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Gene expression during sex determination reveals a robust female genetic program at the onset of ovarian development

Serge Nef [a,*], Olivier Schaad [b], Nancy R. Stallings [c], Christopher R. Cederroth [a], Jean-Luc Pitetti [a], Guillaume Schaer [a], Safia Malki [d], Michel Dubois-Dauphin [e], Brigitte Boizet-Bonhoure [d], Patrick Descombes [b], Keith L. Parker [c], Jean-Dominique Vassalli [a]

[a] *Department of Genetic Medicine and Development, University of Geneva Medical School, 1211 Geneva 4, Switzerland*
[b] *Genomics Platform, National Center of Competence in Research Frontiers in Genetics, 1211 Geneva 4, Switzerland*
[c] *Department of Internal Medicine, University of Texas Southwestern Medical Center, Dallas, Texas 75390-8857, USA*
[d] *Institut de Génétique Humaine, CNRS UPR1142, 141 rue de la Cardonille, 34396 Montpellier Cedex 5, France*
[e] *Department of Geriatrics, Biology of Aging Laboratory University of Geneva, 1211 Geneva 4, Switzerland*

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Abstract

The primary event in mammalian sexual development is the differentiation of the bipotential gonads into either testes or ovaries. Our understanding of the molecular pathways specifying gonadal differentiation is still incomplete. To identify the initial molecular changes accompanying gonadal differentiation in mice, we have performed a large-scale transcriptional analysis of XX and XY Sf1-positive gonadal cells during sex determination. In both male and female genital ridges, a robust genetic program is initiated pre-dating the first morphological changes of the differentiating gonads. Between E10.5 and E13.5, 2306 genes were expressed in a sex-specific manner in the somatic compartment of the gonads; 1223 were overexpressed in XX embryos and 1083 in XY embryos. Although sexually dimorphic genes were scattered throughout the mouse genome, we identified chromosomal regions hosting clusters of genes displaying similar expression profiles. The cyclin-dependent kinase inhibitors *Cdkn1a* and *Cdkn1c* are overexpressed in XX gonads at E11.5 and E12.5, suggesting that the increased proliferation of XY gonads relative to XX gonads may result from the overexpression of cell cycle inhibitors in the developing ovaries. These studies define the major characteristics of testicular and ovarian transcriptional programs and reveal the richness of signaling processes in differentiation of the bipotential gonads into testes and ovaries.

Keywords: Sf1; Gonads; Sex determination; Ovary; Testis; Gene regulation

Introduction

Gonadal development is unique in embryology because a common precursor, the bipotential gonad, can assume two divergent fates, forming either testes or ovaries. In mice, the bipotential gonads arise shortly before embryonic day 10.5, as evidenced by a thickening of the coelomic epithelium adjacent to the mesonephros; they contain somatic cells and primordial germ cells (Brennan and Capel, 2004; McLaren, 2003). Commitment of the bipotential gonad to form testes is dictated by a single gene on the Y chromosome, designated SRY for *S*ex-determining *r*egion, *Y* chromosome. *SRY* encodes a DNA binding protein of the high mobility group (HMG) box family that is both necessary and sufficient to direct testis determination (Koopman et al., 1991). In the absence of SRY, both XX and XY gonads develop along the ovarian pathway (Capel et al., 1993). Sry expression in the XY gonads starts at the 11–12 tail somite (ts) stage just after embryonic day 10.5 (E10.5) and peaks at 18 ts (E11.5), 36 h prior to the earliest histologic evidence of testis differentiation and finally declines towards 28 ts (E12.5) (Bullejos and Koopman, 2001; Hacker et al., 1995; Jeske et al., 1995). In a cell-autonomous fashion, Sry promotes male development by directing the supporting cell lineage to form Sertoli cells instead of follicular cells (Palmer

* Corresponding author.
E-mail address: Serge.Nef@medecine.unige.ch (S. Nef).

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and Burgoyne, 1991; Sekido et al., 2004). The Sertoli cells, in turn, initiate cellular and morphological changes such as testis cord formation, migration of cells from the mesonephros, vascularization and Leydig cell differentiation (for review, see Brennan and Capel, 2004).

Although the cellular processes of male sexual differentiation have been studied extensively, the specific molecular mechanisms by which gonads are formed and, in particular, the nature of the cascade of putative transcriptional events initiated by Sry remain poorly understood. Key regulators of development of the bipotential gonad in both sexes include steroidogenic factor 1 (SF1), Wilm's tumor 1 (WT1), Lhx9 and Emx2 (for review, see Capel, 2000). In the case of *Sf1* null mice, gonadal development is initiated, but the gonadal primordium regresses by apoptosis at E12.5, indicating that Sf1 is required for the differentiation and/or the maintenance and growth of the somatic cells in the developing gonad (Luo et al., 1994). Over the past years, several key regulators of the male pathway have been identified. These include Sox9 (Kent et al., 1996; Morais da Silva et al., 1996), Dmrt1 (Raymond et al., 1998, 2000), Fgf9 (Colvin et al., 2001), Dhh (Bitgood et al., 1996), Sox8 (Chaboissier et al., 2004) and Dax1 (Meeks et al., 2003). However, the molecular pathogenesis of 46 XY gonadal dysgenesis, 46 XY male to female sex reversal or 46 XX true hermaphroditism remains enigmatic in more than 75% of patients, suggesting that other genes affecting sex determination are yet to be discovered (see review by Cotinot et al., 2002).

One of the earliest consequences of Sry expression is increased proliferation of somatic cells, resulting in a dramatic increase in the size of embryonic testes relative to ovaries. Treatment of E11 mice with proliferation inhibitors blocks the formation of testis cords and male sex differentiation, establishing the essential role of this increased cell proliferation in the male pathway (Schmahl and Capel, 2003). However, the identity of the proteins mediating the XY-specific proliferation or the XX-specific quiescence of the differentiating gonads remains elusive.

Insights into the molecular events in early ovarian development are even more rudimentary. For more than 50 years, female development was considered the "default" pathway due largely to the observation that SRY triggers the male pathway, while the female pathway proceeds in the absence of male-promoting signals. A current model of female sex determination suggests that the stable commitment to the ovarian pathway occurs when the germ cells enter meiosis (Yao et al., 2003). The autonomous entry of the germ cells into meiosis initiates the ovarian pathway and blocks testis development. SRY opposes this pathway by initiating cord formation prior to meiosis, thereby sequestering germ cell inside testis cords and blocking meiosis. Although a female-determining gene analogous to SRY has not been identified, it is obvious that a specific program of ovarian organogenesis is somehow triggered. Unfortunately, very little is known about the molecular regulation of early ovarian development. Only a handful of genes with a female-specific expression have been linked directly to the earliest events in ovarian development and function: follistatin (Shimasaki et al., 1989), Wnt4 (Vainio et al., 1999), Bmp2 (Zhang and Bradley, 1996), Dax1 (Swain et al., 1996) and GDF9 (Elvin et al., 1999a,b). Thus, there is a compelling need to define additional genes that mediate ovarian development and to define molecular milestones that herald specific stages in these processes.

In view of the differential development of the bipotential gonad in response to a single genetic switch (i.e., the presence of absence of SRY), gonadal development provides an excellent opportunity to identify genes involved in differential organogenesis. Genes with sexually dimorphic expression patterns have been identified by either differential display (Tohonen et al., 1998), subtractive hybridization (McClive and Sinclair, 2003; Menke and Page, 2002) or cDNA microarrays (Grimmond et al., 2000; Smith et al., 2003). Due to technical limitations, these experiments assayed only a small fraction of the transcriptome and were performed at stages (E12.5 and E13.5) when testicular or ovarian differentiation is already well underway. In addition, they included both the somatic and the germ cell compartments of the developing gonads. The goal of the present study was to identify and characterize the transcriptional changes in somatic cells that accompany the differentiation of the bipotential gonads into either testes or ovaries. We surveyed the expression profile of approximately 34,000 genes in the somatic compartments of XX and XY gonads prior to, during and after sex determination (E10.5–E13.5) and identified a large number of genes that exhibit sexually dimorphic expression before the first morphological changes of the differentiating gonad. We have thus defined the major characteristics of distinct transcriptional programs within the somatic compartments of male and female gonads that underlie testicular and ovarian differentiation.

Materials and methods

Isolation of Sf1/eGFP-positive cells and RNA extraction

C57/B6 CBAJ female mice were bred with Sf1/eGFP transgenic male mice. The mice were maintained on a 12L:12D cycle. Adult females were time-mated and checked for the presence of vaginal plugs the next morning (day post-coitum 0.5). On the relevant days of gestation (dpc 10.5, 11.0, 11.5, 12.5 and 13.5), pregnant females were sacrificed, and the presence of the Sf1/eGFP transgene in the embryos was assessed under a fluorescent binocular microscope for the presence of strong GFP fluorescence in the urogenital ridges. Urogenital ridges from individual embryos were dissected, digested with trypsin/EDTA and filtered through a 40 μm cell strainer to generate single cell suspensions. GFP-positive cells were sorted using a FACS Vantage SE, collected in 96-well plates containing 50 μl of RNA lysis buffer from Qiagen RNeasy kit and stored at −70°C until needed. Accurate staging of embryos between 10.5 and 12.5 dpc was performed by counting the tail somites (ts). Embryos at 8 ts (±2 ts) were considered as E10.5, 14 ts (±2 ts) as E11.0, 19 ts (±2 ts) as E11.5 and 30 ts (±3 ts) as E12.5. Routine sexing of the embryos was performed by PCR to check for the presence or the absence of two Y-chromosome-specific genes *Sry* (15,869: 5′ CAG CCC TAC AGC CAC ATG AT 3′; 15,870: 5′ GAG TAC AGG TGT GCA GCT CTA 3′) and *Zfy* (16,541: 5′ CAG AAC CCT TTG GTA CAC TG 3′; 16,542: 5′ AAC ACC ACT CTC AAG AGT AG 3′). GFP-positive cells were pooled according to the developmental stage and genetic sex of each embryo. Total RNAs were extracted using RNeasy micro kit from Qiagen according to the

manufacturer's protocol. RNA integrity and quantity were assessed using RNA 6000 nanochips with an Agilent 2100 bioanalyzer.

RT-PCR assays performed with 120 ng of totRNAs from male or female cells at E13.5 that are positive or negative for GFP were used to assess cellular specificity and purity of the GFP^+ populations purified by FACS. Total RNAs were reverse-transcribed with the Omniscript RT-kit from Qiagen according to manufacturer's instructions, and 1/20th of cDNA template was used as template for each PCR reaction. Amplification of *Gapdh*, *Sf1*, *Cyp11a*, *Insl3*, *AMH* and *Oct4* was achieved with a classical 30 cycles program using the following primers: *Gapdh* primers 15,024 5′ TCC ATG ACA ACT TTG GCA TTG 3′ and 15,025 5′ CAG TCT TCT GGG TGG CAG TGA 3′; *Sf1* primers; 20,112 5′ AAA TTC CTG AAC AAC CAC AGC 3′; 20,113 5′ GCA TCT CAA TGA GAA GCT TG 3′; *Cyp11a* primers, 20,116 5′ AGT GGC AGT CGT GGG GAC AGT 3′; 20,117 5′ TAA TAC TGG TGA TAG GCC GCC 3′; *Insl3* primers, 15,725 5′ CGG GAT CCA CCA TGC GCG CGC CGC TGC TA 3′; 15,726 5′ TCA GTG GGG ACA CAG ACC CAA 3′; *AMH* primers, 20,130 5′ ACT GGG AGG AAC CCC TAT TA 3′; 20,131 5′ GCG CAG ATC TAC ACT CAG CT 3′; *Oct4* primers, 20,114 5′ CTC AGC CTT AAG AAC ATG TG 3′; 20,115 5′ TTC TCT TGT CTA CCT CCC TT 3′; *Irx3* primers, 20,193 5′ GGA CCA ACA GGC CTT TCC 3′; 20,194 5′ CAC AAC GAA GGA ACC TCA CA 3′.

Microarray probe labeling and hybridization

Approximately 50,000–100,000 GFP^+ cells were needed to obtain the minimum 50 ng of total RNA required for the generation of a single probe for the Affymetrix GeneChip analysis. The numbers of embryos necessary for the isolation of 100,000 cells are ~20 at E10.5, ~10 at E11.0 and E11.5 and around 5 at E12.5 and E13.5. We employed a small-scale protocol from Affymetrix to reproducibly amplify and label total RNA. In short, 50–100 ng of RNA was converted into double-stranded cDNA using a cDNA synthesis kit (Superscript, Invitrogen) with a special oligo$(dT)_{24}$ primer containing a T7 RNA promoter site added 5′ to the poly(T) tract. Following the first cRNA amplification by in vitro transcription (IVT) using the Ambion MEGAscript T7 kit, 400 ng of cRNA was once more reverse-transcribed, and biotinylated cRNAs were generated from double-strand cDNAs using an IVT labeling kit from Affymetrix. For each probe, 20 μg of the 2nd amplification biotinylated cRNA was fragmented and hybridized to Mouse Genome 430 2.0 Array (Affymetrix, High Wycombe, UK) by using Affymetrix GeneChip Fluidics Station 450 and standard protocols. For each condition, three independent sets of totRNA were isolated and used as a template for probe generation. These triplicates were performed to minimize the effects of biological variability. We explored 5 different time points (E10.5, E11.0, E11.5, E12.5 and E13.5) either with male or female Sf1-positive cells, thus requiring a total of 30 sets of 430 2.0 arrays. GeneChips were incubated at 45°C for 16 h with biotin-labeled cRNAs probes and then washed and stained using a streptavidin–phytocoerythrin (SAPE) conjugate with antibody amplification as described in Affymetrix protocol.

Normalization

Prior to analysis, the data were normalized (scaled) to correct for variations in mRNA/totRNA ratio, yield of the IVT, amounts of cRNA probe applied to the microarrays, using the Affymetrix GCOS 1.1 software. Basically, the output of each array was multiplied by a factor in order to make a robust average equivalent to arbitrary target intensity (i.e., 100). The robust average intensity of an array was calculated by averaging all the signal intensities on the array, excluding the highest 2% and lowest 2% of the values. Normalized values were then exported and analyzed with GeneSpring and Matlab. As a complement, we carried out a Robust MultiArray Analysis (RMA) 3,4 using a quantile normalization and analyzed the set data using GeneSpring software (Silicon Genetics, Redwood City, CA) and Matlab 7© (Math Works, Inc., MA).

Selection of differentially expressed genes

To identify differentially expressed transcripts, pairwise comparison analyses were carried out with Affymetrix GCOS 1.2. Each of the experimental samples (n = 3) was compared with each of the reference samples (n = 3), resulting in nine pairwise comparisons. This approach, which is based on the Mann–Whitney pairwise comparison test, allows the ranking of results by concordance, as well as the calculation of significance (P value) of each identified change in gene expression (Hubbell et al., 2002). Genes for which the concordance in the pairwise comparisons exceeded a threshold (e.g., 60%) were considered to be statistically significant. A 77% cutoff in consistency of change (at least 7 of 9 comparisons were either increased or decreased) was then applied to identify potential dimorphic-regulated genes. Only genes that satisfied the pairwise comparison test and displayed ≥1.5-fold change in expression were selected for further study. This conservative analytical approach was used to limit the number of false-positives. Regulated genes were organized and visualized into using the GeneSpring software (Silicon Genetics, Redwood City, CA).

Gene clustering

Sexually dimorphic genes were clustered according to their expression in the different mouse tissues by using the Matlab compiled version 3.3.20 version of the super paramagnetic clustering (SPC) algorithm (Blatt et al., 1996; Getz et al., 2000). Before each clustering operation, the expression values were normalized using RMAexpress version 0.3 (http://stat-www.berkeley.edu/users/bolstad/RMAExpress/RMAExpress.html), RMAexpress is a GUI version of the RMA, which is the Robust Multichip Average (Bolstad et al., 2003; Irizarry et al., 2003). It consists of three steps: a background adjustment, quantile normalization and finally summarizations measured is log2 expression value, then log2 expression value was centered and normalized for each genes at the end of the process, the average of each gene will be 0, and its standard deviation equal to 1 as described (Liu et al., 2002).

Chromosome analysis

The chromosomal analysis has been performed using Matlab programming language. The Chromosomal position of the Affymetrix probe set was obtained from the Affymetrix website (http://www.affymetrix.com), the length of each chromosome was obtained from the Ensembl website (www.ensembl.org). For a selected gene, we draw a bar at a position along a line representing the chromosome length. Probe sets specifically enriched in male and female were represented with blue and red bars, respectively.

Whole-mount in situ hybridization (WISH)

Whole-mount in situ hybridization was carried out as described (Gerard et al., 1996). Briefly, gonads were dissected in PBS, fixed overnight in 4% paraformaldehyde at 4°C, washed in PBS, and then dehydrated in graded methanol solution and store at −20°C in 100% methanol until needed. Plasmids containing cDNAs of the relevant candidate genes were linearized and then used as template to generate digoxigenin-labeled antisense riboprobes. Expression profiles were analyzed at E10.5, E11.5, E12.5 and E13.5 using at least two pairs of embryos of each sex at each stage per candidate gene.

Depletion of germ cells by busulfan treatment

Gonads depleted of germ cells were generated by injecting pregnant females with 50 mg/kg busulfan at 9.5 dpc and then harvesting embryos at E13.5. The germ cell marker Oct4 was used as probe to check that germ cells were completely depleted using WISH (Figs. 3C and D).

Immunohistochemistry

Embryos at E10.5, E11.5, E12.5 and E13.5 were fixed overnight at 4°C in 4% paraformaldehyde/PBS, rinsed in PBS during the following day and finally embedded in paraffin. Five-micrometer sections were incubated overnight at 4°C with a GFP antibody (Molecular Probes, 1/200). GFP was detected by fluorescent imaging with an Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes, 1/2000).

Results

Isolation of Sf1$^+$ somatic cells from both male and female genital ridges

To specifically isolate somatic cells from male and female gonads, we used Sf1/eGFP transgenic mice expressing eGFP under the control of 50 kb of the mouse steroidogenic factor 1 (Sf1) promoter (Stallings et al., 2002). *Sf1* is expressed in the somatic compartments of developing genital ridge of both male and female embryos starting at E9.0 (Ikeda et al., 1994), both in supporting cell precursors (future Sertoli and granulosa cells) and steroidogenic cell precursors (future Leydig and theca cells). In order to accurately determine which cells within the XX and XY urogenital ridges express eGFP, we performed immunohistochemistry (IHC) with an antibody against GFP on Sf1/*eGFP* gonads at E10.5, E11.5, E12.5 and E13.5 (see Fig. 1). GFP was specifically expressed in the somatic compartment, including the coelomic epithelium, of both XX and XY gonads. Most somatic cells of genital ridges appeared to express GFP, although there is a range of intensity of expression: for example, Leydig cells tend to express more GFP that Sertoli cells. In contrast, no signal was detected in germ cells (see arrows in Fig. 1). Overall, the developmental profile of the Sf1/*eGFP* transgene closely parallels the expression of the endogenous Sf1 gene in the developing gonads during the period of sex determination (E10.5–E12.5) (see Fig. 1 and Stallings et al., 2002).

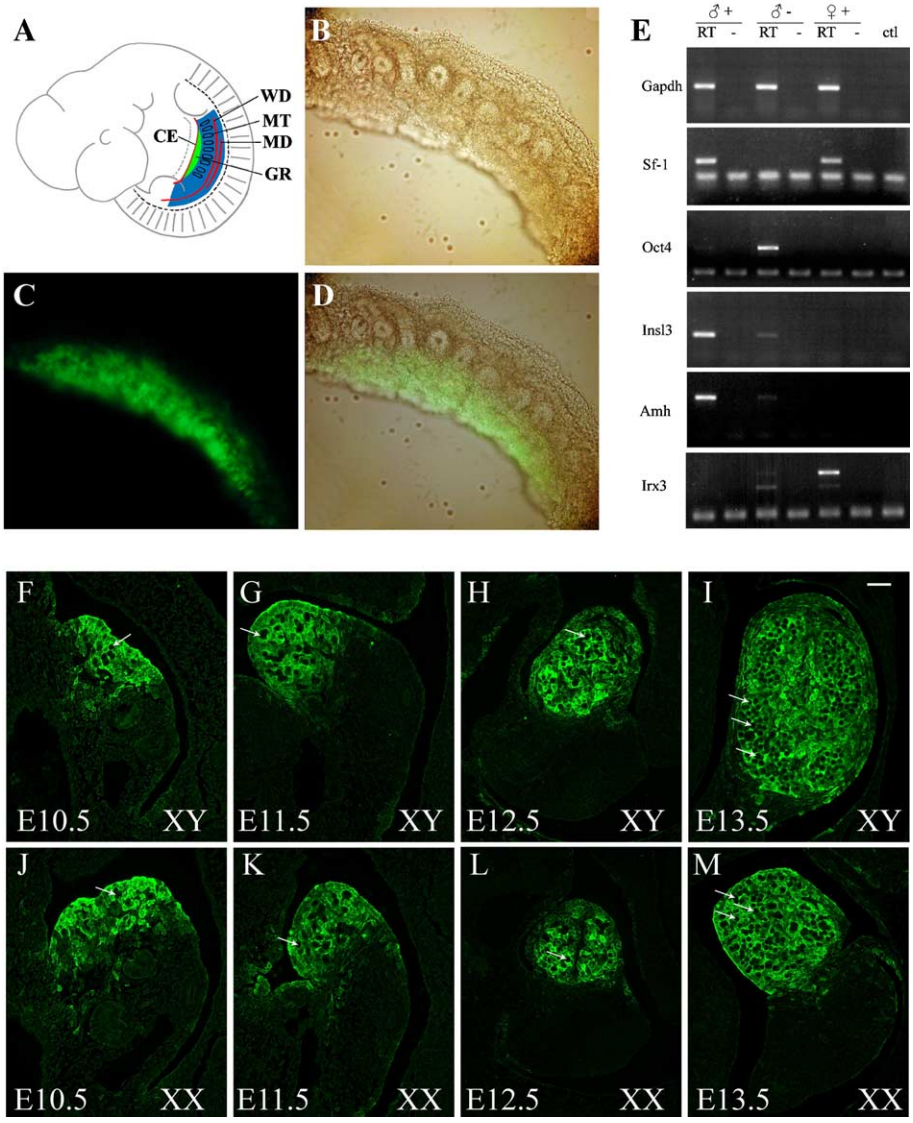


Fig. 1. Expression of the Sf1/GFP transgene in the somatic compartment of the genital ridge and the subsequent isolation of Sf1-positive cells. (A) Schematic drawing of a E10.5 embryo with the genital ridges colored in green. (B) Bright field view of a dissected *Sf1* EGFP genital ridge at E10.5, its corresponding fluorescent photomicrograph (C) and the merged picture (D). (E) RT-PCR performed with total RNAs from male (♂) or female (♀) cells at E13.5 that are positive (+) or negative (−) for GFP. As expected, GFP$^+$ cells expressed *Sf1* as well as somatic cell markers such as *AMH*, *Insl3* or *Irx3* but not the germ cell markers *Oct4*. GFP immunohistochemistry indicates that GFP is exclusively expressed in the somatic cells of male (F–I) and female (J–M) urogenital ridges at E10.5 (F, J), E11.5 (G, K), E12.5 (H, L) and E13.5 (I, M). Note the absence of GFP expression in germ cells (arrows). Scale bar: 50 μm. Abbreviations: WD, wolffian ducts; MT, mesonephric tubules; MD, müllerian ducts; GR, genital ridges; CE, coelomic epithelium.

Urogenital ridges from single Sf1/*eGFP* transgenic embryos were dissected then dissociated to produce single cell suspensions and sorted into GFP^+ and GFP^- population using a FACS Vantage SE. We collected in average 5000 GFP^+ cells from the genital ridges of single embryos at E10.5. This number increased to ~10,000 at E11.0–E11.5, ~18,000 at E12.5 and ~25,000 at E13.5. At E13.5, the difference in size between XY and XX gonads, caused by increased proliferation and cell recruitment from the mesonephros, was visible, and we obtained ~25% more GFP^+ cells from male genital ridges than their female counterparts.

In control experiments, we found that cross contamination of $eGFP^+$ by $eGFP^-$ cells was <2% (Supplementary Fig. 1). To confirm cellular specificity, we compared gene expression profiles of $eGFP^+$ and $eGFP^-$ pools at E13.5 by RT-PCR (Fig. 1E). Both male and female $eGFP^+$ fractions expressed *Sf1* but not the male $eGFP^-$ fraction, suggesting that we had specifically isolated $Sf1^+$ somatic cells. Indeed, classical markers for male Sf1-positive cells, i.e., transcripts expressed in Sertoli (*AMH*) and Leydig cells (*Insl3*, *Cyp11a*), were detected in the male $eGFP^+$ fraction, while the early somatic ovarian marker *Irx3* was expressed specifically in the female $eGFP^+$ fraction. As expected, *Oct4*, the germ cell marker, was excluded from $eGFP^+$ fractions, although a very faint band can be observed in the male $eGFP^+$ fraction. It is therefore formally possible that highly expressed genes specifically expressed in contaminating $eGFP^-$ cells may be included in our list of dimorphic genes.

Identification of male and female genetic programs

To identify the initial molecular changes associated with testicular and ovarian differentiation, we selected 5 different time points (E10.5, E11.0, E11.5, E12.5 and E13.5) for analysis of male and female $Sf1^+$ somatic cells. Gene expression profiling was performed with high-density Affymetrix GeneChip® Mouse Genome 430 2.0 microarrays containing over 34,000 well-substantiated mouse genes. Each of the time points was performed in triplicate to minimize the effect of biological variability. A total of 30 separate arrays were used, and microarray data including the expression profile of every sexually dimorphic genes, chromosomal maps and hierarchical clusterings are all conveniently accessible through the Nef laboratory website at http://www.medecine.unige.ch/recherche/research_groups/nef/. The entire database is also available via ArrayExpress (http://www.ebi.ac.uk/arrayexpress/), a public repository for microarray data from the European Bioinformatics Institute and the Microarray Management and Analysis System (MIMAS), a microarray data repository developed by members of the Swiss Array Consortium (SAC) (http://www.mimas.unibas.ch).

We found that almost half of the probe sets/genes (47%) contained on the Affymetrix microarrays were expressed by the $Sf1^+$ cell population, 10% of these (2306 genes) exhibited a sexually dimorphic expression. Among the 2306 genes displaying $\geq$1.5-fold differences between the somatic compartment of male and female gonads during the critical stages of gonadal determination, 1083 of these were upregulated in the male gonad and 1223 in female gonad (Fig. 2). For simplicity, the terms "gene" and "probe set" are used synonymously, although this is not totally accurate since some genes are represented more than one time by different probe sets on the array. At E10.5, corresponding to the onset of *Sry* expression in XY genital ridges, only 14 genes displayed a dimorphic expression in somatic cells of the gonads: 8 were expressed at a higher level in males and 6 in females. Twelve hours later, male upregulated genes increased to 11, while female upregulated genes remained at 6. At the peak of Sry expression (E11.5), dimorphic genes increased to 86: 61 were upregulated in males and 25 in females. Genes specifically upregulated in males and females numbered 592 and 546 respectively at E12.5 and almost doubled to 919 in males and 1029 in females by E13.5.

Male and female transcriptomes prior to gonadal differentiation

Prior to gonadal determination (E10.5), only a handful of genes exhibited sex-specific expression (see Table 1). At this stage, all genes identified by our transcriptome analysis with male-specific expression localize within a 1 megabase (Mb) region on the Y chromosome (*Smcy*, *Uty*, *Ddx3y*, *Eif2s3y*), while all genes with female-specific expression localize on the X chromosome (*Xist*, *Utx*, *Erdr1*). Interestingly, out of the 1060 probe sets located on the X chromosome, 506 were expressed at similar levels both in male and female gonads, while only 3 genes escaped X-inactivation and were upregulated in the female. In contrast to *Xist*, a non-coding gene required for X inactivation, and *Utx* (Greenfield et al., 1998; Heard, 2004), the Erythroid differentiation factor 1 gene (Erdr1) (Dormer et al., 2004) has never been reported to escape X inactivation. The *Erdr1* gene is located on the distal part of the X chromosome (XqF5, BAC RP24-143B12). Classical and quantitative RT-PCR experiments confirmed the female overexpression pattern (approximately 2-fold) both in gonadal tissue at E10.5, E11.5, E12.5 and E13.5 as well as in the brain and liver at E13.5 (data not shown) which indicates that *Erdr1* is broadly expressed and escapes X inactivation. For all these X- and Y-specific genes described above, sexually dimorphic expression was maintained at each developmental stage thereafter (data not shown). Except for Erdr1, the dimorphic expression of these sex chromosome-specific genes has also been reported in both mouse (E10.5; Dewing et al., 2003) and human (Vawter et al., 2004) brains, suggesting that their sexually dimorphic expression is not gonad-specific but is probably ubiquitous. The absence of autosomal genes with a sex-dimorphic expression at E10.5 is consistent with the fact that sex determination has not yet occurred. From this perspective, the E10.5 data provide a baseline to identify genes with a dimorphic expression at later stages of gonadal development.

Identification of the male-specific genetic program

Twelve hours after the initiation of *Sry* expression (E11.0), 4 additional genes showed an overexpression in the somatic

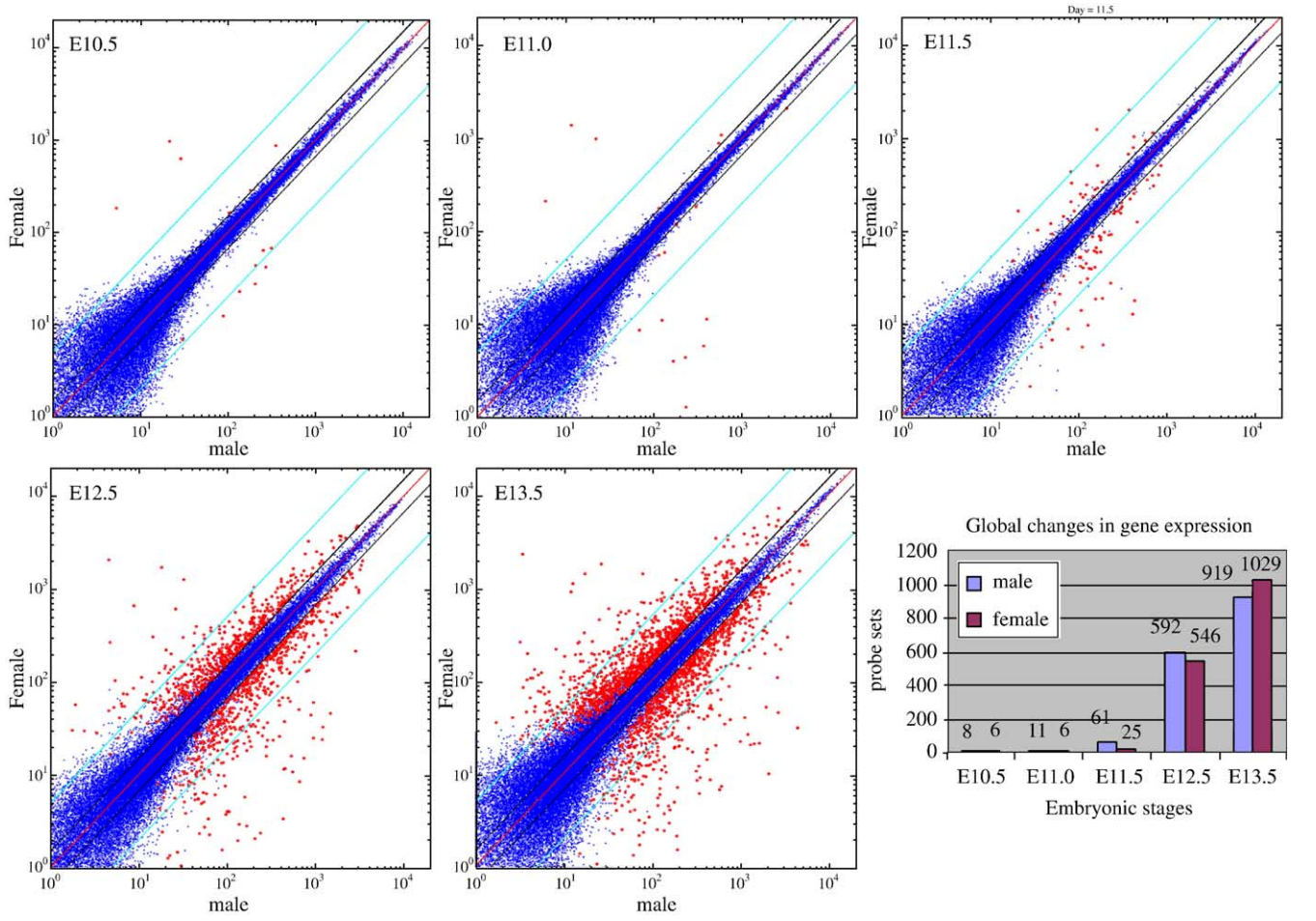


Fig. 2. Global changes in gene expression during gonadal differentiation indicate that both male and female genetic programs are initiated as early as E11.5. Scatterplot analysis representing the systematic comparison of the log2-scaled expression signals from male and female somatic transcriptomes at E10.5, E11.0, E11.5, E12.5 and E13.5 (A–E). Blue dots represent genes with expression levels statistically similar both in male and female gonads, while red dots represent genes whose expression profiles were found to be sexually dimorphic (>1.5-fold change, black diagonals). (F) Overall summary of sexually dimorphic genes during the critical stages of sex determination.

compartment of the male gonads (see Supplementary Table 1). These are: one IMAGE clone (Affymetrix ID 1455451), one Riken clone (Affymetrix ID 1438511), the X-linked lymphocyte-regulated 3a gene (Xlr3a, Affymetrix ID 1420357) and the G-protein-coupled receptor 37 gene (Gpr37, Affymetrix ID 1450875). All four genes demonstrated a transient male-

Table 1
Sexually dimorphic genes at E10.5

Gene name	Gene symbol	Affy ID	GenBank	Chromosome	Fold change
Male enhanced gene expression (probe sets) at E10.5					
Selected mouse cDNA on the Y	Smcy	1424903	AF127244	Y	33.47
Eukaryotic translation initiation factor 2, subunit 3	Eif2s3y	1417210	NM_012011	Y	25.79
DEAD (Asp–Glu–Ala–Asp) box polypeptide 3	Ddx3y	1426438	BB667072	Y	24.38
Ubiquitously transcribed tetratricopeptide repeat gene	Uty	1426598	BB742957	Y	13.15
DEAD (Asp–Glu–Ala–Asp) box polypeptide 3	Ddx3y	1452077	AA210261	Y	9.21
DEAD (Asp–Glu–Ala–Asp) box polypeptide 3	Ddx3y	1426439	AJ007376	Y	7.47
–, –, –		1447329	AI506532	?	4.75
RIKEN clone:4930534N17		1444668	AV268386	?	5.77
Female enhanced gene expression (probe sets) at E10.5					
Inactive × specific transcripts	Xist	1427262	L04961	X	61.97
Inactive × specific transcripts	Xist	1436936	BG806300	X	35.54
Inactive × specific transcripts	Xist	1427263	R74734	X	29.21
Erythroid differentiation regulator 1	Erdr1	1439200	BE686792	X	2.29
Erythroid differentiation regulator 1	Erdr1	1452406	AJ007909	X	2.24
Ubiquitously transcribed tetratricopeptide repeat gene	Utx	1427235	AW536493	X	1.53

specific upregulation at E11.0. Particularly interesting is the *Gpr37* gene, which encodes an orphan G-protein-coupled receptor shown to be expressed in brain tissues and testis (Marazziti et al., 1997, 1998). Increased expression of Gpr37 at E11.0 and E11.5 in the male somatic compartment was confirmed by whole-mount in situ hybridization (data not shown).

At E11.5, a developmental stage corresponding to the peak of *Sry* expression, we observed a dramatic increase in the number of genes preferentially expressed in the male somatic compartment: 61 genes had higher expression in male gonads (see Table 2). These genes include early male markers such as *Sox9*, *Dhh* or *Fgf9* but also male-specific markers previously identified at later stages of testicular differentiation (*Cst9*, *Spp1*, *Mmd2*, *Aard*, *Cbln1*, *Cbln4*, *Rgn*). Numerous genes with a male-specific expression have not previously been described as sexually dimorphic, including the transcription factor *Etv5*, an intracellular FGF receptor antagonist *Spry4* and the metalloproteinase *Adamts16* (see Table 2 for details).

At E12.5 and E13.5, the supporting cell precursors of the male gonads have already differentiated in Sertoli cells, and the fetal Leydig cells first appear in the developing testis. This differentiation is consistent with a massive change in gene expression: genes upregulated during testis determination increased in number from 61 at E11.5 to 587 at E12.5 (see Supplementary Table 2) to 919 at E13.5 (see Supplementary Table 3). Concomitant with the appearance of Leydig cells around E12.5 and E13.5, genes encoding enzymes involved in steroidogenesis underwent a drastic, male-specific increase in expression. This includes genes such as *StAR* (4- and 22-folds higher in males versus females at E12.5 and E13.5 respectively), *Cyp11a1* (8- and 394-fold higher), *Cyp17a1* (35- and 246-fold higher), *Cyp26b1* (12- and 45-fold higher), *Hsd3b1* (12- and 12-fold higher), *Hsd11b2* (5- and 6-fold higher) and *Hsd17b7* (3-fold higher at E13.5).

To assess the efficiency of the transcriptome analysis at identifying the genes upregulated in the somatic compartment of testis, we scanned our data for genes known to be expressed in Sertoli or/and Leydig cells. Our GeneChip profile analysis detected all previously described male-specific genes expressed at E11.5 and E12.5; this includes genes such as *Cystatin 9*, *AMH*, *Hedgehog interacting protein* (*Hhip*), *Nexin1*, *Vanin 1*, *Insl3*, *Renin1*, *tescalcin*, *Cyp26b1*, *Pak3*, *tdl*, *GDNF*, *Dtna*, *Patched* and *Maestro* (see Grimmond et al., 2000; Menke and Page, 2002; Smith et al., 2003; Tohonen et al., 1998), with the notable exception of *Sry*. To explore this apparent discrepancy, we performed quantitative RT-PCR for *Sry* using as templates our 2nd amplification biotinylated cRNAs used as probes for microarray labeling. As reported in the literature, we indeed observed a strong peak of *Sry* expression at E11.5 in male eGFP$^+$ cells (data not shown). Thus, the *Sry* probe sets of the 430 2.0 microarray probably do not efficiently detect *Sry* transcripts. Overall, the expression profiles of known male markers were similar in our microarrays analyses to what have been reported in the literature, further validating our results (see Supplementary Fig. 2). Of the 1083 genes displaying an increased expression in somatic cells of developing testes, only a few have been reported previously. Taken together, these results suggest that we have identified more than 1000 genes that had not as yet been associated with testis differentiation and that these represent a large proportion of the genes whose expression is male-specific in gonadal somatic cells.

A robust female-specific genetic program is initiated as early as E11.5

At both E10.5 and E11.0, only 6 genes located on the X chromosome were overexpressed in the female somatic cells, suggesting that differentiation of the female gonad has not yet started. By E11.5, the initiation of a female-specific genetic program was detected with the upregulation of 25 genes (see Table 2). 24 h later, the number of genes upregulated during ovarian development increased drastically, reaching 546 genes at E12.5 and 1029 genes at E13.5 (see Supplementary Tables 2 and 3 respectively). The identification of this large cohort of genes whose expression is upregulated during early ovarian development constitutes a major, and somewhat unexpected, finding and suggests that an important genetic program is initiated within the somatic compartment of the developing ovary well before the first histological evidence of ovarian differentiation. To assess whether we achieved a complete catalogue of the genes upregulated in the somatic compartment of ovary, we scanned our transcriptome data for the small number of genes reported to be expressed in female gonad somatic cells. Our expression profile analyses detected all of the genes known to be expressed in the somatic compartment of the developing ovary at E11.5 and E12.5, including *Wnt4*, *follistatin* (Shimasaki et al., 1989), *Bmp2* (Zhang and Bradley, 1996) and *Dax1* (Swain et al., 1998) (see Supplementary Fig. 2). For more than 1000 of the female-specific genes identified here, our data provide the first evidence that they are expressed in somatic cells of the developing female gonad.

The complex molecular mechanisms that establish gonadal differentiation must be coordinated by modules or networks of genes that share common function (e.g., proliferation, cellular organization, etc). An example is given by the structural proteins keratins, markers of tissue organization, that revealed differences in their expression depending on the sex of the gonads. In XX genital ridges, the first changes at the cellular and structural level occur between E12 and E13.5 with the formation of loose cord-like structures referred to as ovigerous cords (Konishi et al., 1986; Odor and Blandau, 1969). Ovigerous cords are composed of a cluster of primordial germ cells surrounded by somatic cells with a mesenchymal appearance. We found that keratins (K) 7, 8, 18 and 19 are overexpressed in the developing ovaries from E11.5 onward (data not shown). It has been shown previously that K8, K18 and K19 are expressed in the somatic compartment of the ovigerous cords during fetal life and in primordial follicles (Appert et al., 1998). These dimorphic expression profiles

Table 2
Sexually dimorphic genes at E11.5

Gene ID	GenBank	Gene symbol	Gene name	Chromosome	Fold change
Male overexpressed genes (probe sets) at E11.5					
1417210	NM012011	Eif2s3y	Eukaryotic translation initiation factor 2, subunit 3	Y	256.00
1426438	BB667072	Ddx3y	RIKEN cDNA 8030469F12	Y	126.04
1452077	AA210261	Ddx3y	DEAD (Asp–Glu–Ala–Asp) box polypeptide 3	Y	49.64
1457582	BB528233	Uty	Ubiquitously transcribed tetratricopeptide repeat gene, Y chromosome	Y	26.81
1426598	BB742957	Uty	Ubiquitously transcribed tetratricopeptide repeat gene	Y	25.40
1424903	AF127244	Smcy	Selected mouse cDNA on the Y	Y	20.95
1418744	NM021344	tesc	Tescalcin	5	15.40
1426439	AJ007376	Ddx3y	DEAD (Asp–Glu–Ala–Asp) box polypeptide 3	Y	12.41
1449285	NM009979	Cst9	Cystatin 9	2	7.18
1460693	BG074456	Col9a3	Procollagen, type IX, alpha 3	2	6.60
1420786	NM011253	RbmY1a1	RNA binding motif protein, Y chromosome, family 1, member A1	Y	6.30
1457945	BM121819		*Mus musculus* transcribed sequences	?	6.02
1460734	BG074456	Col9a3	Procollagen, type IX, alpha 3	2	6.02
1434959	AV367068	Dhh	Desert hedgehog	15	4.96
1434528	AV256613	Aard	Alanine- and arginine-rich domain containing protein	15	4.74
1444038	BI076720		*Mus musculus* transcribed sequence	X	4.70
1438654	AV269411	Mmd2	Monocyte to macrophage differentiation-associated 2	5	4.00
1424950	BI077717	Sox9	SRY-box containing gene 9	11	3.76
1422247	NM009484	Uty	Ubiquitously transcribed tetratricopeptide repeat gene, Y chromosome	Y	3.73
1433607	AV343573	Cbln4	Cerebellin 4 precursor	2	3.56
1449254	NM009263	Spp1	Secreted phosphoprotein 1	5	3.51
1448852	NM009060	Rgn	Regucalcin	X	3.38
1451538	BC024958	Sox9	SRY-box containing gene 9	11	3.13
1445669	BB080456	Spry4	Sprouty homolog 4	18	2.89
1439604	AV319357	Adamts16	A disintegrin-like and metalloprotease with thrombospondin type 1 motif, 16	13	2.70
1457270	AI506234		RIKEN cDNA B230343A10	11	2.66
1434653	AV026976	Ptk2b	PTK2 protein tyrosine kinase 2 beta	14	2.64
1438718	AW046863	Fgf9	Fibroblast growth factor 9	14	2.58
1423287	AA016422	Cbln1	Cerebellin 1 precursor	8	2.56
1448021	AA266723		*Mus musculus* transcribed sequences	3	2.54
1423286	BQ174436	Cbln1	Cerebellin 1 precursor	8	2.50
1423288	AA016422	Cbln1	Cerebellin 1 precursor protein	8	2.35
1448330	NM010358	Gstm1	Glutathione *S*-transferase, mu 1	3	2.33
1418028	NM010024	Dct	Dopachrome tautomerase	14	2.23
1447707	BB367174	Pde2a	Phosphodiesterase 2A, cGMP-stimulated	7	2.23
1424393	BC026584	Adhfe1	Alcohol dehydrogenase, iron containing, 1	1	2.23
1425627	J03952	Gstm1	Glutathione *S*-transferase, mu 1	3	2.19
1453345	AK014427		RIKEN cDNA 3830408G10	4	2.19
1424392	BC026584	Adhfe1	Alcohol dehydrogenase, iron containing, 1	1	2.19
1433454	BB621938		Expressed sequence AW539457	2	2.18
1416416	NM010358	Gstm1	Glutathione *S*-transferase, mu 1	3	2.14
1449109	NM007706	Socs2	Suppressor of cytokine signaling 2	1	2.14
1434099	BB752393	Casp7	Caspase 7	1	2.14
1430943	AK020088		RIKEN cDNA 6030498E09	5	2.13
1418507	BB244736	Socs2	Suppressor of cytokine signaling 2	10	2.11
1428142	AK003461	Etv5	ets variant gene 5	4	2.08
1416411	NM008183	Gstm2	Glutathione *S*-transferase, mu 2	3	1.88
1433453	BB621938		Expressed sequence AW539457	2	1.88
1424477	BC019731		Hypothetical protein MGC30809	5	1.82
1450875	BQ175510	Gpr37	G-protein-coupled receptor 37	6	1.77
1416468	NM013467	Aldh1a1	Aldehyde dehydrogenase family 1, subfamily A1	19	1.75
1428834	AK012530	Dusp4	Dual specificity phosphatase 4	8	1.74
1418412	NM009413	Tpd52l1	Tumor protein D52-like 1	10	1.74
1436990	AA038464		RIKEN cDNA 1620401E04	10	1.68
1423858	BC014714	Hmgcs2	3-Hydroxy-3-methylglutaryl-Coenzyme A synthase 2	3	1.66
1418237	NM009929	Col18a1	Procollagen, type XVIII, alpha 1	10	1.64
1419072	NM026672		RIKEN cDNA 0610005A07	3	1.62
1448894	NM008012	Akr1b8	Aldo–keto reductase family 1, member B8	6	1.61
1450744	NM138953	Ell2	Elongation factor RNA polymerase II 2	13	1.56
1437132	BB535494	Nedd9	Neural precursor cell expressed, developmentally downregulated gene 9	13	1.54
1416600	AF260717	Dscr1	Down syndrome critical region homolog 1	16	1.54

Table 2 (*continued*)

Gene ID	GenBank	Gene symbol	Gene name	Chromosome	Fold change
Female overexpressed genes (probe sets) at E11.5					
1427262	L04961	Xist	Inactive × specific transcripts	X	26.40
1436936	BG806300	Xist	Inactive × specific transcripts	X	16.89
1427263	R74734	Xist	Inactive × specific transcripts	X	11.76
1421365	NM008046	Fst	Follistatin, Fst, NM_008046 // follistatin	13	6.65
1434458	BB444134		Follistatin 1 precursor	13	4.29
1420771	NM011470	Sprr2d	Small proline-rich protein 2D	3	3.05
1418517	NM008393	Irx3	Iroquois related homeobox 3	8	2.99
1429814	AV232168		*Mus musculus* transcribed sequence	19	2.62
1416871	NM007403	Adam8	A disintegrin and metalloprotease domain 8	7	2.54
1439200	BE686792	Erdr1	Erythroid differentiation regulator 1	X	2.30
1457110	BB440150		*Mus musculus* transcribed sequences	19	2.26
1452406	AJ007909	Erdr1	Erythroid differentiation regulator 1	X	2.09
1415777	NM018874	Pnliprp1	Pancreatic lipase related protein 1	19	2.06
1428089	AK014285		RIKEN cDNA 3200001I04	14	2.03
1424638	AK007630	Cdkn1a	Cyclin-dependent kinase inhibitor 1A (P21)	17	2.03
1451095	BC005552	Asns	Asparagine synthetase	6	1.90
1419431	NM_007950	Ereg	Epiregulin	5	1.87
1435155	BI455486	Cgn	Cingulin	3	1.84
1433988	BG075755		RIKEN cDNA C230098O21	8	1.81
1433966	AV212753	Asns	Asparagine synthetase	6	1.81
1436978	AV273409	Wnt9a	Wingless-type integration site 9A	11	1.73
1440150	AI893889	Tgm3	Transglutaminase 3, E polypeptide	2	1.62
1437113	BM228590	Pld1	Phospholipase D1	3	1.62
1434170	BB083162		RIKEN cDNA A230038L21	X	1.61
1422804	NM011454	Serpinb6b	Serine (or cysteine) proteinase inhibitor, clade B, member 6b	13	1.58
1456307	BB746807		Clone IMAGE:3596174	8	1.54
1452366	AV371987		RIKEN cDNA 4732435N03	8	1.53

represent part of the molecular mechanisms put in place to mediate proper development, differentiation and interaction of the various cell types of the ovary.

Validation of expression profiles by RT-PCR and whole-mount ISH

To confirm the expression profiles of sexually dimorphic genes identified with our microarray analysis, we selected 26 genes (including equal proportions of male and female-overexpressed clones, see Supplementary Table 4) for further study. We performed either quantitative RT-PCR (data not shown) and/or whole-mount in situ hybridization (WISH) with XX and XY gonads at E10.5, E11.5, E12.5 and E13.5. Whole-mount ISH was found to be more informative than quantitative RT-PCR assays, mainly because it reveals the spatio-temporal expression pattern of each selected gene. In the case of male-specific candidates, WISH refined our analysis by determining in which somatic cell types the explored genes were expressed: expression within testicular cords indicates a Sertoli-specific expression, while interstitial expression suggests a Leydig-specific expression. Not only were we able to confirm the sexual dimorphism between male and female gonads (Figs. 3 and 6), but we also found that expression profiles revealed by microarray analyses faithfully reflected the expression profiles generated by quantitative RT-PCR or WISH (Fig. 6). Overall, the analyses of these of 26 genes strikingly validated the expression profiles generated by microarrays.

To verify the somatic expression of this subset of testis and ovarian genes, we analyzed their expression in E13.5 busulfan-treated embryonic gonads. Exposure to busulfan completely eliminates germ cells (Merchant, 1975), as confirmed in our studies by the abrogation of expression of the germ cell marker *Oct4* (Figs. 3C and D). Busulfan treatment did not cause marked changes in the expression of *Cdkn1a*, *Daam2*, *Tcf12*, *Runx1*, *Irx3* and *Gpr49*, in female gonads or of *Cbln1*, *Cbln4*, *Rerg*, *Tesc*, *Nedd9* and *Gpr37* in male gonads, confirming the somatic expression of these 14 genes and suggesting that it is largely independent of the presence of germ cells. The expression of *Wnt9a* appeared to be upregulated in busulfan-treated female gonads, while *Grb14* expression was down-regulated, but not completely ablated, in male busulfan-treated gonads. Thus, interactions between the germinal and the somatic compartments may affect the expression of these two genes; alternatively, *Grb14* may be coexpressed by germ and somatic cells.

Hierarchical clustering of dimorphic genes

To obtain a broad overview of the expression profiles of the 2306 sexually dimorphic genes, we generated a hierarchical clustering of these genes. This yields a dendrogram that arranges the genes in tight clusters according to similar patterns of expression. Examination of the clusters showed that once sexual dimorphism is initiated, the dimorphism remains at later stages. In Fig. 4, we present a hierarchical clustering of the 88 probe sets that displayed a sexually dimorphic expression

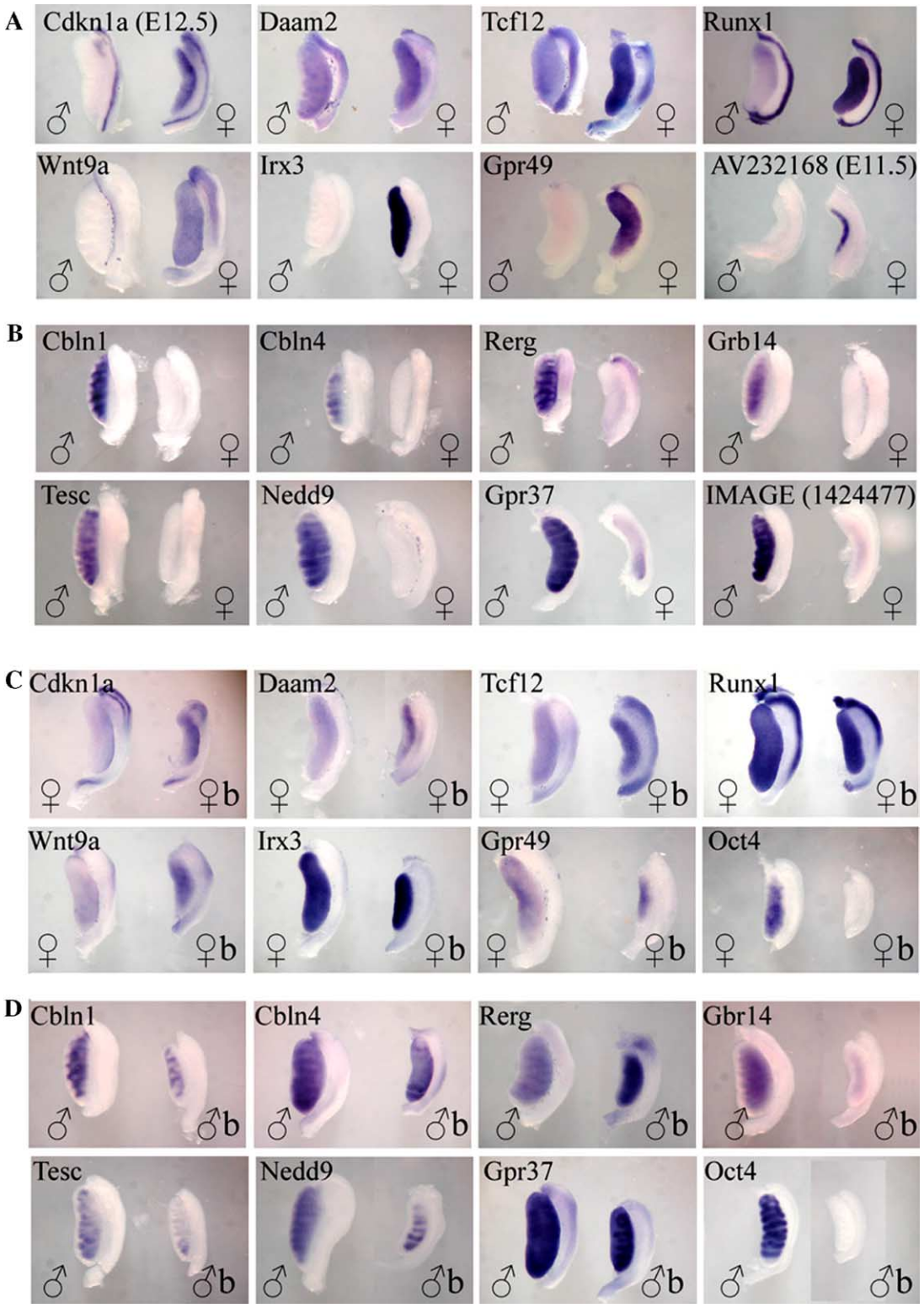


Fig. 3. Validation of expression profiles by whole-mount ISH. A selection of 8 female (A) and 8 male (B) overexpressed genes initially identified by our transcriptome analysis was selected, and overexpression either in ovary or testis was confirmed by WISH. To rule out germ cell-specific expression of candidate genes, expression of 7 female (C) and 7 male (D) overexpressed candidate genes was assessed in busulfan-treated testes (♂b) or ovaries (♀b). Oct4, a germ-cell-specific marker, has been used as an internal control to confirm germ cell depletion in busulfan-treated gonads. Embryonic stage is 13.5 except when otherwise noted.

pattern at E11.5 and show that 72% of these genes maintain the dimorphism a later stages. This analysis confirms previous hypotheses formed by examination of gonadal expression of known male- or female-specific genes and indicates that only a small subset of genes (e.g., *Sry* or *Gpr37*) exhibit a transient pattern of sexually dimorphic expression.

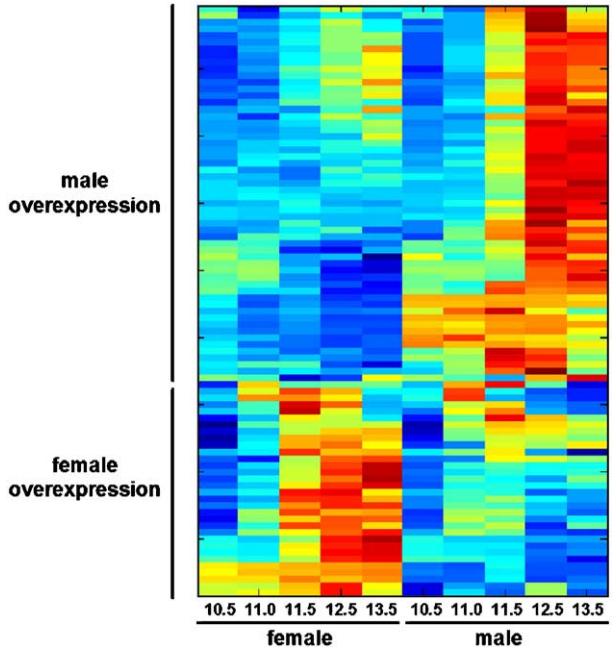


Fig. 4. Hierarchical clustering of 88 probe sets with male or female dimorphism at E11.5. Genes whose expression is dimorphic at E11.5 usually remain dimorphic at later stages. Each line is a gene, and each column corresponds to a specific stage and sex. Heatmap: expression signal intensities are shown in red to yellow to blue indicating high, medium and low expression, respectively.

Chromosomal map of ovarian- or testicular-specific genes

To assess whether chromosomal location may correlate with sex-specific expression, we designed a Matlab computer program to map on the mouse genome all relevant probe sets or genes displaying either a male- or a female-specific expression. Fig. 5 illustrates the chromosomal localization of genes displaying a sexually dimorphic expression at E13.5. At this stage, male or female overexpressed genes were distributed widely in the mouse genome; however, we observed some clusters of male- or female-specific genes. For example, a gene cluster on a 2.2 megabase region of the long arm of chromosome 1 contains 31 genes, three of which (e.g., *Renin1*, *Rassf5* and *Prst*) exhibited strikingly similar, male-specific expression patterns. A second gene cluster within a 1.5 megabase region of the distal part of chromosome 7 contains 11 genes, three of which (e.g., *Kcnq1* (*Hvlqt1*), *Phlda2* and the Est AU040576) exhibited a female-specific expression starting at E12.5. Interestingly, *Kcnq1*, *Phlda2* together with *Slc22a11*, *Cdkn1c* ($p57^{Kip2}$) and *Ascl2* (*Mash2*) are maternally expressed/paternally silenced genes clustered in an imprinted domain associated with the human disease Beckwith–Wiedermann syndrome (BWS) (Algar et al., 1999; Engel et al., 2000). These examples point towards common regulatory elements shared by genes within a given locus to control the expression of testicular- or ovarian-specific genes.

Dimorphic genes potentially involved in gonadal differentiation or function

From the set of genes displaying a dimorphic expression, we chose to further study the gonadal expression of a small number of these based on their potential involvement in sex determination and/or gonadal function (Fig. 6). The G-protein-coupled receptor Grp37 was expressed in both male and female gonads at E11.5, E12.5 and E13.5; in males, where the gene was expressed at higher levels, testicular cords were labeled, consistent with Sertoli cells expression. *Cbln1* (Urade et al., 1991) is a secreted neuropeptide implicated in steroid secretion (Mazzocchi et al., 1999); *Cbln1* expression was male-specific,

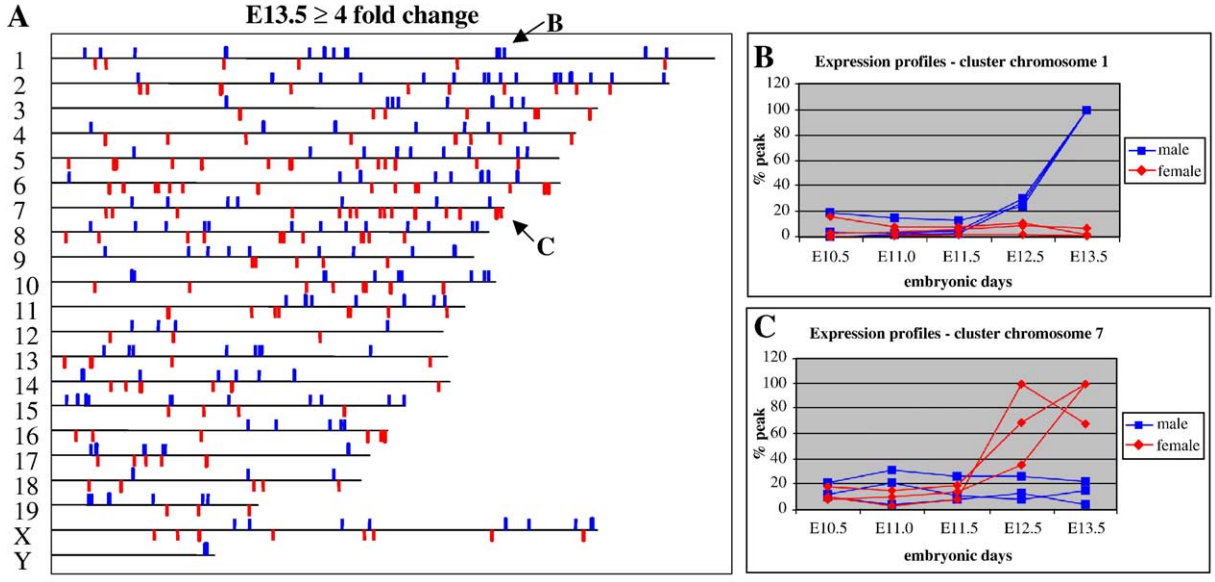
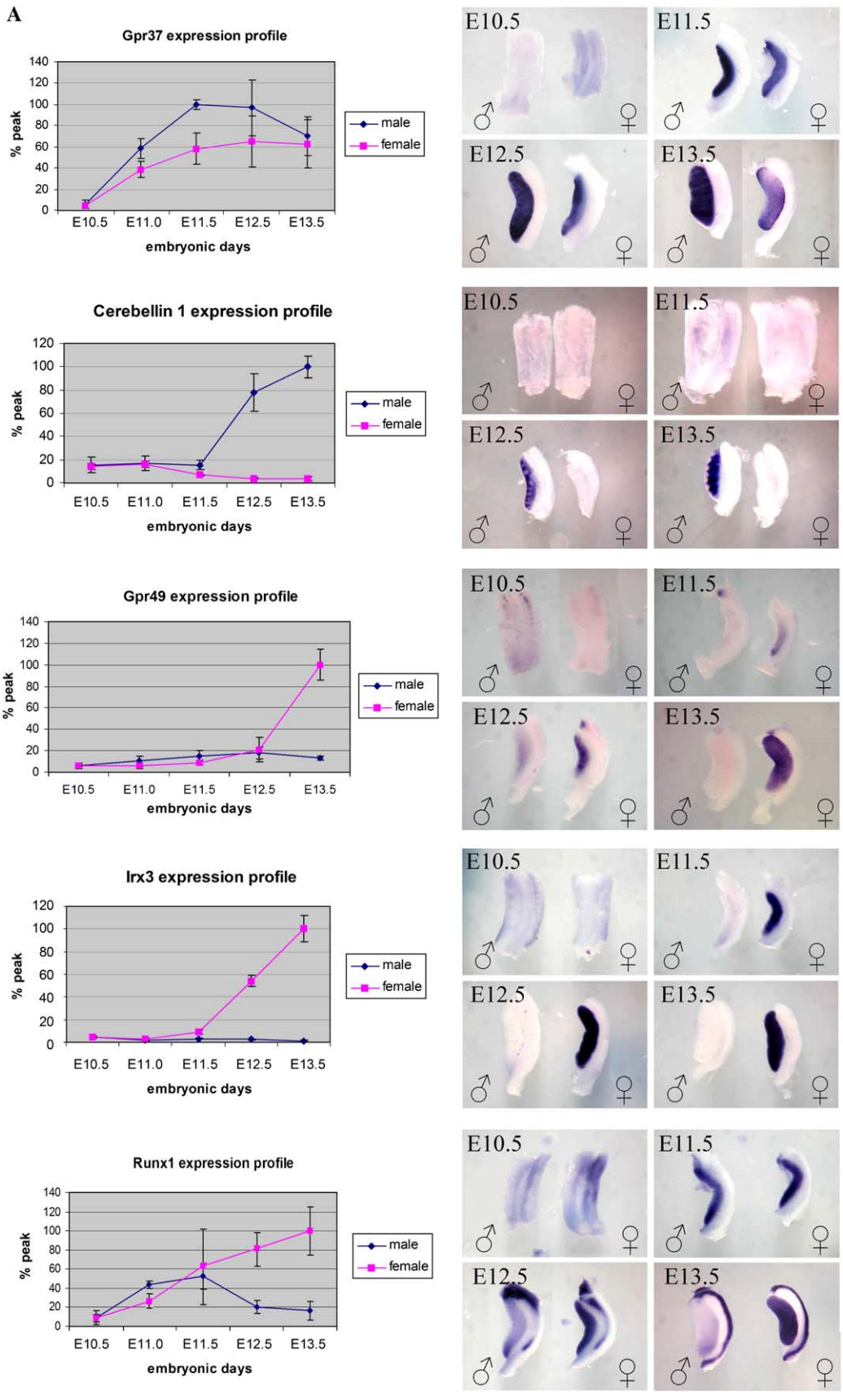


Fig. 5. Chromosomal map of genes exhibiting sexually dimorphic expression at E13.5. Sexually dimorphic genes are scattered throughout the genome with the exception of some discrete chromosomal region that host clusters of differentially expressed genes. Female and male overexpressed genes (≥4-fold change) are represented respectively by red and blue bars. Example of expression profiles of genes located within a male (B) or a female (C) cluster demonstrated strikingly similar expression patterns.


A
Gpr37 expression profile
% peak
embryonic days
E10.5
E11.0
E11.5
E12.5
E13.5
male
female
Cerebellin 1 expression profile
Gpr49 expression profile
Irx3 expression profile
Runx1 expression profile
E10.5
E11.5
E12.5
E13.5

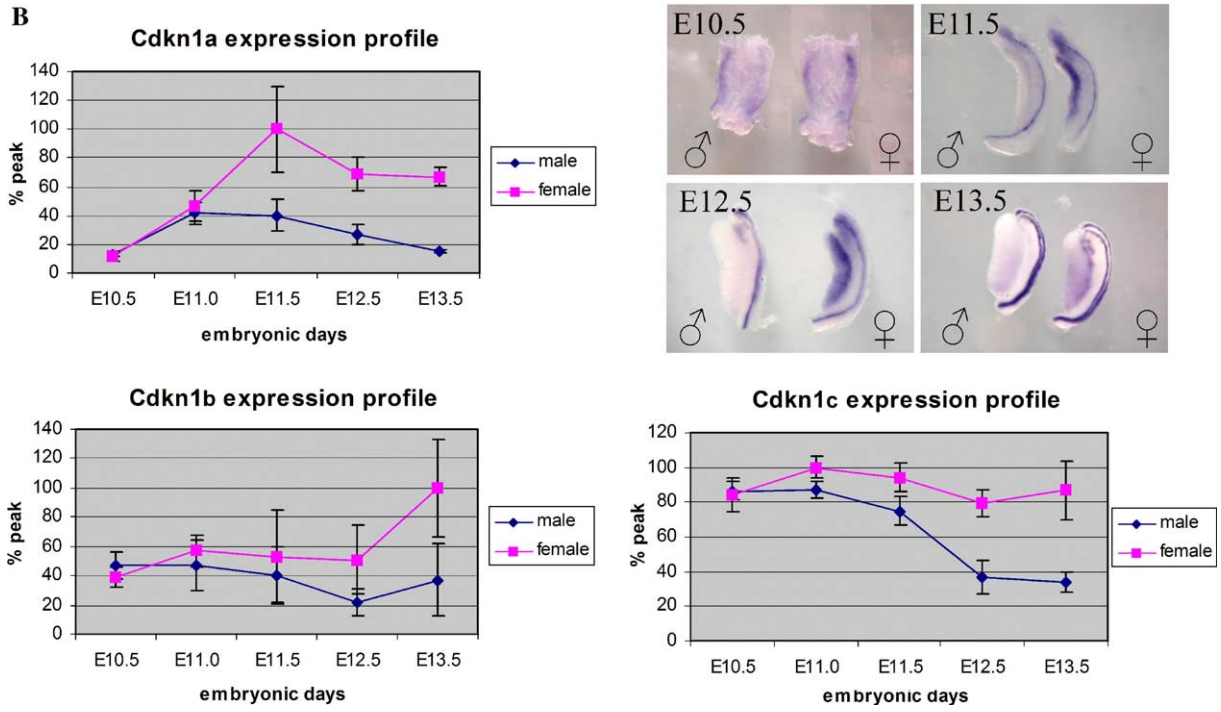


Fig. 6. Expression profiles of potentially relevant genes for gonadal differentiation or function as determined by microarrays and by WISH. (A) Two male (Gpr37 and Cbln1) and three female (Gpr49, Irx3 and Runx1) overexpressed genes have been selected as examples. (B) Cyclin-dependent kinase inhibitors *Cdkn1a*, *Cdkn1b* and *Cdkn1c* are overexpressed in developing ovaries. *Cdkn1a* (p21) exhibited a female-specific expression at E11.5 and E12.5 as determined by microarray and by WISH. *Cdkn1c* (p57) expression is reduced in XY gonads and remains relatively constant in XX gonads, while *Cdkn1b* (p27) became overexpressed in females starting at E13.5. For the Affymetrix expression profiles, the peak expression level is set as 100%, and the expression levels at other time points are normalized to percentage of peak levels (% peak). Bars represent the standard deviation.

and transcripts were detected in interstitial cells from E11.5 onward, suggesting a Leydig cell-specific expression. Gpr49 is a G-protein-coupled receptor of the LH/FSH family with an expression restricted to ovarian tissue beginning at E11.5. Irx3 is a transcription factor involved in patterning and differentiation of the nervous system; strong expression was detected specifically in ovaries, starting at E11.5. Finally, Runx1/AML1 is another transcription factor involved in controlling proliferation and differentiation; although occurring initially at similar levels in male and female genital ridges, *Runx1* expression became restricted to the ovaries and mesonephric ducts starting at E12.5.

One of the earliest morphological changes that occur during sex determination is a drastic size increase of XY gonads relative to XX gonads. This variation results from a higher rate of proliferation that could reflect activation of proliferation in testes or inhibition of proliferation in ovaries. In general, withdrawal from the cell cycle by inactivation of cyclin-dependent kinases is achieved by physical association with cyclin-dependent kinase inhibitors such as Cdkn1a$^{p21/Cip1/Waf1/Sdi1/Cap20}$, Cdkn1b$^{p27/Kip1}$ and Cdkn1c$^{p57/Kip2}$ (Kiyokawa and Koff, 1998; Sherr and Roberts, 1999). Sorting the differentially expressed genes according to gene ontology classification revealed that genes involved in cell cycle regulation were overexpressed in developing ovaries. In particular, we found that the cyclin-dependent kinase inhibitor *Cdkn1a* (p21) was overexpressed in XX as compared to XY gonads starting at E11.5 and E12.5, that *Cdkn1b* (p27) was upregulated in developing ovaries both at E12.5 (2.3-fold) and E13.5 (2.7-fold) and that *Cdkn1c* (p57) expression remained strong in ovaries but decreased in the developing testes (21% reduction at E11.5, 54% at E12.5 and 61% at E13.5) (Fig. 6B). These findings suggest that female-specific overexpression of Cdkn inhibitors may explain the reduced proliferation and size of ovary versus testis at the time of sex determination. We hypothesize that Sry may downregulate—either directly or indirectly—the expression of Cdkn inhibitors such as *Cdkn1a*, *Cdkn1b* and *Cdkn1c* in XY somatic cells, thereby allowing Sertoli cells to proliferate and divert the XY gonad from the ovarian fate.

Discussion

The mouse is an attractive model system to investigate the mammalian male and female genetic programs required for the uncommitted genital ridge to become either a testis or an ovary. Indeed, it has been well established that initiation of these alternative programs depends on the presence or absence of a single genetic switch, i.e., expression of Sry, and that morphologic gonadal differentiation occurs during a short time window of approximately 48 h thereafter. Furthermore, the cells that are critical for the process of sex determination, i.e., the somatic cells of the uncommitted gonad, can be purified from the genital ridges of embryos carrying a GFP-encoding transgene under the control of the promoter of the Sf1 gene. Sf1 is expressed in both male and female genital ridge somatic

cells, throughout the critical window of sex determination, in the precursors of the steroidogenic and supporting cell lineages (Stallings et al., 2002), the latter being essential for the initiation of the testicular differentiation by Sry (Palmer and Burgoyne, 1991). Our experimental data have convincingly established that the GFP^+ cell fractions used in our profiling experiments consisted almost exclusively of somatic $Sf1^+$ cells.

A striking finding in our studies is the scale of both male- and female-specific genetic programs in gonadal somatic cells. We found that almost half of the genes (47%) contained on the Affymetrix microarrays are expressed by the $Sf1^+$ cell population of which 10% of them exhibited a sexually dimorphic expression (2306 genes). We have identified more than 1100 genes that display a sexually dimorphic pattern with a difference in expression $\geq$1.5-fold at E12.5 (592 are overexpressed in testis and 546 in ovaries). This number reaches approximately 1900 genes at E13.5 (919 overexpressed in testes and 1029 in ovaries). Even when the selection criteria are more stringent, i.e., by increasing the fold changes (see Table 3), the number of genes exhibiting a sexual dimorphism remains far above what has previously been identified. Both the scale and the temporal patterns of sexually dimorphic genes are a direct consequence of multiple specific genetic programs that are triggered by the differentiation of Sertoli and Leydig cell in the testis and granulosa and theca cells in the ovary, all of these cells expressing *Sf1*.

Gonads develop as ovaries in the absence of a Y chromosome, and the prevalent view is that Sry actively diverts the uncommitted gonad toward the testicular fate via a male-specific genetic program. The ovarian fate has thus been considered as the "default" pathway of gonadal differentiation. But, even if it arises through a "default" pathway, it is obvious that the ovary must also follow a genetic program of differentiation (Eicher and Washburn, 1986); however, almost nothing is known about the ovarian program. Our transcriptome analyses have identified robust genetic programs in both male and female genital ridges that are initiated prior to the first morphological changes in the indifferent gonads. Considering the number of genes with male or female overexpression at E11.0 and E11.5 (61 and 25 respectively) as well as the average fold changes in their expression, it appears that the male genetic program is set in motion slightly earlier and/or more vigorously than its female counterpart.

One of the most obvious morphological changes following gonadal differentiation is the size difference between XX and XY gonads; by E13.5, XY gonads are approximately twice the size of XX gonads. In males, the size increase is due in part to XY-specific cell migration from the mesonephros and in part due to an increase in cell proliferation in XY gonads. In females, this conversely can be viewed as a reduced proliferative index in the genital ridge. In fact, the earliest identified physiological difference between XY and XX gonads is an increase in cell proliferation of $Sf1^+$ cells underlying the coelomic epithelium in male embryos (Schmahl et al., 2000). Recent studies (Schmahl and Capel, 2003) have shown that cell proliferation is necessary for testis determination as inhibition of proliferation in XY gonads within an 8-h period of gonadal development (~E11.2) leads to ovarian differentiation. This finding suggests that proliferation within a developmental window is essential to initiate the male pathway. *Sry* must therefore either (1) activate genes and signaling pathways involved in male-specific proliferation in XY gonads or (2) repress inhibitory signals blocking cell proliferation and differentiation of supporting lineages. Our observation that Cdk inhibitors are overexpressed in the developing ovaries coincident with sex determination and gonadal development supports the second hypothesis. Reduced proliferation in XX genital ridges, a female-dependent characteristic, could therefore be the result of increased expression of the 3 Cdk inhibitors (Cdkn1a, b, c), inhibiting cdk activity and causing $Sf1^+$ cells to cease proliferation. Sry may downregulate—either directly or indirectly—the expression of Cdkn inhibitors such as *Cdkn1a*, *Cdkn1b* and *Cdkn1c* in XY somatic cells, thereby allowing Sertoli cells to proliferate and divert the XY gonad from the ovarian fate. If correct, this hypothesis requires that Cdk inhibitors have to be expressed in the supporting cell lineage of XX gonads at the time of sex determination. Reports indicate that both *Cdkn1a* and *Cdkn1b* are expressed in granulosa cells and cooperate for the exit of differentiating granulosa cells from the cell cycle (Jirawatnotai et al., 2003). Mice lacking *Cdnk1b* are sterile due to hyperproliferation of granulosa cells during luteinization (Kiyokawa et al., 1996; Tong et al., 1998).

For the first time, we have mapped the chromosomal localization of genes exhibiting ovarian or testicular-specific expression. If we except the undifferentiated stage at E10.5, when sexually dimorphic genes are specifically located on X and Y chromosomes, the genes exhibiting either a testicular or ovarian-specific profile are generally scattered throughout the whole genome. Nevertheless, some discrete chromosomal regions contain clusters of either male or female overexpressed genes. In some cases, genes located within these clusters

Table 3
Number of genes (probe sets) classified as sexually dimorphic according to fold changes in expression

Fold Changes	E10.5		E11.0		E11.5		E12.5		E13.5	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
$\geq$1.5	8	6	11	6	61	27	592	546	919	1029
$\geq$2	8	5	8	3	46	15	343	286	562	640
$\geq$3	8	3	7	3	23	6	201	124	298	289
$\geq$4	8	3	7	3	16	5	129	62	235	196
$\geq$5	7	3	7	3	13	4	100	49	182	131

exhibited similar expression profiles, suggesting that these genes may share common regulatory elements (see Fig. 5 for clusters on chromosomes 1 and 7). Strikingly, these male- or female-specific genes are interspaced by other genes within the cluster that are not sexual dimorphic. In the case of the variant-specific group of genes on chromosome 7, these genes are part of a cluster of imprinted genes mapping a synthenic region corresponding to the human chromosome 11p15.5 whose dysregulation results in the Beckwith–Wiedermann syndrome (Koufos et al., 1989).The sexual dimorphic expression of *Kcnq1*, *Phlda2* and the Est AU040576 discovered by chromosome mapping analysis represents an additional layer of gene regulation together with the regulatory element controlling imprinting within this cluster. These in silico data mining analyses could provide information concerning the identification of genes involved in sexual ambiguities and/or infertility. For instance, genes with both a sexually dimorphic expression profile and a chromosomal locus known to be affected in patients with sexual ambiguity and/or sterility will represent first choice candidates.

We have analyzed in more detail the expression profile of 5 genes selected for the potential roles in gonadal differentiation or endocrine functions (see Fig. 6A). Cerebellin1 is a secreted 16-amino-acid peptide widely expressed in the central nervous system, including the hypothalamus, and in the adrenal medulla (Satoh et al., 1997). Cbln1 has been reported to stimulate steroid secretion from adrenal cortex in vivo and in vitro (Albertin et al., 2000; Mazzocchi et al., 1999). We found that *Cbln1* is upregulated in Leydig cells of the developing testis starting at E12.5 (see also Menke and Page, 2002), and extrapolation with its reported function in the adrenal gland suggests that Cbln1 may also regulate steroidogenesis in Leydig cells. Interestingly, Cbln4, another member of the Cerebellin family, is expressed in the developing testis but in Sertoli cells starting at E11.5 (Fig. 3 and data not shown). Gpr37 has been selected for its transient male-specific upregulation at E11.0. *Gpr37* encodes an orphan G-protein-coupled receptor shown to be expressed in brain tissues and testis (Marazziti et al., 1998). *Irx3* belongs to a homeobox gene family composed of 6 members (*Irx1–6*) (Houweling et al., 2001). *Irx* genes are involved in the patterning and regionalization of differentiation. Irx3 is known to direct the neuronal fate of progenitors in the ventral neural tube (Briscoe et al., 2000). We found that Irx5 is also specifically expressed in the developing ovary starting at E11.5 (data not shown). *Irx3* and *Irx5* genes (chromosome 8) are separated by 550 kb of intergenic sequences, and thus far no function has been reported for Irx3 and 5 in the developing gonad. Another candidate is Gpr49, also known as Lgr5, a G-protein-coupled receptor belonging to the FSH, LH and TSH receptor family. Our expression profile demonstrated a female-specific expression starting at E12.5 (Fig. 6A). In the adult ovary, Gpr49 is expressed in the follicles (Hermey et al., 1999). Targeted deletion of Gpr49 in mice leads to neonatal lethality (Morita et al., 2004) and so far, no report has assessed ovarian function and development in *Gpr49* mutant mice. Finally, Runx1, also known as AML1, encodes a transcription factor belonging to a family defined by the runt domain and that comprises three members (*Runx1, Runx2* and *Runx3*). *Runx1* is involved in controlling the proliferation and differentiation of cells during development (Coffman, 2003). In *Drosophila*, the Runx1 orthologue (runt) has been implicated in processes such as sex determination and neurogenesis (Duffy and Gergen, 1991; Duffy et al., 1991). Homozygous *Runx1* knockout mice die at E12.5 from an early block in blood development (Okuda et al., 1996). So far, no function has been reported for Runx1 in the developing gonad.

Another study reporting gene expression profiles in male and female differentiating gonads has been published very recently (Small et al., 2005). Although conceptually similar, there are a number of differences in the two studies. The present work used $Sf1^+$ somatic cells purified from E10.5, E11.0, E11.5, E12.5 and E13.5 embryos. In contrast, the published study analyzed RNA prepared from dissected genital ridges at E11.5 (which certainly included the mesonephros) and whole gonads at later stages (E12.5, E14.5, E16.5 and E18.5). Our data thus concern the subset of gonadal cells in which sexually dimorphic gene expression is initiated and focus more tightly on the 48 h time window when critical events in gonadal sex determination and sex differentiation occur. A considerable advantage of working with $Sf1^+$ cells is to optimize the detection of specific genes expressed within the population where Sry is thought to be acting by avoiding dilution by RNAs originating from other cell populations. Comparing the average signals obtained in the two studies for the 10 most highly expressed female-specific genes at E11.5 and E12.5, our results were systematically higher (between 4- and 9-fold; data not shown). We also detected sexually dimorphic genes at earlier stages and usually with greater differences between sexes. For example, female over-expressed genes such as *Fst*, *Irx3*, *Wnt4, Cdkn1a* and *Bmp2* and male overexpressed genes such as *Tesc*, *Dhh*, *Fgf9* and *Cbln1* were already sexually dimorphic in our study at E11.5, while such differences did not become detectable until 24 h later in the whole gonad profiling experiment of Small et al. We also provide relevant additional data for a number of dimorphic genes revealed in our study. In particular, WISH on gonads from normal or busulfan-treated embryos has allowed us to localize transcripts to specific cell lineages.

Conclusion

This study describes the male and female genetic programs triggered in the somatic compartment of the developing gonads during a critical period of sex determination and differentiation. It describes the expression profiles of more that 2300 genes that exhibit either a male or a female-specific expression pattern and should serve as an expression profile library for further studies in the field of sex determination, testicular and ovarian development and functions. Our studies further provide valuable clues regarding potential candidate genes involved in mechanisms of normal sex determination, ovarian and testicular development and their endocrine and reproductive functions. We identify new molecular markers to trace the development, differentiation and interactions of the various cell types of the ovary that should provide the long-needed

tools to dissect the molecular regulation mediating ovarian development.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2005.09.008.

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