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New Candidate Genes Identified for Controlling Mouse Gonadal Sex Determination and the Early Stages of Granulosa and Sertoli Cell Differentiation[1]

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ABSTRACT

Mammalian gonadal sex-determining (GSD) genes are expressed in a unique population of somatic cells that differentiate into granulosa cells in XX gonads or Sertoli cells in XY gonads. The ability to efficiently isolate these somatic support cells (SSCs) during the earliest stages of gonad development would facilitate identifying 1) new candidate GSD genes that may be involved in cases of unexplained abnormal gonad development and 2) genes involved in the earliest stages of granulosa and Sertoli cell differentiation. We report the development of a unique mouse carrying two transgenes that allow XX and XY mice to be distinguished as early as Embryonic Day 11.5 (E11.5) and allow SSCs to be isolated from undifferentiated (E11.5) and early differentiated (E12.5) fetal gonads. The Mouse Genome 430v2.0 GeneChip (Affymetrix) was used to identify transcripts exhibiting a sexual dimorphic expression pattern in XX and XY isolated SSCs. The analysis revealed previously unidentified sexually dimorphic transcripts, including low-level expressed genes such as *Sry*, a gene not identified in other microarray studies. Multigene real-time PCR analysis of 57 genes verified that 53 were expressed in fetal gonads in a sexually dimorphic pattern, and whole-mount in situ hybridization analysis verified *4930563E18Rik*, *Pld1*, and *Sprr2d* are expressed in XX gonads, and *Fbln2*, *Ppargc1a*, and *Scrn1* are expressed in XY gonads. Taken together, the data provide a comprehensive resource for the spatial-temporal expression pattern of genes that are part of the genetic network underlying the early stages of mammalian fetal gonadal development, including the development of granulosa and Sertoli cells.

fetal gonads, gene expression, mammalian, sex determination, somatic support cells

INTRODUCTION

Sexual differentiation in mammals begins when the pair of bipotential gonadal primordia (i.e., the genital ridges) initiates development as ovaries in XX individuals or testes in XY individuals. Failure of the gonads to differentiate properly can lead to disorders of sexual development, including partial or complete gonadal sex reversal (e.g., XX testes and XY ovaries) followed by abnormal differentiation of the internal and external secondary reproductive structures (reviewed in Hughes [1] and Siklar et al. [2]). Testis development comprises an extensive pathway initiated by the expression of the Y-linked gene *Sry* [3, 4] followed by the upregulation of the transcription factor *Sox9* [5, 6]. Ovarian development appears to rely on a complex interaction of the signaling molecules *Wnt4* and *Rspo1*, which activate beta-catenin and prevent *Sox9* upregulation [7–9]. The molecular events that are critical for differentiation of XX and XY bipotential gonads occur in a specific population of somatic cells, the somatic support cells (SSCs).

The SSCs originate from coelomic epithelium cells covering the genital ridge [10]. The SSCs differentiate as granulosa cells in the XX gonad and Sertoli cells in the XY gonad [11]. In the developing ovary, precursor SSCs differentiate into fetal granulosa cells that 1) associate with germ cell clusters to form ovigerous cords, 2) likely play a role in the breakdown of these cords, and 3) form primordial follicles at the time of birth [12]. In the developing testis, precursor SSCs express *Sry*, leading to their proliferation and differentiation into Sertoli cells [10, 11]. Fetal Sertoli cells are essential regulators of testicular organogenesis: They prevent primordial germ cells from entering meiosis [13, 14] and induce migration of cells from the adjacent mesonephros into the fetal testis [15], where they differentiate into steroidogenic (Leydig) and vascular endothelial cells [15, 16].

The key role of the precursor SSC population during gonad development is evident by the observation that these cells express genes involved in gonadal sex determination (GSD). Genes such as *Sry*, *Sox9*, *Gata4*, *Zfpm2* (*Fog2*), *Wnt4*, *Fgf9*, *Nr0b1* (*Dax1*), *Nr5a1* (*Sf1*), *Rspo1*, and *Wt1* originally were identified as GSD genes because when mutated, they caused abnormal gonadal development, including sex reversal (reviewed in Brennan and Capel [17], Park and Jameson [18], and Wilhelm et al. [19]). The fact that many cases of human gonadal sex reversal remain unexplained indicates that additional GSD genes await discovery. We reasoned that the rate of GSD gene discovery would improve if the gene expression profile of SSCs was determined during the early stages of gonad development. We chose to conduct these experiments in mice because the necessary genetic tools were available. Not only would the planned approach provide a powerful resource for identifying additional GSD genes, it would provide a list of potential genes to examine in cases of unexplained human abnormal GSD.

Two previous studies have revealed the expression profile of isolated *Sf1-EGFP*-positive gonadal somatic cells from mouse XX versus XY fetal gonads [20, 21]. *Sf1* is expressed in XX and XY SSCs. Because *Sf1* is expressed in additional cell types, including precursor steroidogenic cells, using the *Sf1*-

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EGFP transgene introduced the possibility that expression of genes unique to the SSCs was masked in the mixed cell population. For example, neither study detected the expression of the critical GSD gene *Sry*, known to be expressed in precursor SSCs.

We report here a comparison of the gene expression profile in XX versus XY SSCs isolated from Embryonic Day 11.5 (E11.5) and E12.5 gonads. This was accomplished by creating a unique mouse carrying an *Sry-EGFP* transgene expressed only in SSCs and an X-linked trangene that distinguishes XX and XY individuals by eye pigmentation. A microarray analysis of the transcriptional profile of E11.5 and E12.5 XX versus XY SSCs identified the spatial-temporal sexual dimorphic expression pattern of many new genes. Real-time RT-PCR analysis verified that 53 of 57 genes tested were expressed in a sexual dimorphic pattern, and whole-mount in situ hybridization (WISH) analysis confirmed the expression pattern of six genes in XX and XY fetal E11.5, E12.5, and E13.5 gonads. Combined, these data provide an important resource for determining the genetic network controlling fetal gonad development and differentiation.

MATERIALS AND METHODS

The Jackson Laboratory is American Association for Laboratory Animal Science accredited, and all animal procedures were approved by The Jackson Laboratory Animal Care and Use Committee.

Mouse Strains

FVB.Cg-Tg(Tyr)3412ARpw Tg(Sry-EGFP)92Ei. To allow XX and XY SSCs to be efficiently isolated from fetal gonads, we constructed a special FVB inbred strain carrying two transgenes. The origin of both transgenes and the method used to construct this strain are presented below. For brevity, this strain is designated FVB-Tg3412A,Tg92.

FVB/NJ-Tg3412A. Visual inspection of the gonads cannot be used to determine the sex of individual fetuses prior to E12.5. To circumvent this problem, we obtained FVB-Tg3412A transgenic mice from Dr. Richard Woychik, then at the Oak Ridge National Laboratory (Oak Ridge, TN). FVB-Tg3412A transgenic mice were created by injecting a tyrosinase minigene (1.95-kb cDNA sequence; Tg3412A) into FVB/N fertilized eggs [22, 23]. Normally, FVB/N mice are homozygous for the albino allele of the tyrosinase gene, and thus are unpigmented. Because Tg3412A inserted into the X chromosome, FVB females carrying a single copy of Tg3412A had a mosaic pigmented pattern, whereas FVB females homozygous for Tg3412A and males hemizygous for Tg3412A were pigmented. By mating normal (albino) FVB/NJ females to FVB/NJ males carrying Tg3412A, the sex of each offspring can be determined as early as E11: XY offspring lacked eye pigmentation (Tg3412A absent), whereas XX offspring displayed a ring of pigment (Tg3412A present) at the outer edge of the eye (Supplemental Fig. S1; all Supplemental Data are available online at www.biolreprod.org). The FVB/N-Tg3412A strain was maintained so that Tg3412A remained segregating.

C57BL/6J-Tg(Sry-EGFP)92Ei. To isolate SSCs from undifferentiated gonads, we used an *Sry-EGFP* transgene (hereafter called Tg92). Tg92, which consists of the 5′ regulatory region of the *Sry* gene (base pairs 542-8304) driving an *EGFP* reporter gene, is expressed specifically in precursor XX and XY SSCs [11]. (Because the *Sry* open reading frame is absent, Tg92 does not cause testicular development in XX mice.) Tg92 was produced in and maintained in a homozygous state on the C57BL/6J strain background.

FVB-Tg3412A,Tg92. To construct FVB-Tg3412A,Tg92, an F(1) female, produced by mating a C57BL/6J-Tg92 male to an FVB-Tg3412A female, was backcrossed to an FVB/NJ male. Because knowing the chromosomal position of Tg92 would facilitate distinguishing homozygous from heterozygous Tg92 mice, a genome scan was performed on the backcross (N2) offspring. Tg92 was located on chromosome 5 between *D5Mit255* and *D5Mit197* (data not shown). (Tg3412A was mapped on the X chromosome between *DXMit187* and *DXMit122*.) An N2 mouse carrying Tg92 and the B6-derived alleles of the *D5Mit* loci flanking Tg92 was mated to an FVB mouse, and this mating scheme was continued. At backcross generation N9, a female carrying Tg92 and the B6-derived alleles of *D5Mit255* and *D5Mit197* was mated to an FVB-Tg3412A male (to reintroduce Tg3412A), and their N10 offspring were typed to identify individuals carrying Tg92 and the B6-derived alleles of *D5Mit255* and *D5Mit197*. An N10 female and a male meeting these criteria were mated, and their N10F2 offspring were typed to identify those who carried Tg3412A and were homozygous for B6 alleles of the Tg92 flanking markers (thus homozygous for Tg92). A female and male meeting these criteria were selected and used to establish the FVB-Tg3412A,Tg92 strain. As in the case for the original FVB-Tg3412A strain, Tg3412A was kept segregating in this newly constructed strain.

Embryo Staging and Tissue Collection

Timed matings, determined by the presence of a vaginal plug, were used to identify fetuses within a development period. Because individual developmental variation occurs among littermates, more precise embryonic staging was used to determine the developmental stage of each fetus. Fetuses younger than E13 were staged by counting tail somites (ts) distal to the hind limb buds (approximately eight ts corresponds to E10.5, approximately 18 ts to E11.5, and approximately 28 ts to E12.5) [24]. Fetuses E13 and older were staged according to fore and hind limb bud morphology [25]. Gonad-mesonephros complexes (E11.5) or gonads (E12.5 and older) were collected and pooled according to phenotypic sex as determined by eye pigmentation. XX and XY genotypes were confirmed retrospectively by PCR analysis of tail lysate using a Y-chromosome-specific sequence [26, 27].

Isolation of EGFP-Positive Precursor SSCs

XX and XY SSCs were isolated as described previously [28]. Briefly, E11.5 and E12.5 gonad-mesonephros complexes from same-stage, same-sex fetuses were pooled and placed in 1.5-ml centrifuge tubes containing RNAse-free PBS. A single-cell suspension was obtained by enzymatic digestion with trypsin-ethylenediaminetetraacetic acid (Gibco, Carlsbad, CA), DNaseI, and gentle pipetting of the partial digest. The resulting cell suspension was filtered through a sterile mesh (122-μm pore size), and the filtrate containing single cells was kept on ice until further use (less then 30 min).

EGFP-positive XX and XY SSCs were isolated from the single-cell suspensions using a FACSVAntageSE/DiVa option flow cytometer (BD Biosciences, San Jose, CA) as described in Bouma et al. [28]. EGFP-positive XX and XY cells were collected in 100 μl of Extraction Buffer (Arcturus, Mountain View, CA) and stored at −80°C until further analysis.

RNA Isolation

Single cells for microarray analysis. Total RNA was isolated from EGFP-positive XX and XY SSCs using the PicoPure RNA isolation kit (Arcturus) according to the manufacturer's instructions. Approximately 500 XX and 1100 XY EGFP-positive cells were used per sample at E11.5, and 4600 XX and 800 XY EGFP-positive cells were used per sample at E12.5.

Whole gonads for real-time RT-PCR analysis. Gonad-mesonephros complexes (E11.5) and gonads (E12.5 and E13.5) were collected from FVB/NJ XX and XY fetuses. Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions, with minor modifications as described previously [27].

Microarray Analysis

Total RNA quantity and quality were determined using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Rockland, DE) and the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA), respectively. Isolated total RNA from each sample was used to generate amplified, biotin-labeled cDNA using a TargetAmp 2-Round Aminoallyl-aRNA Amplification Kit (Epicenter Biotechnologies, Madison WI). All protocols were conducted according to the manufacturer's instructions.

A total of 12 Mouse Genome 430v2.0 GeneChip arrays (Affymetrix, Santa Clara, CA) were used (three XX and three XY for E11.5 and E12.5 isolated EGFP-positive SSCs). The GeneChip arrays were scanned using a laser confocal slide scanner (GeneChip Scanner 3000; Affymetrix). The quality of the arrays was assessed, and images were quantified using GeneChip Operating Software (GCOS v1.2; Affymetrix). Probe-level data were imported into the R software environment (www.r-project.org [29]), and R/affy software was used to normalize the data, perform background subtraction, and obtain summary expression values for each probe [30, 31]. Statistical analysis was conducted as described previously [28], and statistical significance was determined at $q \leq 0.10$ (false discovery rate [FDR] $\leq$ 10%) and $q \leq 0.01$ (FDR $\leq$ 1%) for E11.5 and E12.5, respectively.

Real-Time RT-PCR

Multigene real time RT-PCR analysis was performed on total RNA isolated from gonad-mesonephros complexes (E11.5) and fetal gonads (E12.5, E13.5) to validate differential expression of genes identified by microarray analysis.

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TABLE 1. Fifty significantly differentially expressed transcripts between E11.5 XX and XY SSCs exhibiting the greatest fold change.

E11.5 XX > XY				E11.5 XY > XX			
Probe set ID	Gene symbol[a]	Fold change	Chromosome	Probe set ID	Gene symbol[a]	Fold change	Chromosome
1427262_at	*Xist*	136.7	X	1417210_at	*Eif2s3y*	95.6	Y
1415832_at	*Agtr2*	14.3	X	1420786_a_at	*Rbmy1a1*	71.6	Y
1450079_at	***Nrk***	10.4	X	1438718_at	*Fgf9*	57.0	14
1416286_at	***Rgs4***	9.3	1	1424903_at	*Kdm5d*	40.2	Y
1455851_at	***Bmp5***	8.9	9	1449742_at	***AA522020***	29.6	Y
1453351_at	*Tbx20*	8.4	9	1426438_at	*Ddx3y*	29.1	Y
1455735_at	*Ap1s3*	6.5	1	1419758_at	***Abcb1a***	26.1	5
1456389_at	*Zeb2*	6.2	2	1436279_at	*Slc26a7*	22.0	4
1435386_at	*Vwf*	5.9	6	1424950_at	*Sox9*	16.5	11
1419599_s_at	***Ms4a6d***	5.8	19	1426598_at	*Uty*	12.4	Y
1442029_at	*Kcnq1*	5.6	7	1419080_at	*Gdnf*	8.5	15
1447426_at	***Appl2***	4.8	10	1460336_at	*Ppargc1a**	8.1	5
1439059_at	***BC031748***	4.8	X	1441909_s_at	***Nipal4***	6.9	11
1416832_at	***Slc39a8***	4.7	3	1444038_at	*AU015836*	6.7	X
1421883_at	***Elavl2***	4.5	4	1438654_x_at	*Mmd2*	6.3	5
1456402_at	***A330076H08Rik***	4.2	7	1441213_at	*BC021891*	6.2	8
1453211_at	***Ccdc89***	4.0	7	1420589_at	***Has3***	5.8	8
1429905_at	*Lhx9*	3.9	1	1418744_s_at	*Tesc*	5.2	5
1441469_at	*Dock4*	3.8	12	1439500_at	*Scrn1**	5.1	6
1438930_s_at	***Mecp2***	3.8	X	1432901_at	*Etl4*	4.9	2
1444875_at	***Ppp2ca***	3.7	11	1418872_at	***Abcb1b***	4.5	5
1436791_at	*Wnt5a*	3.5	14	1423407_a_at	*Fbln2**	4.5	6
1425918_at	*Egln3*	3.4	15	1445669_at	*Spry4*	4.4	18
1452365_at	*Csgalnact1*	3.3	8	1442019_at	***BB627097***	4.1	11
1434645_at	***C530008M17Rik***	3.2	5	1443013_at	*AW552930*	4.0	13
1428629_at	***Zfp518***	2.6	19	1434528_at	*Aard*	4.0	15
1423621_a_at	***Slc33a1***	2.6	3	1457270_at	*Gas7*	3.8	11
1442312_at	***Tbl1xr1***	2.5	3	1438470_at	*Socs2*	3.8	10
1416688_at	***Snap91***	2.5	9	1427062_at	***Rbbp8***	3.7	18
1435452_at	***Tmem20***	2.4	19	1418723_at	***Lpar3***	3.7	3
1447547_at	*Ltbp1*	2.4	17	1425807_at	*BC021891*	3.4	8
1420443_at	***Pcdhb19***	2.4	18	1415871_at	*Tgfbi*	3.3	13
1434286_at	***Trps1***	2.4	15	1455794_at	*Smtnl2*	3.3	11
1434425_at	***Tchh***	2.3	3	1434275_at	*Nkd2*	3.2	13
1417549_at	***Zfp68***	2.3	5	1455136_at	***Atp1a2***	3.2	1
1422890_at	*Pcdh18*	2.3	3	1421425_a_at	***Rcan2***	3.0	17
1434621_at	***Tmem204***	2.3	17	1434873_a_at	*Acap1*	3.0	11
1429478_at	***6720463M24Rik***	2.3	14	1453191_at	*Col27a1*	3.0	4
1454937_at	***B630005N14Rik***	2.2	6	1437621_x_at	*Phgdh*	2.8	7
1450117_at	***Tcf3***	2.2	6	1450579_x_at	*Sry*	2.7	Y
1457830_at	***AV247312***	2.2	13	1424652_at	***Fam176a***	2.6	6
1434414_at	***Foxred2***	2.2	15	1418746_at	***Pnkd***	2.6	1
1436854_at	***Trpc2***	2.2	7	1449251_at	***Ndp***	2.6	X
1449051_at	*Ppara*	2.2	15	1425837_a_at	***Ccrn4l***	2.5	3
1436278_at	***BM934468***	2.2	X	1418061_at	***Ltbp2***	2.5	12
1429579_at	***6330407I18Rik***	2.2	9	1446914_at	***C80425***	2.5	2
1430569_at	***Ttc9c***	2.2	19	1434092_at	***Atg9b***	2.4	5
1427736_a_at	***Ccrl2***	2.2	9	1448700_at	*G0s2*	2.4	1
1435113_x_at	***Stmn3***	2.1	2	1454070_a_at	***Ddhd1***	2.4	14
1456126_at	*Malt1*	2.1	18	1432332_a_at	***Nudt19***	2.4	7

[a] Transcripts in bold are newly identified sexually dimorphic expressed transcripts in SSCs that were not identified in *Sf1-EGFP* positive XX and XY somatic cells isolated from E11.5 gonads (http://www.medecine.unige.ch/recherche/research_groups/nef/microarrays.php [20]; [21]).
* Transcripts examined by WISH analysis.

For each time point and each sex, tissues from five fetuses were used. Initially, primer pairs were designed for 73 genes significantly differentially expressed between XX and XY SSCs at E11.5 and/or E12.5. After validating primer specificity (i.e., single band after PCR amplification and sequencing of PCR products), 57 genes were considered for further analysis (primer sequences provided in Supplemental Table S1). To examine the expression of these 57 genes during mouse fetal gonadal development, multigene real-time RT-PCR analysis was conducted as described previously [27]. The housekeeping genes *Rn18s* rRNA and *Gapdh* were included in the real-time PCR assay, as well as *Sry* and *Dhh* (testis specfic) and *Fst* (ovary specific). Fold change and statistically significant difference were determined using Global Pattern Recognition (GPR) software v2.0 and the Student *t*-test [27, 32].

Whole-Mount In Situ Hybridization

Analysis by WISH was performed to examine the localization pattern of 16 genes identified as differentially expressed between XX and XY gonads by microarray analysis and real-time RT-PCR, and for which no previous WISH data were available. FVB or (B6 × FVB)F(1) fetuses were collected at E11.5, E12.5, and E13.5, and a piece of tail was placed in lysate buffer (see above) for later determining the presence of the Y chromosome. For each transcript, localization was examined in gonads from three XX and three XY fetuses at each time point. After removing the head, the body below the forelimbs was opened, and internal organs were dissected away to expose the gonads. The body/gonads were then fixed in 4% parafomaldehyde (PFA) in PBS at 4°C overnight. After removing the PFA, the body/gonads were washed twice with

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TABLE 2. Fifty significantly differentially expressed transcripts between E12.5 XX and XY SSCs exhibiting the greatest fold change.

E12.5 XX > XY				E12.5 XY > XX			
Probe set ID	Gene symbol[a]	Fold change	Chromosome	Probe set ID	Gene symbol[a]	Fold change	Chromosome
1428089_at	***Slitrk1***	96.1	14	1417210_at	*Eif2s3y*	150.5	Y
1427263_at	*Xist*	82.1	X	1460693_a_at	*Col9a3*	104.8	2
1434458_at	*Fst*	66.4	13	1428571_at	*Col9a1*	79.7	1
1432031_at	*4930563E18Rik**	48.3	18	1434099_at	*Casp7*	63.1	5
1449991_at	***Cd244***	41.6	1	1419080_at	*Gdnf*	58.0	15
1435314_at	*Tph2*	40.7	10	1444038_at	***AU015836***	51.5	X
1454926_at	***Sphkap***	40.2	1	1426438_at	*Ddx3y*	49.0	Y
1460084_at	*BB473929*	39.1	—	1434959_at	*Dhh*	45.2	15
1446308_at	*1700106J16Rik*	37.2	11	1435486_at	*Pak3*	45.1	X
1417122_at	*Vav3*	33.3	3	1436870_s_at	*Afap1l2*	44.5	19
1457352_x_at	***Svopl***	32.1	6	1428142_at	*Etv5*	43.5	10
1456557_at	*Ccdc30*	30.0	4	1423860_at	*Ptgds*	37.1	2
1452366_at	*Csgalnact1*	29.9	8	1424903_at	*Kdm5d*	35.1	Y
1460604_at	***Cybrd1***	29.4	2	1438068_at	*BB251859*	35.0	5
1420771_at	*Sprr2d**	29.1	3	1438654_x_at	*Mmd2*	34.7	5
1442379_at	***Fam196b***	27.6	11	1433787_at	***Nell1***	34.3	7
1455056_at	*Lmo7*	27.0	14	1435554_at	***Tmcc3***	33.5	10
1420433_at	***Taf7l***	25.9	X	1427168_a_at	*Col14a1*	33.4	15
1440452_at	*Drp2*	25.6	X	1433607_at	*Cbln4*	31.8	2
1443913_at	*BG071002*	23.3	—	1416666_at	*Serpine2*	31.7	1
1418536_at	***LOC386462***	23.2	17	1455664_at	***Rtn4rl1***	29.4	11
1441429_at	***Irs4***	22.5	X	1451712_at	*8030411F24Rik*	29.1	2
1434411_at	*Col12a1*	21.7	9	1451236_at	*Rerg*	28.3	6
1436659_at	*Dclk1*	20.4	3	1423271_at	*Gjb2*	28.2	14
1418517_at	*Irx3*	20.0	8	1440339_at	***Enpp1***	27.9	10
1437112_at	*Pld1**	19.0	3	1438408_at	*Ankrd56*	27.7	5
1419719_at	*Gabrb1*	18.6	5	1454622_at	***Slc38a5***	27.0	X
1435095_at	*C030009O12Rik*	18.5	5	1438718_at	*Fgf9*	27.0	14
1450065_at	*Adcy7*	18.3	8	1443287_at	*Gm1337*	26.7	2
1417504_at	*Calb1*	17.2	4	1421114_a_at	*Epyc*	26.4	10
1429814_at	*Pank1*	17.2	19	1418743_a_at	*Tesc*	25.4	5
1435578_s_at	*Dab1*	17.0	4	1456509_at	*1110032F04Rik*	25.2	3
1433988_s_at	*C230098O21Rik*	16.7	8	1443322_at	*AV328597*	24.1	—
1438532_at	***BB024475***	16.7	1	1455436_at	***Diras2***	23.9	13
1436319_at	*Sulf1*	16.5	1	1418497_at	*Fgf13*	23.5	X
1451264_at	***Frmd6***	16.1	12	1418813_at	*Serpina5*	23.0	12
1455595_at	***Ugt2b36***	15.8	5	1434606_at	*Erbb3*	22.2	10
1452670_at	*Myl9*	15.7	2	1456823_at	***Gm70***	21.7	12
1449319_at	*Rspo1*	15.5	4	1448955_s_at	*Cadps*	21.7	14
1436405_at	***Dock4***	15.4	12	1456076_at	***Defb19***	21.0	2
1436361_at	***Vgll2***	15.3	10	1417408_at	***F3***	20.3	3
1419324_at	*Lhx9*	15.1	1	1434873_a_at	*Acap1*	20.3	11
1423072_at	*6720475J19Rik*	14.8	19	1434528_at	*Aard*	20.2	15
1447839_x_at	*Adm*	14.5	7	1435693_at	*Mall*	20.0	2
1436853_a_at	*Snca*	13.9	6	1445669_at	*Spry4*	19.7	18
1422836_at	*Mbnl3*	13.8	X	1436279_at	***Slc26a7***	19.7	4
1456960_at	*Adk*	13.6	14	1441213_at	*BC021891*	18.9	8
1427044_a_at	*Amph*	13.4	13	1448507_at	***Efhd1***	18.4	1
1440534_at	*BB177862*	13.1	1	1433532_a_at	*Mbp*	18.2	18
1449641_at	*AI508733*	13.1	14	1420504_at	*Slc6a14*	18.1	X

[a] Transcripts in bold are newly identified sexually dimorphic expressed transcripts in SSCs that were not identified in *Sf1-EGFP* positive XX and XY somatic cells isolated from E12.5 gonads (http://www.medecine.unige.ch/recherche/research_groups/nef/microarrays.php [20]; [21]).
* Transcripts examined by WISH analysis.

PBS/0.1% Tween 20 (PBT) at 4°C, dehydrated through a methanol series at room temperature, and stored in 100% methanol at −20°C for up to 2 mo.

Primers were designed to PCR amplify a fragment of each gene to be analyzed by WISH (Supplemental Table S2). The PCR fragments were cloned into pGEM-T easy (Promega, Madison, WI) and sequenced using the M13F primer (GCCAGGGTTTTCCCAGTCACGA). The DNA insert and flanking regions of the plasmid containing the T7 and SP6 promoters were amplified using PCR with M13F and M13R (GAGCGGATAACAATTTCACACAGG) primers to generate a riboprobe template.

Digoxigenin (DIG)-labeled riboprobes were synthesized by in vitro transcription using a DIG RNA Labeling Kit (Roche Applied Sciences, Indianapolis, IN). The in vitro transcription reaction, containing 1 μl of 10× transcription buffer, 2 μl of DIG nucleotide triphosphate labeling mix, 2 μl of RNA polymerase (T7 or SP6 depending on orientation of the DNA insert), and ~0.5–1 μg of riboprobe template DNA in 10 μl total volume, was incubated at 37°C for 1 h. The DNA template was degraded by adding 2 μl of DNaseI and incubating at 37°C for 15 min. The enzymatic reactions were stopped by adding 2 μl of 0.2 M EDTA, and the riboprobe was precipitated by adding 3.4 μl of 2 M sodium acetate and 55 μl of 100% ethanol, and incubating at −20°C for at least 30 min. After centrifugation at 15 800 × g for 10 min at 4°C, the riboprobe was washed with 70% ethanol, resuspended in 100 μl of RNase-free water, and stored at −20°C.

The WISH protocol used was based on the alternate protocol for mouse and chicken embryos as described in *Current Protocols in Molecular Biology* [33]. Briefly, the body containing the gonads was washed twice with PBT on ice and was rehydrated through a graded methanol series at room temperature. After digestion with 10 μg/ml proteinase K in PBT, body/gonads were washed and postfixed in 0.2% glutaraldehyde/4% PFA and were incubated overnight in prehybridization solution (50% deionized formamide, 5× saline sodium citrate [SCC], 0.1% Tween 20, 0.5% 3-[(3-cholamidopropyl)dimethylammonio]-1-

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TABLE 3. Expression of selected gene markers for precursor granulosa cells (XX SSCs) and precursor Sertoli cells (XY SSCs).

Gene symbol	Higher in E11.5 SSCs (fold change)	Higher in E12.5 SSCs (fold change)
XY		
Amh	1.1 (NS)	2.8
Cyp26b1	1.6 (NS)	4.3
Fgf9	57.0	15.0
Sox9	16.5	15.8
Sry	2.7	NS
XX		
Fst	2.7 (NS)	66.4
Irx3	1.4 (NS)	20.0
Rspo1	1.3 (NS)	15.5
Wnt4	1.7 (NS)	4.7

NS = not significant at $q \leq 0.10$ (E11.5) or $q \leq 0.01$ (E12.5).

propanesulfonate [CHAPS], 5 mM EDTA, 50 μg/ml yeast RNA, and 50 μg/ml heparin). This was followed by an overnight incubation in hybridization solution (50% deionized formamide, 5× SCC, 1% SDS, 50 μg/ml yeast RNA, and 50 μg/ml heparin) containing 1 μg/ml DIG-labeled riboprobe. Riboprobe detection was performed by incubating body/gonads overnight in preblock solution (10% sheep serum, 1% bovine serum albumin, and 0.1% Tween 20) containing 1:2000 dilution of a DIG antibody conjugated to alkaline phosphatase Fab fragments (Roche Applied Sciences). The next day, body/gonads were incubated in 1 ml of BM Purple (Roche Applied Sciences) at room temperature in the dark. The color reaction was monitored periodically with the aid of a dissecting microscope, and the reaction was stopped by washing six times in PBT. Body/gonads were stored in PBT containing EDTA at 4°C until photographed. Prior to photography, gonad-mesonephros complexes were dissected free from the adhering tissue.

RESULTS

Isolation of Precursor XX and XY SSCs and Microarray Analysis

The mouse genome 430v2.0 GeneChip was used to examine the expression profile of isolated E11.5 and E12.5 XX and XY SSCs. Raw expression values for each data set are available at the National Center for Biotechnology Information Gene Expression Omnibus (GSE18211). At E11.5, 238 probes were differentially expressed (level significance $q < 0.10$) between XX and XY SSCs, with 103 probe IDs higher in XX and 135 probe IDs higher in XY SSCs. At E12.5, 5374 probes were expressed significantly ($q < 0.01$) differentially between XX and XY SSCs, with 2547 probe IDs higher in XX and 2827 probe IDs higher in XY SSCs. The 50 transcripts exhibiting the highest fold change are shown in Tables 1 and 2, and include known GSD genes. Importantly, 34 of the 50 transcripts and 19 of the 50 transcripts expressed at significantly higher levels in E11.5 XX and XY SSCs, respectively, were not noted as differentially expressed in *Sf1-EGFP*-positive XX versus XY cells isolated from fetal E11.5 gonads [20, 21]. Similarly, 14 of 50 and 12 of 50 transcripts expressed at significantly higher levels in E12.5 XX and XY SSCs, respectively, were not reported as differentially expressed in *Sf1-EGFP*-positive XX versus XY somatic cells from E12.5 fetal gonads [20, 21].

Successful isolation of a pure XX and XY SSC population from E11.5 and E12.5 fetal gonads was confirmed by examining the expression of several genes known to be expressed in these cells (Table 3). Importantly, *Sry* (initiates testicular development) was identified as expressed significantly higher in E11.5 XY compared with XX SSCs. *Sry* was not identified previously as differentially expressed in *Sf1-EGFP*-positive XX and XY cells isolated from E11.5 gonads [20, 21]. In addition, germ cell-specific genes (e.g., *Pou5f1*) were not expressed, as determined by an "Absent" call using

TABLE 4. Significantly different expressed genes (GPR score ≥0.4; fold change ≥2.0) according to real-time RT-PCR analysis.[a]

Gene symbol	GPR score	Fold change[b]
E11.5 XX vs XY		
4930563E18Rik	0.620	3.2
Dclk1	0.580	2.0
Gdnf	0.840	−5.2
Sprr2d	0.560	3.4
Sry	1.000	−800.3
E12.5 XX vs XY		
4930563E18Rik	0.891	24.0
BC021891	0.913	−10.2
BB114398	0.761	4.6
Bmp5	0.435	2.7
Col14a1	0.761	−2.5
Csgalnact1	0.457	3.6
Cybrd1	0.739	3.1
Dhh	0.978	−226.8
Dock4	0.761	3.9
Fam196b	0.891	16.4
Fbln2	0.565	−4.6
Fst	0.978	106.5
Gdnf	0.674	−8.2
Mmd2	0.913	−9.3
Pak3	0.913	−9.5
Pld1	0.761	3.7
Ppargc1a	0.630	−2.1
Rerg	0.913	−5.8
Scrn1	0.674	−2.2
Slitrk1	0.848	12.9
Sprr2d	1.000	4599.1
Spry4	0.565	−2.6
Sry	0.957	−45.8
Taf7l	0.826	15.3
Vwf	0.457	2.2
E13.5 XX vs XY		
4930563E18Rik	0.929	28.2
Agtr2	0.476	2.2
AV370141	0.929	−13.5
BB114398	0.667	2.8
Col14a1	0.714	−3.1
Csgalnact1	0.595	3.4
Cybrd1	0.571	2.6
Dclk1	0.571	5.3
Dhh	0.952	−66.3
Dock4	0.619	2.9
Etl4	0.667	−2.9
Etv5	0.690	−2.3
Fam196b	0.857	18.1
Fst	0.976	97.7
Gdnf	0.929	−24.0
Irs4	0.643	4.1
Mmd2	0.905	−17.5
Pak3	0.690	−4.6
Pld1	0.667	2.7
Ppargc1a	0.476	−2.3
Rerg	0.738	−5.3
Scrn1	0.571	−2.0
Slc26a7	0.429	−2.1
Slitrk1	0.786	7.1
Spry4	0.429	−2.2
Sry	0.857	−10.6
Taf7l	0.667	4.6
Zeb2	0.619	−2.1

[a] Significance (indicated by GPR score) and fold change were determined using Global Pattern Recognition v2.0 [26, 32].
[b] Positive and negative fold changes indicate higher expression in XX and XY gonads, respectively.

Affymetrix GCOS analysis (data not shown). Finally, similar to previous studies identifying sexually dimorphic expressed transcripts in fetal gonads, several sex chromosome-linked genes were identified (Tables 1 and 2). For example, the X-linked gene *Xist* was expressed significantly higher in E11.5

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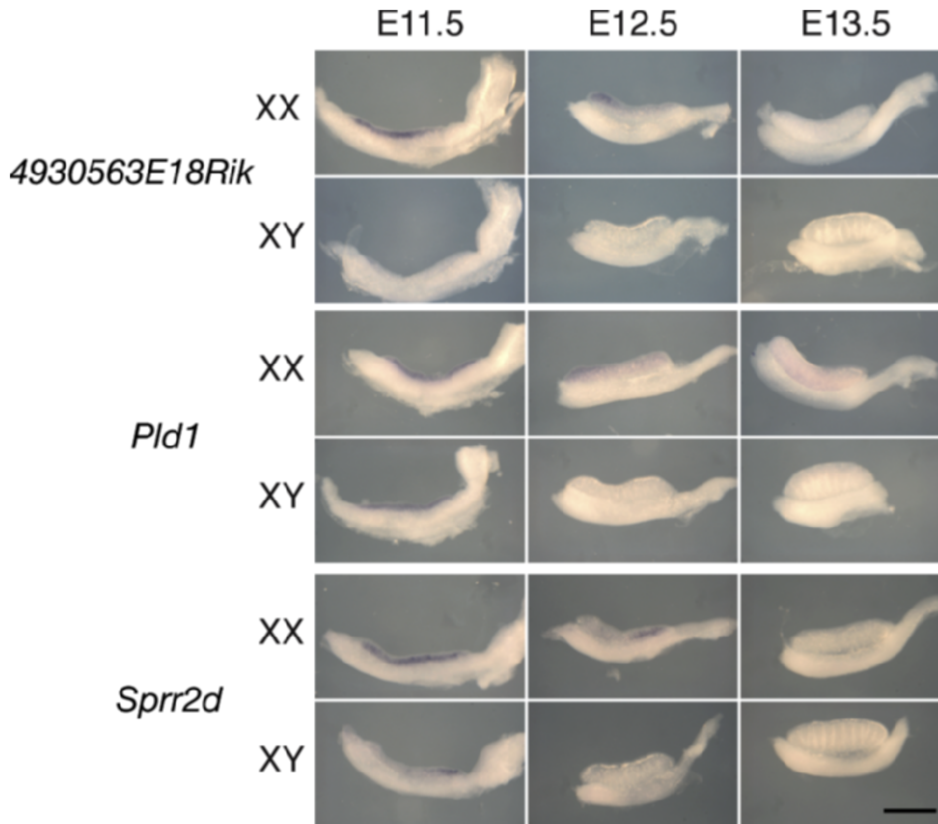

FIG. 1. Transcripts preferentially expressed in XX gonads as revealed by WISH. The anterior end of the gonad faces left. Contrast was enhanced using Adobe Photoshop. Bar = 250 μm.

and E12.5 XX SSCs, whereas the Y-linked genes *Eif2s3y*, *Ddx3y*, and *Kdm5d* (*Jarid1d*) were expressed significantly higher in E11.5 and E12.5 XY SSCs.

Sexual Dimorphic Expression of Genes: Real-Time RT-PCR

To confirm the sexual dimorphic expression of 57 genes identified by microarray analysis as expressed differentially between XX and XY SSCs at E11.5 or E12.5, multigene real-time PCR assay was performed on RNA isolated from gonad-mesonephros complexes (E11.5) and fetal gonads (E12.5, E13.5; see *Materials and Methods*). Table 4 lists the genes expressed at significantly different levels (GPR $\geq$ 0.400; fold change $\geq$ 2.0) between XX and XY gonads and, as expected, the testis-specific genes *Sry* and *Dhh* were expressed at significantly higher levels in XY gonads, whereas the ovary-specific gene *Fst* was expressed at significantly higher levels in XX gonads. At E11.5, when XX and XY genital ridges are morphologically indistinguishable, real-time PCR analysis revealed significantly higher expression of *Gdnf* in XY and significantly higher expression of *4930563E18Rik*, *Dclk1* (*Dcamkl1*), and *Sprr2d* in XX genital ridges (Table 4). After the initiation of both the fetal ovarian and testicular developmental pathways, the number of genes exhibiting significant different expression patterns between XX and XY gonads increased at E12.5 and E13.5. As noted in Table 4, a total of 53 of the 57 genes tested were differentially expressed, validating the data obtained from our microarray experiment.

Sexual Dimorphic Expression of Genes: WISH

The spatial-temporal expression pattern of six transcripts, that exhibited a sexual dimorphic expression profile according to our microarray and real-time RT-PCR data was examined by WISH. The *4930563E18Rik*, *Pld1*, and *Sprr2d* transcripts were clearly expressed in E11.5 XX genital ridges, whereas only faint staining was observed in XY genital ridges for *4930563E18Rik* and *Sprr2d* (Fig. 1). At E12.5, *4930563E18Rik* and *Pld1* expression was detected in the ovary at the anterior position, whereas *Sprr2d* expression was evident at a posterior position (Fig. 1). No expression was observed for these transcripts in E12.5 and E13.5 testes, and expression was barely detected in E13.5 ovaries (Fig. 1). Tissue treated with sense probes showed no staining for *Sprr2d* and *Pld1*, whereas ubiquitous staining of the XX and XY gonads and mesonephroi was observed for *4930563E18Rik* (data not shown).

Expression of *Fbln2*, *Ppargc1a*, and *Scrn1* was preferentially observed in XY compared with XX gonads. Distinct staining was evident in E11.5 XY genital ridges, whereas expression was faint or diffuse in XX genital ridges (Fig. 2). Distinct staining was observed for all three transcripts within testicular cords of E12.5 and E13.5 testes, whereas staining was absent in ovaries. In addition, strong staining for *Fbln2* was observed in the anterior end of E12.5 and E13.5 XX and E13.5 XY mesonephroi (Fig. 2). Similarly, the anterior end of E12.5 and E13.5 XY mesonephroi exhibited intense staining for *Srcn1*. No staining was observed for sense probes *Fbln2*, *Ppargc1a*, and *Scrn1* (data not shown).

DISCUSSION

Transcriptome Analysis of Isolated Somatic Cells from Mouse Genital Ridges

Since the discovery of the mammalian testis-determining gene *Sry* in 1990 [3, 4], many studies have focused on identifying additional genes important for fetal gonadal development and differentiation. Initial genome-wide approaches used whole fetal gonads sampled at different developmental time points and identified transcripts differen-

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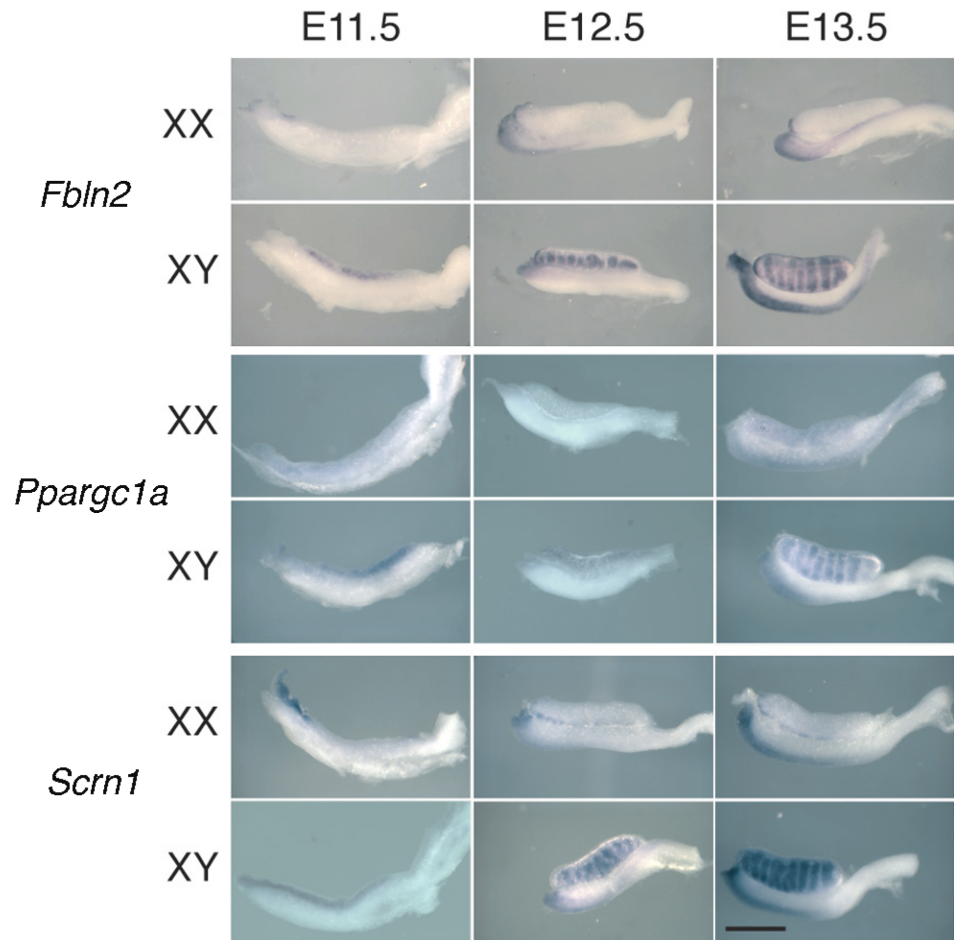

FIG. 2. Transcripts preferentially expressed in XY gonads as revealed by WISH. The anterior end of the gonad faces left. Contrast was enhanced using Adobe Photoshop. Bar = 250 μm.

tially expressed between XX and XY gonads [34–38]. A potential disadvantage of using a whole-gonad approach is that information regarding the cellular localization for any gene is lost. Two recent studies strove to overcome the whole-gonad problem by determining the sexually dimorphic expression pattern of isolated somatic cells obtained from undifferentiated and early-differentiated XX and XY gonads. In both studies, the isolation of somatic cells was accomplished by employing an *Sf1-EGFP* trangene, allowing *Sf1-EGFP*-positive cells to be analyzed [20, 21]. These data did provide a better picture of differentially expressed transcripts in XX and XY gonadal somatic cells. However, because the transcription factor *Sf1* is expressed in cells of the steroidogenic lineage (i.e., XX theca cells and XY Leydig cells) and the support cell lineage (SSCs; i.e., XX granulosa cells and XY Sertoli cells), the data did not distinguish which genes were expressed in which cell lineage. Given that all currently identified GSD genes are expressed in fetal SSCs (e.g., *Sry*, *Sox9*, *Wnt4*, *Fst*), it is possible that GSD genes awaiting discovery are expressed in SSCs, thus making a case for using a model that allows SSCs to be separated from the remaining gonadal cells. Having an isolated, purified SSC population available also is important for overcoming detection problems for genes expressed at low levels or in few SSC cells, such as the *Sry* gene.

In the mouse, *Sry* is expressed in XY SSCs between E10.5 and E12.5, with peak expression levels at E11.5 [24]. *Sf1-EGFP*-positive cells isolated from E10.5, E11.5 [20, 21], and E12.5 [20] XX and XY gonads failed to exhibit differential expression of *Sry* [20, 21], suggesting that 1) the probes present on the Mouse Genome 430v2.0 GeneChip are not sensitive enough to detect *Sry* expression or 2) expression in SSCs was masked by the presence of additional *Sf1*-positive cells. Our data rule out the suggestion that the probes present on the Affymetrix chip fail to detect *Sry*. As shown in Tables 1–3, *Sry-EGFP*-positive XX and XY SSCs isolated from undifferentiated E11.5 gonads detected significant differential *Sry* expression between XX and XY SSCs, a result in concert with the finding of others that *Sry* is at peak expression levels in mouse E11.5 gonads [24]. In addition, expression differences for *Sry* were still evident at E12.5, a time when *Sry* expression is diminishing in fetal XY gonads. Taken together, our results for *Sry* suggest that the differential gene expression profile obtained for isolated *Sf1-EGFP*-positive cells masked detection of some genes expressed in SSCs. This argument is strengthened when examining the first 50 transcripts exhibiting the greatest fold difference between XX and XY SSCs. As noted in Tables 1 and 2, we identified a number of transcripts that were not identified as differentially expressed in *Sf1-EGFP*-positive XX versus XY cells (see published data resource at www.medecine.unige.ch/recherche/research_groups/nef/microarrays.php [20]). In addition, Tables 1 and 2 include transcripts that were not identified as upregulated in E11.5 compared with E10.5 XX and XY *Sf1-EGFP*-positive isolated cells [21].

Both *Sf1-EGFP* and *Sry-EGFP* genome profiling experiments comparing isolated XX to XY fetal gonadal somatic cells revealed sexual dimorphic expression of several genes known to play a role in fetal ovarian and testicular development and differentiation. These include significantly higher expression of *Sox9*, *Amh*, *Dhh*, *Cyp26b1*, and *Fgf9* in fetal testes, and significantly higher expression of *Rspo1*, *Wnt4*, and *Fst* in fetal ovaries (Tables 1–3). During ovarian development, one of the

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most dramatically upregulated genes in fetal XX SSCs is *Fst* (Table 3). Previous studies have demonstrated that *Fst* is necessary to prevent development of a coelomic blood vessel and promote primordial germ cell survival [39, 40]. Thus, early upregulation of *Fst* in XX SSCs is likely necessary to both antagonize the testicular developmental pathway and promote the ovarian developmental pathway.

During the initiation of fetal testicular development, *Sry* is transiently expressed in a subset of somatic cells that will differentiate into fetal Sertoli cells. Additional somatic cells are recruited to develop as Sertoli cells through PGD2-mediated upregulation of *Sox9* [41], although it is unclear whether these cells transiently expressed *Sry*. It is possible that by using the *Sry-EGFP* transgene, we isolated a subset of XY SSCs that differentiate into fetal Sertoli cells. However, considering that most precursor Sertoli cells express *Sry*, the half-life of EGFP allows *Sry*-positive cells to be identified and isolated beyond the period of their endogenous expression pattern [28], and known GSD genes were successfully identified, we suggest that using the *Sry-EGFP* transgene allows the majority of XX and XY SSCs to be isolated.

We conclude that the transcriptional profile comparison of isolated *Sry-EGFP*-positive XX versus XY SSCs presented here provides a complementary, important resource for the identification of novel genes important for mammalian fetal gonadal development and differentiation.

Expression Pattern of Identified Genes by Real-Time PCR and WISH

Multigene real-time PCR analysis and WISH were conducted to provide further insight into the temporal-spatial expression and localization pattern of transcripts identified in the microarray experiment. The relative, quantitative expression difference was confirmed for 53 transcripts between XX and XY gonads during the process of XX and XY fetal gonadal differentiation, adding to the growing list of sexual dimorphic expressed genes associated with fetal ovarian and testicular development. Four (*Drp2*, *Kcnq1*, *Slc39a8*, and *Tmcc3*) of 57 transcripts were not confirmed by real-time PCR, because the relative levels in gonad-mesonephros complexes (E11.5) and whole gonads (E12.5 and E13.5) were considered not expressed (Ct values > 37). It is possible that for these four transcripts, expression levels are very low and are confined to just the SSCs, thus preventing their detection in whole tissues using real-time PCR.

Analysis by WISH was performed to examine localization patterns of transcripts for which no previous WISH data had been reported. The localization pattern of nine transcripts (*Cybrd*, *Slitrk1*, *Gas7*, *EG574403*, *Taf7l*, *Slc26a7*, *Bmp5*, *Gdnf*, and *Tbx20*) was not determined using WISH. One possible reason for this failure is that WISH is not sensitive enough to detect transcripts expressed at very low levels. Because expression of *Sry* (expressed only in precursor SSCs) has been examined using WISH [42], it also is possible that technical issues related to the WISH procedure and/or the design of the DIG-labeled RNA probes prevented their detection.

Expression of *4930563E18Rik*, *Pld1*, and *Sprr2* was noted in XX genital ridges as early as E11.5 but was barely detectable by E13.5. This period of expression corresponds to when primordial germ cells enter the gonad, continue proliferation, and then enter meiosis. Currently, a function for these genes in regulating fetal gonadal development and differentiation is unknown.

Expression of *Pld1* and *Sprr2d* was previously reported in adult mouse ovaries and uteri, respectively [43, 44]. *Pld1* (phospholipase D) encodes a membrane-associated enzyme involved with phosphatidic acid formation and functions as a signaling molecule regulating processes such as cell proliferation, differentiation, and secretion [45]. Kim et al. [43] examined expression of *Pld1*/PLD1 in various mouse tissues and demonstrated PLD1 localization to granulosa cells of growing follicles, suggesting a role in regulation of follicular growth. *Sprrd2* belongs to the small proline-rich gene family encoding a structural protein associated with the formation of protective barriers provided by stratified squamous epithelium [46], and recently was demonstrated to exhibit a center-to-pole expression pattern during fetal ovarian development [47]. Expression of *Pld1* and *Sprrd2* during the earliest stages of fetal ovarian development is a new finding and suggests that both genes are important novel regulators of pre-granulosa cell development.

Expression of *Fbln2*, *Ppargc1a*, and *Scrn1* was detected preferentially in XY genital ridges as early as E11.5, and expression was prominent within the testicular cords and the anterior portion of the adjacent mesonephros at E13.5. *Fbln2* (fibulin-2) encodes an extracellular matrix protein with calcium binding and epidermal growth factor domains. Previously, *Fbln2* expression was observed in embryonic testes and appeared to localize to tunica cells covering the testis and gonocytes [48]. Recently, Sicot et al. [49] generated *Fbln2* knockout mice and reported no effect on fertility. However, a possible compensatory upregulation of *Fbln1* was noted, suggesting functional redundancy between *Fbln2* and *Fbln1*. *Ppargc1a* (peroxisome proliferative-activated receptor gamma, coactivator 1 alpha; also known as *Pgc1*) previously was not described as having a sexual dimorphic expression pattern in mouse fetal gonads. *Ppargc1a*, a transcriptional coactivator, is implicated in regulating energy metabolism and adaptive thermogenesis [50]. Furthermore, *Ppargc1a* can interact with and coactivate several steroid hormone receptors, including the thyroid hormone and estrogen receptors, regulating downstream target genes. Although the function of *Fbln2*, *Ppargc1a*, and *Scrn1* in fetal testis development is unclear, their prominent expression as early as E11.5 indicates they play important roles in regulating testicular growth and/or morphogenesis.

We report the successful isolation of precursor SSCs from XX and XY fetal gonads during the critical stage of GSD; namely, the time when the fetal ovarian and testicular developmental pathways are initiated and precursor SSCs differentiate into fetal pre-granulosa cells and fetal Sertoli cells in XX and XY gonads, respectively. Transcriptional genome profiling of the XX and XY SSCs revealed sexual dimorphic expression of known GSD genes, including *Rspo1* and *Sry*. Moreover, this approach allowed the detection of low-expressing genes in SSCs that previously were not detected by microarray analysis of isolated gonadal somatic cells containing more than just SSCs. In addition, profiling the expression of genes in SSCs now allows additional sexually dimorphic genes to be explored as to their role in GSD and in fetal granulosa and Sertoli cell development. Both multigene real-time RT-PCR and WISH confirmed preferential expression of transcripts during fetal ovarian or testicular development, supporting the idea that these genes play important roles in mammalian gonadal differentiation. Finally, the data presented provide a comprehensive resource of the spatial-temporal expression pattern of genes that are part of the genetic

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network underlying mammalian fetal gonadal development and differentiation.

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