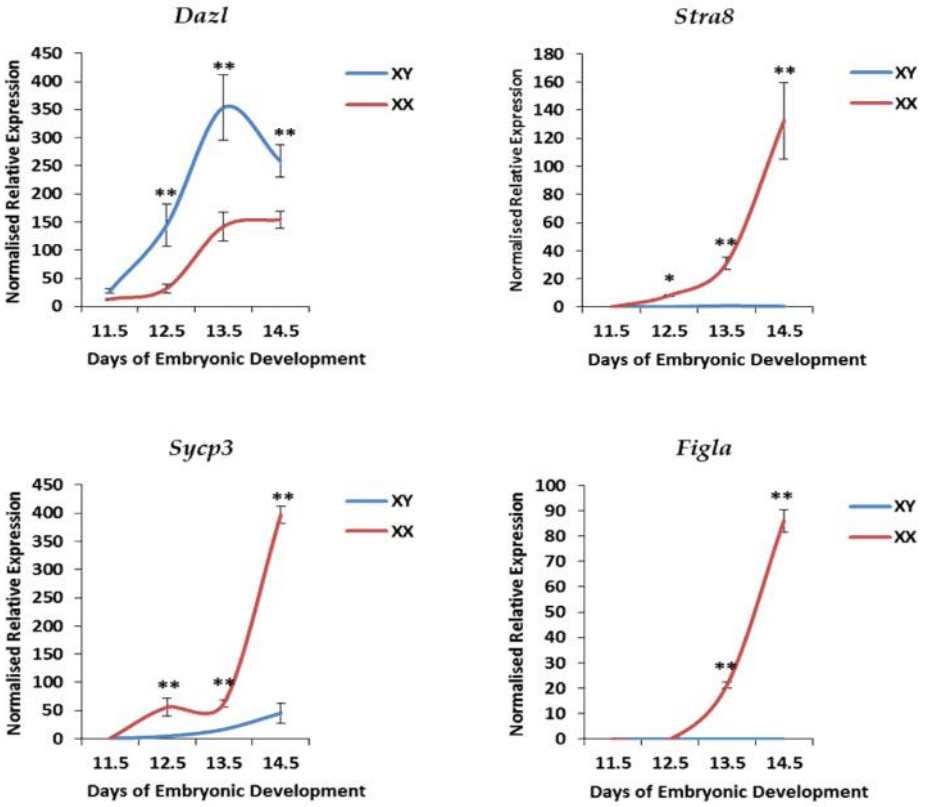


Fig. 6: Relative expression of genes associated with germ cell development and differentiation in the murine XY and XX gonads at E11.5 - E14.5. The expression levels were normalized to *18S rRNA*. Values are given as Mean ± SD (n=). Student's T-test was used to show statistical significance, * indicates $p< 0.05$ and ** indicates $p< 0.0005$.

Sycp3

No S*ycp3* transcripts were detected in the XX or XY gonads at E11.5. In the XX gonads S*ycp3* mRNA was detected at E12.5 and the levels were detected to be identical at E13.5. The expression of *Sycp3* dramatically increased at E14.5. In the XY gonads, low but detectable levels of *Sycp3* were observed on E13.5 andby E14.5 the levels were found to be marginally increased (Fig 6).

Figla

Figla mRNA was expressed only in the XX gonads (Fig 6). The expression in the XX gonads is initiated at E13.5 with the expression peaking at E14.5. There was a 4 fold higher expression of *Figla* on E14.5 as compared to at E13.5.

Discussion

In the present study, we have determined the expression of twenty genes known to be associated with sex determination in mouse. The results reveal that there are dynamic changes in the pattern of their expression during the course of gonad development. *Sox9* is the most consistent marker to study testicular fate, whereas*Wnt4* and *Lh*x9 have emerged as best markers for ovarian soma.

Studying alterations in gene expression is essential to understand the processes that are important for development. Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) is the most widely used and a robust method to study gene expression. However, there are several variations including difference in the amount of starting material, enzymatic efficiencies and differences between tissues or cells in overall transcriptional activity during different stages of development that need to be normalized. A scientifically accepted way to control most variables is normalization against internal control/reference genes. A prerequisite for a suitable reference gene is the knowledge of its expression which is not just robust, but also stable and not sexually dimorphic during the course of gonad development. Two previous studies (Bouma et al., 2004; Svingen et al., 2009) have compared the expression profiles of several reference genes. Bouma, *et al* (2004) recommended the use of global pattern recognition (GPR) program where multiple reference genes could be used to obtain reliable quantification of changes in gene expression. While most reliable, use of GPR is not very practical owing to small amounts of tissues and limited quantities of RNA obtained from gonads. The limitation is further exacerbated when mutants are used and large amounts of information are to be obtained from restricted RNA quantities. Thus GPR is not a practical solution. In an attempt to identify optimal housekeeping gene(s), Svingen*et al*(2009) analysed transcript abundance of ten frequently employed normalizing genes of which *18S rRNA*, *Rsp29*, *Tbp* and *Sdha* were found to be least sexually dimorphic and stably expressed between E11.5 toE14.5 and hence were recommended as suitable reference genes. However, Svingen*et al (*2009)used whole gonad-mesonephi complex; in the present study we have separated the gonad from the mesonephros. Thus, we first determined a suitable housekeeping gene which could be used for normalization. Corroborating with the observations of Svingen*et al*(2009), in the present study where only gonads were used for RNA isolation, we observed that *18s rRNA* is the most robust and consistently expressed in the developing mouse gonads. *Sdha*, though was not sexually dimorphic, its levels were often lower than many genes and there were some variations in its expression across development. *Gapdh*, although used by many authors as normalising gene, was found it to be sexually dimorphic with higher expression in the XX gonads as compared to XY gonads. *Gapdh* mRNA levels also had maximum variability across development. Thus, based on our data, we recommend use of *18s rRNA* as an appropriate housekeeping/ normalizing/ reference gene for qPCR analysis during gonadal development.

In mouse, the gonad develops by E9.5 and is bipotential until E10.5. In the XY embryos, *Sry* gene is transcribed in the somatic cells by E10.5 and the Sertoli cells are specified by E11.5. At E12.5, the gonad is committed towards testicular fate. In absence of *Sry*, the somatic cells differentiate towards the ovarian pathway and the granulosa cells are specified by E13.5 (Sekido and Lovell-Badge, 2013; Eggers et al., 2014; She and Yang, 2014). In an attempt to study the expression profiles of genes involved in gonad development, we performed qRT-PCR using RNA isolated from gonads between E11.5 to E14.5. Only those pools where the RNA was of high quality and gonad enriched (as assessed by RT-PCR for gonad vs mesonephros markers) were included. The results revealed that amongst the somatic cell differentiation markers, except for *Lhx*9, the sexual dimorphism in expression of most genes was as expected. *Sox9*, *Fgf9*, *Amh* and *Dmrt1* were XY dominant while *Wnt4*, *Rspo1*, *B-Catenin* and *Foxl2* were XX dominant. *Lhx9* has been reported to be expressed in the developing gonads at E9.5 in the somatic cells both in the XX and XY gonads (Birk et al., 2000). Using gonad-mesonephros complexes, Bouma,*et al*(2004)reported the expression of *Lhx*9 to be XY dominant, whereas in this study we found it to be XX dominant. At present we do not have an explanation of such contrasting finding; differences in housekeeping genes used could be a possibility.

Comparing the expression profiles of the somatic cell markers, we observed that *Amh*, *Cyp26b1* and *Dmrt1* are XY exclusive during the period of gonadal development. However all the three are expressed later in development (E12.5 for *Amh*and *Cyp26b1*and E13.5 for *Dmrt1*). This data is consistent with the earlier reported (Munsterberg and Lovell-Badge, 1991; Raymond et al., 1999; Kashimamada et al., 2011)and suggest that these genes can be reliably used as terminal markers of testicular fate. However, if one desires to study sexual dimorphism during early

development, these genes cannot be used. Both *Sox9* and *Fgf9*, as expected, were XY dominant right from E11.5, *Fgf*9 was less abundant and had high standard deviations as compared to *Sox9*. *Gata4* is an early somatic cell marker and it specifies the pre-Sertoli cells that express *Sry*(Vigner et al., 1998). However its expression is sexually dimorphic only by E12.5 and more robust change is observed at E13.5. Based on our analysis, *Sox9* is the best marker to study the Sertoli cell differentiation during period of sex determination; we suggest use of *Sox9* and *Amh*/*Dmrt1* as a best combination for evaluation of Sertoli cell fate during development.

In case of XX gonads, we found that with the exception of *Foxl2*, all the genes were also expressed in the XY gonads. Like *Dmrt1* in case of XY gonads, *Foxl2* is only detected in the XX gonads from E13.5 and would be a reliable terminal marker of ovarian fate. During early development, *Wnt4* was found to be sexually dimorphic from E11.5, *Rspo1* and *B-Catenin* were dimorphic only E12.5 onwards. This observation is intriguing, as based on studies in the knockout mice *Rspo1* is reportedly upstream of *Wnt4* (Tomizuka et al., 2008). However, in this study we found that *Wnt4* becomes sexually dimorphic a day earlier (E11.5) as compared to *Rspo1* (E12.5). While knockout studies are a formal proof in determination of the proximal and distal members of a cascade, is possible that during sex determination, similar to the *Sox9-Fgf9* loop (Sekido and Lovell-Badge, 2013; Eggers et al., 2014; She and Yang, 2014); *Wnt4*also may be induced earlier than *Rspo1* in the XX gonads, but its subsequent maintaining may be *Rspo1* dependent. Our studies have revealed that beyond the known genes, *Lhx9* has emerged to be a novel gene involved in ovarian development. Since *Lhx9* is sexually dimorphic and expressed in an XX dominant manner during entire window of sex determination, we speculate that it might also function in ovarian development. Based on our results, expression of *Wnt4* and *Lhx9* are the most reliable and robust markers to test the ovarian fate in the developing mouse gonads during the period of sex differentiation. To summarize, our results revealed that *Amh* and *Dmrt*1 are exclusively expressed in the XY gonads; their expression appears only at E12.5 and E13.5 respectively, indicating that they can be the best markers to determine terminal testicular differentiation. *Sox9* appeared as the best marker that could be used for studying testicular fate as early as E11.5 until E14.5. In case of ovarian soma, *Wnt*4 and *Lhx*9 were consistently XX dominant from E11.5 to E14.5 and had minimal variability. *Foxl2* is XX exclusive but appears only at E13.5 and hence could be used as terminal marker for ovarian fate.

During gonad development, once the somatic cell fate is established the interstitial cell fate is recognized where the leydig cells differentiate in the XY gonads (Griswold and Behringer, 2009). *Sf1* in the bipotential gonads specifies pre-Sertoli cells that express *Sry* and *Sox9*(Sekido and Lovell-Badge, 2008). Eventually, interstitial cells are specified cells as leydig cell precursor under *Sf1* that become *Cyp17* positive (Barsoum et al., 2013). Consistent with this, *Sf1* was observed to be XY dominant at E12.5 with a robust increase at E13.5. *Cyp17* is a marker of differentiated foetal steroidogenic cells (Scott et al., 2009) and is detectable only by E13.5 in the XY gonads. In the XX gonads, *Sf1* is detected at low levels in XX gonads until E12.5; its expression is undetectable by E13.5. *Cyp17* however is absent XX gonads. Thus both these genes are useful markers to study the steroidogenic cell differentiation by qRT-PCR during gonadal development.

During gonadal development, vascularization of the XY gonads but not the XX gonads is a conspicuous feature (Coveney et al., 2008). Time course live imaging studies have shown that the endothelial cells differentiate in the mesonephros and migrate in to the XY but not the XX gonads by late E12.5 and proper vasculature is established by E13.5 (Coveney et al., 2008). Traditionally, *PECAM* was used as a marker for detecting the vasculature cells in immunofluorescence studies (Jeays-Ward et al., 2003); but it is not vascular cell specific and is also expressed by the germ cells (Wakayama et al., 2003). *Jagged1*, a ligand for NOTCH signalling system, is expressed in the interstetium and is a marker for the migrating endothelial cells from the mesonephros to the gonad (Brennan et al., 2002). *Pdgfr* is essential for testicular cell fate determination and organogenesis as it is responsible for cell proliferation, endothelial cell migration from the mesonephros, vascularization and foetalleydig cell differentiation. *Pdgfr*−/− XY gonads displayed disruptions in the organization of the vasculature and in the partitioning of interstitial and testis cord compartments(Brennan et al., 2003). Thus we analysed the expression of both these markers in the XX and XY gonads. As expected from the imaging studies, in the XY gonads, our results show a significant increase in the expression of *Jagged1*and *Pdgfr*from E12.5 which continue to increase until E14.5, corroborating the increase in the number of endothelial cells. In the developing XX gonads, *Jagged1* and *Pdgfr* are not detectable until E12.5,

but at E13.5their transcripts are detectable but at levels lower than XY gonads.

Important components of the gonads are the germ cells that form the future sperm and the ova in the XY and XX gonads respectively. The germ cells (termed as primordial germ cells) are specified in the extra embryonic mesoderm and migrate via the hind gut and reside in the gonads by E10.5, where they proliferate until E12.5 (Western, 2009; Rossitto et al., 2015). In the XY gonads, the primordial germ cells undergo mitotic arrest, whereas in the XX gonads they enter meiosis and get arrested in the prophase stage to form the oocytes (Hu et al., 2015). While the array of markers for primordial germ cells have been extensively described (Western, 2009; Rossitto et al., 2015; Hu et al., 2015), the first marker to appear in primordial germ cells to make them competent for gametogenesis is *Dazl*(Gill et al., 2011). *Dazl* is required for embryonic germ cell development and survival of XY germ cells prior to E14.5 (Gill et al., 2011). Our results demonstrate that the expression of *Dazl* is identical in both the sexes at E 11.5, but at E 12.5 the expression of *Dazl* increases in the XY gonads and peaks at E13.5. This corresponds to the increased rates of proliferation of the germ cells in the XY gonads between E11.5 to E13.5 (Rossitto et al., 2015; Gill et al., 2011). In the XY gonads, the germ cells cease to proliferate by E14.5 (Western, 2009; Rossitto et al., 2015) when the levels of *Dazl* start reducing. As compared to the XY gonads, the expression of *Dazl* is lower in the XX gonads. Unlike, in the XY gonads, *Dazl* mRNA levels increase in the XX gonads at E13.5 after which their levels remain constant. These observations underscore the fundamental differences in the germ cells of the XY and XX gonads. In the XX gonads on E12.5, the germ cells enter meiosis whereas they undergo mitotic arrest in the XY gonads owing to degradation of retinoic acid due to increased expression of *Cyp26b1*. In the XX gonads, retinoic acid acts on germ cells to induce expression of *Stra8* which is an indispensable requirement for entry of germ cells in to meiosis (Feng et al., 2014). Keeping with these observations, *Stra8* mRNA was detected only in the XX gonads first at E12.5 and its expression gradually increase until E14.5 indicating that meiosis is first initiated in the XX gonads at E12.5 and progressively more germ cells enter meiosis during the course of development. Once the germ cells enter meiosis, the synaptonemal complex protein *Sycp3* gets loaded on to thesynaptonemal complex, a meiosis-specific protein structure essential for synapsis of homologous chromosomes (Rossitto et al., 2015; Feng et al., 2014). Consistent with the *Stra8* data, *Sycp3* mRNA is first detected in the gonads at E12.5 with a robust increase at E14.5 indicating progressive entry of germ cells in to meiosis. However unlike *Stra8* which was exclusively detected in the XX gonads, *Sycp3* mRNA is detected in the XY gonads as well. This observation is intriguing as germ cells enter meiosis in the XY gonads only at puberty. However a previous study has demonstrated expression of *Sycp3* mRNA in the XY foetal gonads (Chuma and Nakatsuji, 2001), the functional significance of this finding is yet unclear.

In summary, from our study we conclude that *Sox*9, *Fgf9*and*Amh* are important for the formation of testis and could be important markers to detect the fate of differentiated Sertoli cells.*Sox9* can be used as markers at E11.5 and *Amh* and/or *Fgf9* will be important markers at E12.5. *Cyp17* can be used as a marker to check the activity of leydig cells and the efficiency of XY gonad's critical function, steroidogenesis. Endothelial cell migration in the XY gonads post E12.5 can be analysed by checking the levels of *Jagged1*. *Dazl*, *Stra8* and *Sycp3* are the markers which will dictate the number and functioning of the germ cells in the XY gonads.

To the best of our knowledge this is a comprehensive and first of its kind study elucidating marker/s to be used at a specific time point of gonadal development to access differentiation of particular cell types and formation of a gonad.

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Conflicts of Interest:

The author declares no conflict of interest.

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