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Sexually dimorphic gene expression in the developing mouse gonad

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Abstract

Over the course of a few days, the bipotential embryonic mouse gonad differentiates into either a testis or an ovary. Though a few gene expression differences that underlie gonadal sex differentiation have been identified, additional components of the testicular and ovarian developmental pathways must be identified to understand this process. Here we report the use of a PCR-based cDNA subtraction to investigate expression differences that arise during gonadal sex differentiation. Subtraction of embryonic day 12.5 (E12.5) XY gonadal cDNA with E12.5 XX gonadal cDNA yielded 19 genes that are expressed at significantly higher levels in XY gonads. These genes display a variety of expression patterns within the embryonic testis and encode a broad range of proteins. A reciprocal subtraction (of E12.5 XX gonadal cDNA with E12.5 XY gonadal cDNA) yielded two genes, *follistatin* and *Adamts19*, that are expressed at higher levels in XX gonads. Follistatin is a well-known antagonist of TGFβ family members while *Adamts19* encodes a new member of the ADAMTS family of secreted metalloproteases.

Keywords: Sex differentiation; Gonad; Mouse; Subtractive hybridization; Testis; Ovary; Sexually dimorphic; Embryo; Mouse

1. Results and discussion

1.1. Isolation of testis genes

To discover gene expression differences between embryonic XX and XY gonads, we performed a PCR-based cDNA subtraction. We began by subtracting E12.5 XY gonadal cDNA with E12.5 XX gonadal cDNA to generate a cDNA pool enriched for testis transcripts. E12.5 is the earliest time at which XX and XY gonads can be distinguished morphologically, and one would expect numerous differences in gene expression to be present at this stage. After four rounds of subtraction, we cloned the resulting cDNA fragments and randomly selected 1078 clones for sequencing. Strikingly, 30% of the cDNA clones originated from four Y chromosome genes (Table 1). These Y chromosome genes are ubiquitously expressed in male tissues (Agulnik et al., 1994; Ehrmann et al., 1998; Greenfield et al., 1996; Mazeyrat et al., 1998). An additional 12% of the clones represent genes previously reported to be upregulated during testis differentiation (Table 1). These genes include *Mullerian inhibiting substance*, *desert hedgehog*, *17-α-hydroxylase*, and *testatin*.

We analyzed the expression of all novel sequences that were represented by two or more cDNA clones. We also examined several known genes that were represented by multiple clones and/or that were deemed unlikely to be ubiquitously expressed based on BLAST searches against the NCBI EST database. RT-PCR expression analysis of these genes on testes and ovaries at E12.5, E13.5, and E14.5 revealed that most are expressed at significantly higher levels in testes than ovaries (Fig. 1, and data not shown). We ultimately identified 19 genes that are upregulated in XY gonads relative to XX gonads by E12.5 (Table 1). Five of these 19 genes are novel. Differential expression of six of the remaining 14 genes has recently been reported by other investigators. When combined with the Y derived cDNAs and other differentially expressed genes mentioned above, at least 80% of the subtracted cDNAs in our testis library were derived from differentially expressed genes.

Whole-mount in situ hybridization of E13.5 testes and ovaries revealed the expression of these 19 genes in different gonadal cell types. Twelve of these genes are expressed within the testicular cords (Table 1 and Fig. 2A–L). Interestingly, one of the twelve, *osteopontin* (*Spp1*), is also expressed in the Mullerian ducts, where it is found at higher levels in the ducts of XX embryos than in those of XY embryos (Fig. 2E). The remaining seven genes are expressed either outside the testicular cords or are expressed in cells located both inside and outside the cords (Fig. 2M–S).

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Table 1
Testis cDNA subtraction: summary of differentially expressed genes

Gene symbol	Gene name	Number of clones[a]	Gonad expression[b]	Comments/references
Dby[c]	*DEAD box, Y*	137	Y	(Mazeyrat et al., 1998)
Eif2s3y[c]	*Eukaryotic translation initiation factor 2, subunit 3, Y*	100	Y	(Mazeyrat et al., 1998)
Smcy[c]	*Selected mouse cDNA, Y*	32	Y	(Agulnik et al., 1994)
Uty[c]	*Ubiquitously transcribed tetracopeptide repeat, Y*	53	Y	(Greenfield et al., 1996)
Cst9[c]	*Cystatin 9 (testatin)*	5	C	(Tohonen et al., 1998)
Cyp17[c]	*Cytochrome p450 17-α-hydroxylase/C17-20 lyase*	23	I	(Greco and Payne, 1994)
Dhh[c]	*Desert hedgehog homolog*	95	C	(Bitgood et al., 1996)
Mis[c]	*Mullerian inhibiting substance*	2	C	(Munsterberg and Lovell-Badge, 1991)
Aard	*Alanine and arginine rich domain containing protein (A5D3)*	47	C	167 a.a., no homology (Blomberg et al., 2002)
Cbln1	*Cerebellin 1 precursor protein*	38	I	(Urade et al., 1991)
Cbln4[d]	*Cerebellin 4 precursor protein*	17	C	New member of cerebellin family
Cdh11[e]	*Cadherin 11*	1	I,M	(Wertz and Herrmann, 2000)
Col9a3[e]	*Procollagen, type IX, α3*	33	C	(Perera et al., 2001)
Cyp26b1	*Cytochrome p450, subfamily 26b, polypeptide1 (p450RAI2)*	6	C,I	Retinoic acid hydroxylase (White et al., 2000)
Dtna	*α-Dystrobrevin*	17	C	(Grady et al., 1999)
Etd[d]	*Embryonic testis differentiation*	7	C	59 a.a., no homology
Hhip	*Hedgehog interacting protein*	11	I	(Chuang and McMahon, 1999)
Loc195443[d]	Novel electronically annotated gene	9	C	390 a.a., contains ankyrin repeats
Mmd2[d]	*Monocyte to macrophage differentiation associated 2*	4	C	247 a.a., 69% identical to MMD1
Pak3	*p21-activated kinase 3*	3	C	(Allen et al., 1998)
Pkr2	*Prokineticin receptor 2* (*G protein-coupled receptor 73-like 1*; *Gpr73l1*)	18	I	(Lin et al., 2002)
Ptgds[e]	*Prostaglandin D2 synthase*	2	C	(Adams and McLaren, 2002)
Ren1[e]	*Renin*	100	I	(Perera et al., 2001)
Serpine2[e]	*Serine (or cysteine) proteinase inhibitor, clade E, member 2 (protease nexin 1)*	2	C	(Grimmond et al., 2000)
Sostl[d]	*Sclerostin-like protein*	4	I	206 a.a., 39% identical to SOST
Spp1	*Secreted phosphoprotein 1 (osteopontin)*	10	C,D	(Liaw et al., 1998)
Tdl[e]	*Testis-specific β-Defensin-like gene*	80	C	(Yamamoto and Matsui, 2002)

[a] A total of 1078 clones were sequenced.
[b] Y, ubiquitously expressed Y chromosome gene; C, testicular cords; I, interstitial cells; M, mesonephros; D, Mullerian duct.
[c] Genes reported to be differentially expressed prior to initiation of this study.
[d] Novel gene.
[e] Differential expression of these genes has recently been reported by other laboratories and was independently determined in this study.

We also wanted to know whether the genes identified here are expressed in tissues other than testis. We examined the tissue distribution of eight genes (including all five novel genes) by performing RT-PCR on a panel of cDNAs derived from 8-day postpartum (8dpp) mice. Though the expression of each gene was restricted to a subset of tissues, *Aard* and *Etd* displayed the greatest testis specificity (Fig. 3). *Aard* was also expressed at low levels in lung, consistent with recent work demonstrating that the orthologous rat gene is expressed in lung tissue (Blomberg et al., 2002). *Etd* is the only gene for which we found no evidence of expression outside the testis. Additional RT-PCR performed on tissues from E9.5 and E10.5 embryos failed to reveal any *Etd* expression (data not shown).

The 12 genes expressed in testicular cords (Fig. 2A–L) are most likely expressed by germ cells or Sertoli cells. We included testes from adult W^v/W^v mice in our RT-PCR analysis to help determine which cell types express these genes. W^v/W^v mice contain a point mutation in the c-kit tyrosine kinase receptor and are severely depleted of germ cells (Nocka et al., 1990). The strong RT-PCR signal observed for most of our genes in W^v/W^v testes suggests their expression is largely independent of germ cells (Fig. 3). To confirm these results, we tested a subset of the testis genes for expression in busulfan-treated embryonic gonads. Exposure of embryos to busulfan virtually eliminates germ cells (Merchant, 1975), and we confirmed this loss by staining for the germ cell specific marker alkaline phosphatase (data not shown). Busulfan treatment did not cause a noticeable reduction in expression of *Cbln4*, *Tdl*, or *Aard*, all of which are expressed in the testicular cords (Figs. 2G–I and 4A–C). Thus, Sertoli cells are the most likely source of

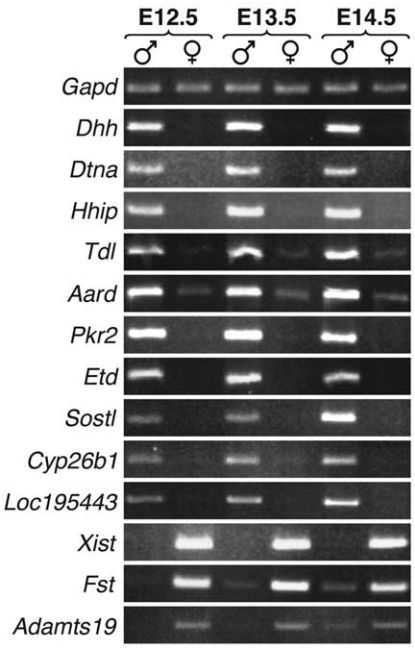

Fig. 1. RT-PCR expression analysis of testis and ovary subtraction products in E12.5, E13.5 and E14.5 testis and ovary. *Gapd* served as a testis/ovary common control. *Dhh* and *Xist* served as testis and ovary controls, respectively.

expression. Nonetheless, we cannot exclude the possibility that germ cells also express these genes.

Only a small number of differentially expressed signaling molecules are known to be present during testis development. It is therefore interesting that two of the five novel genes that we identified, *Sostl* and *Cbln4*, encode proteins with putative secretion signals. *Sostl* (*Sclerostin-like*) is expressed in interstitial cells of the testis and is ~39% identical to the recently identified human *sclerostin* gene (*SOST*), mutation of which results in progressive bone overgrowth (Brunkow et al., 2001). Both genes contain a cysteine-knot motif, a characteristic of many signaling molecules. *Cbln4* is a new member of the cerebellin family of secreted neural peptides that share homology with the C-terminal globular domain of C1q complement subunits (Urade et al., 1991). We also found that another member of the cerebellin family, *Cbln1*, is upregulated in embryonic testes. Despite the similarities of their encoded proteins (77% amino acid identity), *Cbln1* is expressed in testicular interstitial cells, while *Cbln4* is restricted to the testicular cords (Fig. 2G,O). Although the in vivo role of the CBLN1 protein is not known, protease processing yields a hexadecapeptide that is present in both cerebellum and adrenal gland and that is capable of inducing steroid secretion in adrenal gland slices in vitro (Albertin et al., 2000). Of the remaining novel genes, *Mmd2* (*Monocyte to macrophage differentiation associated 2*) is related to a gene (*Mmd1*) encoding a protein with putative transmembrane domains, the predicted product of *Loc195443* contains ankyrin repeats, and *Etd* (*Embryonic testis differentiation associated transcript*) possesses a small open reading frame with no evident similarity to known protein-coding genes.

For several of the genes that we identified as being expressed at greater levels in XY gonads, functional insights have been obtained by other investigators. The CYP26B1 protein acts as a retinoic acid hydroxylase and is capable of hydroxylating all-trans-retinoic acid (White et al., 2000). The hydroxylated metabolites produced by CYP26B1 do not activate any known retinoic acid receptors. Thus, it is believed that CYP26B1 regulates retinoic acid signaling by inactivating all-trans-retinoic acid (Niederreither et al., 2002). Although a role for retinoic acid in embryonic gonads has not been demonstrated in vivo, retinoid receptors are expressed in embryonic gonads (Dufour and Kim, 1999; Morita and Tilly, 1999), and exposure of embryonic testes to retinoic acid in organ culture disrupts cord formation (Livera et al., 2000a). Similar experiments with embryonic ovaries indicate that in vivo or in vitro exposure of retinoic acid promotes germ cell survival, proliferation, and meiotic entry (Livera et al., 2000b; Morita and Tilly, 1999). Our identification of a differentially expressed regulator of retinoic acid activity strengthens the case for the involvement of retinoids in gonadal development.

Mouse *Pkr2* encodes a G-coupled receptor that has recently been identified as a receptor for prokineticin 1 and 2 (PK1 and PK2) (Lin et al., 2002). Interestingly, PK1 (also known as EG-VEGF) has been demonstrated to promote angiogenesis specifically in endothelial cells derived from endocrine glands. It acts by inducing cellular proliferation and migration (LeCouter et al., 2001). Since the vasculature of XX and XY embryonic gonads develop significant differences (Brennan et al., 2002), *Pkr2* expression in interstitial cells of the XY gonad suggests the possible involvement of this gene in the formation of the testis vasculature.

We found that *Hedgehog-interacting protein* (*Hhip*) is also upregulated during testis development. HHIP is thought to attenuate hedgehog signaling by binding to and sequestering hedgehog proteins (Chuang and McMahon, 1999). Two other hedgehog signaling components, *Desert hedgehog* (*Dhh*) and its receptor, *patched*, are upregulated in XY embryonic gonads as well. Targeted deletion of *Dhh* results in male infertility (Bitgood et al., 1996), and recent work has shown that *Dhh* signaling specifies Leydig cell fate (Yao et al., 2002). Thus, the importance of hedgehog signaling in testis development is well established.

In contrast to the genes discussed above, experimental evidence indicates that certain differentially expressed transcripts we identified are not essential for testis development. Specifically, mice deficient in either *Spp1* or *Dtna* are viable and fertile (Grady et al., 1999; Liaw et al., 1998).

1.2. Isolation of ovary genes

In addition to the testis subtraction, we performed a reciprocal experiment in which we subtracted E12.5 XX gona-

dal cDNA with E12.5 XY gonadal cDNA. After four rounds of subtraction, we randomly chose 188 clones for sequencing. Approximately 54% of these cDNA clones originated from *Xist* RNA (Table 2). The non-coding *Xist* RNA is expressed from the inactive X chromosome of female cells and is lacking in males (Brown et al., 1991). Thus, the extreme enrichment of *Xist* in our ovarian subtraction is related to dosage compensation rather than an ovary specific function. We also isolated one cDNA clone derived from *Wnt4*, one of the few genes known to be upregulated in XX gonads relative to XY gonads during sexual differentiation (Vainio et al., 1999).

Other than *Xist*, the only non-housekeeping gene represented by multiple cDNA clones in our ovarian subtraction

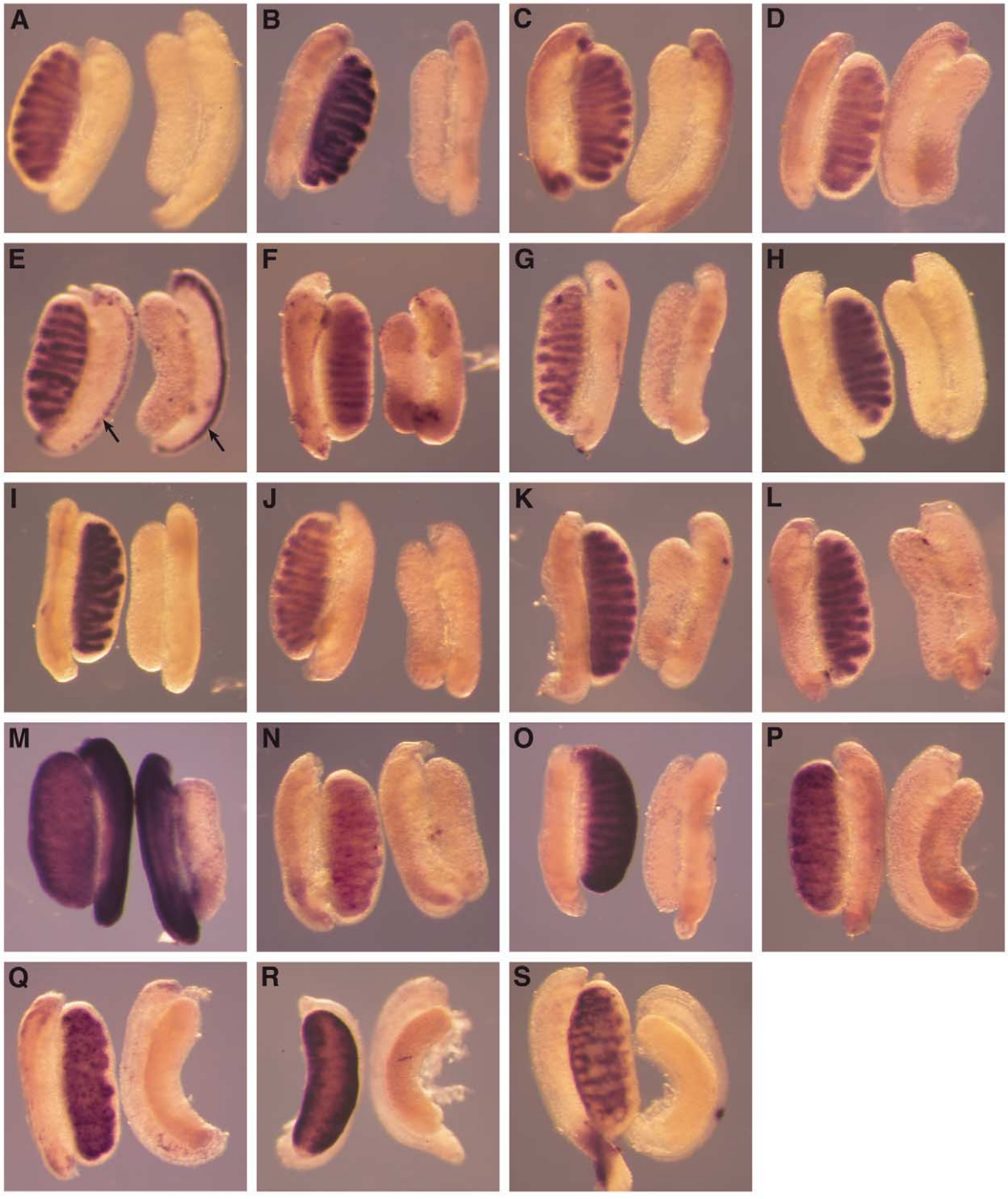

Fig. 2. Whole-mount in situ hybridization of differentially expressed testis genes on E13.5 testes and ovaries. (A) *Col9a3*, (B) *Ptgds*, (C) *Serpine2*, (D) *Dtna*, (E) *Spp1* (arrows indicate expression in mesonephros), (F) *Pak3*, (G) *Cbln4*, (H) *Tdl*, (I) *Aard*, (J) *Etd*, (K) *Mmd2*, (L) *Loc195443*, (M) *Cadh11*, (N) *Hhip*, (O) *Cbln1*, (P) *Pkr2*, (Q) *Cyp26b1*, (R) *Sostl*, and (S) *Ren1*. In all panels, embryonic testes are located on the left and embryonic ovaries on the right.

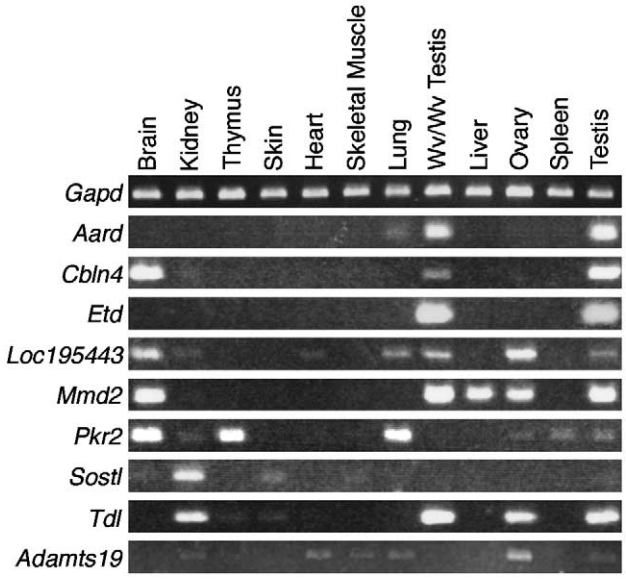

Fig. 3. RT-PCR expression analysis of novel testis and ovary genes on 8 day post partum mouse tissues and germ cell depleted W^v/W^v adult testes. *Gapd* served as a ubiquitously expressed control.

was *follistatin* (Table 2). Examination by RT-PCR confirmed that *follistatin* is expressed more abundantly in ovaries than in testes at E12.5, E.13.5, and E14.5 (Fig. 1). Analysis of ten novel cDNAs, each recovered once in our ovary subtraction, revealed that only one of these is expressed at higher levels in ovary (Fig. 1, and data not shown). This cDNA is derived from *Adamts19*, a new member of the ADAMTS (a disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif) family of secreted metalloproteases. The orthologous human gene has recently been reported elsewhere (Cal et al., 2002).

Whole-mount in situ hybridization confirmed that both *follistatin* and *Adamts19* are expressed more abundantly in embryonic ovaries than in embryonic testes at E12.5, E13.5, E14.5, and E15.5 (Fig. 5). *Follistatin* displayed staining in ovaries at all stages, while no expression was detected in testes (Fig. 5A–D). In contrast, *Adamts19* expression was strongest in anterior and ventral regions of the ovary at E12.5 and E13.5 (Fig. 5E,F) before becoming more uniform (Fig. 5G,H). Faint *Adamts19* expression was evident in embryonic testes at E14.5 and E15.5 (Fig. 5G,H). In busulfan-treated ovaries we observed intense staining for *follistatin* and *Adamts19* (Fig. 4E,F). Thus, these genes are expressed from somatic cells of the ovary. However, we cannot exclude the possibility that germ cells also express these genes.

Follistatin encodes a secreted protein that is capable of binding to and antagonizing the function of multiple members of the TGFβ superfamily, including the activins and certain BMPs (Patel, 1998). *Follistatin* is expressed by numerous cell types both embryonically and postnatally, including the granulosa cells of the ovary (Shintani et al., 1997), and its expression in embryonic gonads has been previously reported (Feijen et al., 1994). However, it was not previously appreciated that *follistatin* expression is sexually dimorphic. Disruption of the *follistatin* gene in mice results in pleiotropic effects and death ensues shortly after birth (Matzuk et al., 1995). Though no gonadal abnormalities were reported, follistatin appears to play regulatory roles in postnatal ovaries and testes (de Kretser et al., 2001; Knight and Glister, 2001). We believe it will be

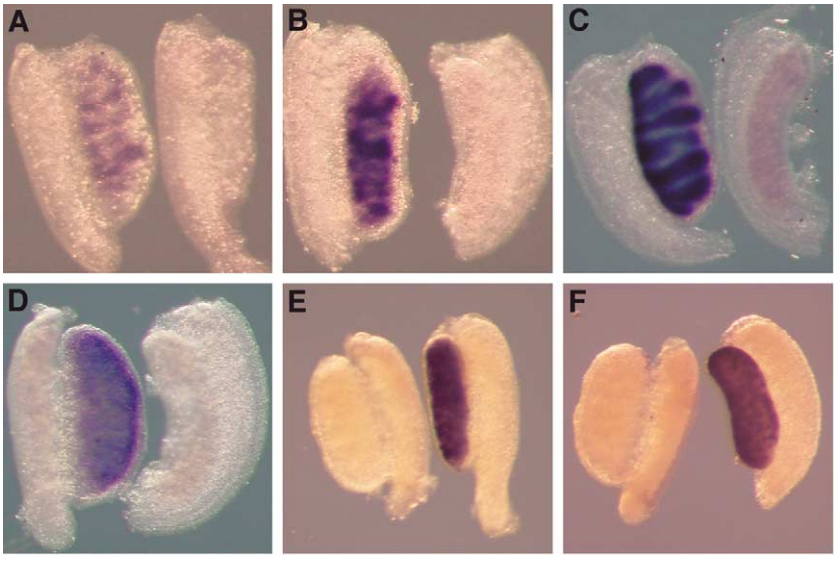

Fig. 4. Expression of testis and ovary genes in busulfan-treated E13.5 testes and ovaries. Whole-mount in situ hybridization of (A) *Cbln4*, (B) *Tdl*, (C) *Aard*, (D) *Cbln1*, (E) *follistatin*, and (F) *Adamts19*. In all panels, embryonic testes are located on the left and embryonic ovaries on the right.

Table 2
Ovary cDNA subtraction: summary of differentially expressed genes

Gene symbol	Gene name	Number of clones[a]	Comments/references
Xist[b]	*Inactive X specific transcript*	101	Required for X inactivation (Brown et al., 1991)
Wnt4[b]	*Wingless-related MMTV integration site 4*	1	Required for ovarian development (Vainio et al., 1999)
Fst	*Follistatin*	13	TGFβ binding protein (Matzuk et al., 1995)
Adamts19	*A disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 19*	1	New member of the ADAMTS family of metalloproteases

[a] A total of 188 clones were sequenced.
[b] Genes reported to be differentially expressed prior to initiation of this study.

worthwhile to reexamine *follistatin* null embryonic ovaries for expression defects using somatic and germ cell markers of ovarian differentiation that have become available.

The second ovarian gene, *Adamts19*, is a new member of the ADAMTS family of secreted metalloproteases (Cal et al., 2002). While *Adamts19* is predominantly expressed in ovary at 8 dpp, low levels of expression were also observed in kidney, heart, skeletal muscle, lung, and testis (Fig. 3). Other members of this family are implicated in a range of processes including the inflammatory response, angiogenesis, and organogenesis (Tang, 2001). At least one mammalian family member, *Adamts1*, is required for female fertility and appears to be involved in ovulation (Espey et al., 2000; Shindo et al., 2000). In addition, gonad formation in *C. elegans* is disrupted when the ADAMTS encoding gene *Gon-1* is mutated (Blelloch and Kimble, 1999). Therefore, expression of *Adamts19* in embryonic and postnatal ovaries makes it an attractive candidate for functional analysis.

1.3. Subtraction assessment

Our testis subtraction successfully recovered 14 genes previously reported to be upregulated in embryonic testis relative to embryonic ovary; these constitute the majority of sexually dimorphic transcripts known to be present in embryonic XY gonads at E12.5. We identified an additional 13 genes, of which five are novel, that have not been previously shown to be differentially expressed. Likewise, our ovary subtraction was successful in identifying four differentially expressed genes, two of which were not known to be sexually dimorphic prior to this study. Identification of these genes should help deepen our understanding of gonadal sex differentiation by providing insights into complexes and pathways operating in differentiating gonads, by serving as markers of gonadal cell types, and by enabling functional analysis. Nonetheless, it seems likely that we identified only a small fraction of the differences that are present. Our testis subtraction did not recover

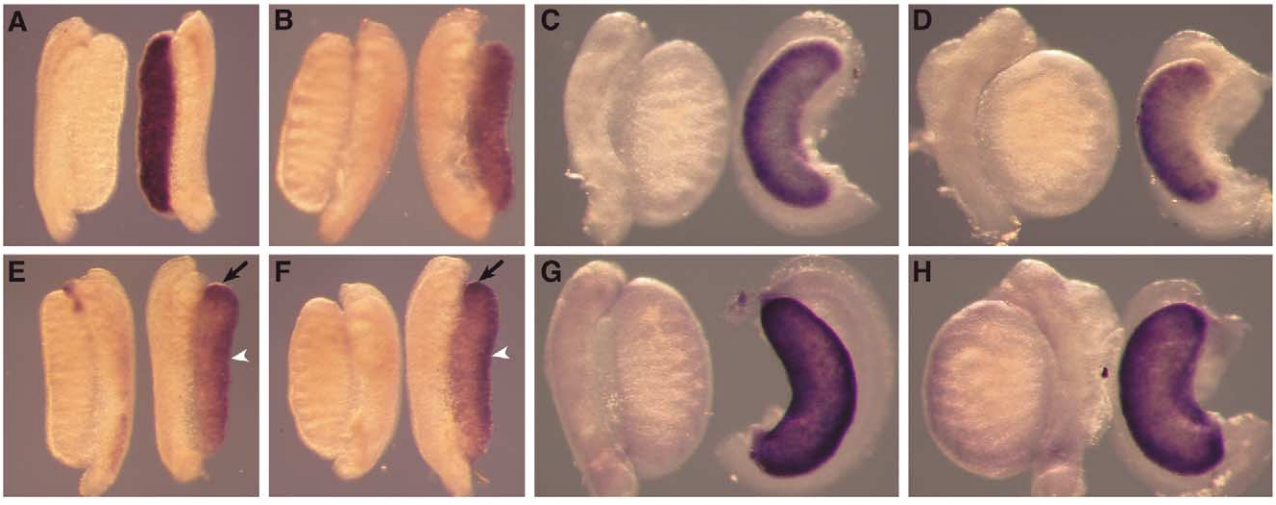

Fig. 5. Whole-mount in situ hybridization of differentially expressed ovary genes in embryonic gonads from E12.5 to E15.5. *Follistatin* expression at (A) E12.5, (B) E13.5, (C) E14.5, and (D) E15.5. *Adamts19* expression at (E) E12.5, (F) E13.5, (G) E14.5, and (H) E15.5. In all panels, embryonic testes are located on the left and embryonic ovaries on the right. Black arrows in (E,F) indicate the anterior tip of the ovaries; white arrowheads indicate the ventral surface of the ovaries.

certain known differentially expressed genes such as *Sox9* (Morais da Silva et al., 1996), *tescalcin* (Perera et al., 2001), *vanin-1* (Bowles et al., 2000; Grimmond et al., 2000), and *patched* (Bitgood et al., 1996). Moreover, our subtractions were biased towards transcripts with large expression differences between XX and XY gonads. Thus, we did not isolate genes such as *Wt1* and *Sf1* that are only moderately more abundant in XY than XX gonads at E12.5 (Nachtigal et al., 1998). Additional approaches will be required to achieve a complete catalog of gene differences.

2. Experimental procedures

2.1. Mice and tissues

C57BL/6 mice from Taconic Farms Inc. (Germantown, NY) were used for all experiments except for those using W^v/W^v mutant mice which were produced through heterozygous matings. Timed matings were performed with the day a vaginal plug was found designated as E0.5. Germ cell depleted gonads were generated by injecting E9.5 pregnant females with 0.2 ml of 6.6 mg/ml busulfan (Sigma) in 50% DMSO (53 mg/kg body weight). For subtraction material E12.5 XY and XX gonads were isolated and the adjacent mesonephroi were removed.

2.2. cDNA subtraction and sequence analysis

We performed a PCR-based cDNA subtraction based on previously reported protocols with substantial modifications (Diatchenko et al., 1996; Lavery et al., 1997). A detailed version of our protocol is available at http://staffa.wi.mit.edu/page/papers/Menke_and_Page_2002/. The subtracted testis and ovary cDNA pools were cloned into the pAMP10 plasmid (GIBCO-BRL) and clones were randomly selected for sequencing. Sequences were organized into contigs using the DNA alignment program Sequencher (Gene Codes Corp) and these contigs were compared against the NCBI databases.

2.3. RT-PCR

Total RNA (1 μg) was reverse transcribed with oligo $d(T)_{18}N$ using Superscript II (GIBCO-BRL) in a total reaction volume of 25 μl. PCR was performed using 0.5 μl of RT as template in a total volume of 20 μl. Primer sequences are available at http://staffa.wi.mit.edu/page/papers/Menke_and_Page_2002/. PCR cycling conditions for all primers were as follows: 94 °C (30 s), 60 °C (30 s), 72 °C (1 min) for 25–30 cycles.

2.4. Whole-mount in situ hybridization

Whole-mount in situ hybridizations were performed essentially as previously described (Wilkinson and Nieto, 1993). Gonads for whole-mount in situ hybridization were dissected out in PBS and fixed overnight at 4 °C in 4% paraformaldehyde. Tissues were subsequently washed and stored at −20 °C in methanol until used. Digoxigenin labeled riboprobes were generated using cDNA fragments cloned into the TA cloning vector pCR2.1-TOPO or pCR4-TOPO (Invitrogen). Plasmids were linearized by restriction digestion and transcribed with T3 or T7 RNA polymerase in the presence of Dig-labeling mix (Roche).

2.5. Full-length cDNA cloning

Full-length cDNA sequences are electronic composites derived from a combination of subtracted cDNA clones, conventional cDNA libraries, and 5′ and 3′ RACE. Three cDNA libraries derived from adult mouse testis were used (Clontech, Stratagene, and one library of our own construction) and one library generated from a mixture of E12.5 XY and XX gonads (of our own construction). 5′ and 3′ RACE was performed from testis and ovary mRNA using the Marathon cDNA Amplification Kit according to the manufacturer's instructions (Clontech).

2.6. GenBank Accession numbers

Cyp26b1, AY134662; *Cbln4*, AY134663; *Mmd2*, AY134664; *Aard*, AY134665; *Sostl*, AY134666; *Etd*, AY134667; *Tdl*, AF532614; and *Adamts19*, AY135183.

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