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Global gene expression analysis in fetal mouse ovaries with and without meiosis and comparison of selected genes with meiosis in the testis

Abstract In order to identify novel genes involved in early meiosis and early ovarian development in the mouse, we used microarray technology to compare transcriptional activity in ovaries without meiotic germ cells at embryonic age 11.5 (E11.5) and E13.5 ovaries with meiosis. Overall, 182 genes were differentially expressed; 134 were known genes and 48 were functionally uncharacterized. A comparison of our data with the literature associated, for the first time, at least eight of the known genes with female meiosis/germ cell differentiation (Aldh1a1, C2pa, Tex12, Stk31, Lig3, Id4, Recql, Piwil2). These genes had previously only been described in spermatogenesis. The microarray also detected an abundance of vesicle-related genes of which four were upregulated (Syngr2, Stxbp1, Ric-8, SytIX) and one (Myo1c) was downregulated in E13.5 ovaries. Detailed analysis showed that the temporal expression of SytIX also coincided with the first meiotic wave in the pubertal testis. This is the first time that SytIX has been reported in non-neuronal tissue. Finally, we examined the expression of one of the uncharacterized genes and found it to be gonad-specific in adulthood. We named this novel transcript "Gonad-expressed transcript 1" (Get-1). In situ hybridization showed that Get-1 was expressed in meiotic germ cells in both fetal ovaries and mature testis. Get-1 is therefore a novel gene in both male and female meiosis.

Keywords Meiotic prophase I · Ovary · Testis · Microarray · Gene expression · Mouse (B6D2/F1)

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Introduction

Meiotic initiation in mammalian female germ cells occurs in fetal life in many species (e.g., mouse), whereas this process in male germ cells normally begins at early puberty (e.g., in mouse testis around 1 week after birth). Female meiosis is initiated shortly after the ovary and testis can be distinguished morphologically in the mouse. Gonadal sex is apparent from embryonic day 12.5 (E12.5; Capel 2000), and in the ovary, the first meiotic cells, i.e., leptonema, are seen from E13.0 and zygonema from E13.5 (Peters 1970; Di Carlo et al. 2000). By the time of birth, almost all oocytes have reached the resting stage (diplonema), and follicle formation has begun. Early meiotic spermatocytes (leptonema) in the mouse are first recognized around day 7–8 post partum and diplonema in the third week of life (Bellve et al. 1997).

The mechanisms involved in the transition of a mitotically dividing germ cell line into meiosis have virtually only been described in yeast (Marston and Amon 2004). In mammals, the genes known to be related to this process and to meiotic prophase I in general are few and have emerged predominantly from laborious techniques such as gene-targeting in mice and mutational and chromosomal analysis in patients with fertility disorders (Rooij and Boer 2003; Critchlow et al. 2004; Tempest and Griffin 2004). With the introduction of differential display methodology, the first successful attempts to undertake genome-wide screenings for these genes were made by Olesen et al. (2004) and Goto et al. (1999). Subsequently, this procedure has been replaced with the more reproducible and swift microarray technology. To date, the microarray technique has demonstrated its efficiency in a range of biological and medical reports, including several studies of spermatogenesis and male meiosis in mice (Ellis et al. 2004; Wrobel and Primig 2005; Hsia and Cornwall 2004). Recently, a microarray-generated expression profile of the mature ovary has been compared with that of newborns, and numerous novel genes in ovarian development and follicle formation have been discovered (Herrera et al. 2005). To our knowledge, three microarray studies have been presented so far that include embryonic ovaries with germ cells in meiotic prophase I (Grimmond et al. 2000; Small et al. 2005; Nef et al. 2005). These studies, however, have been aimed primarily at characterizing new genes in gonadogenesis and sex determination, and transcriptional activity related to female meiosis has not been addressed in particular. We have therefore performed a microarray study comparing the genome-wide expression in E11.5 and E13.5 ovaries with the purpose of identifying novel genes associated with early female meiosis and early ovarian development in the mouse.

Materials and methods

Animals All tissues used in this study were isolated from B6D2/F1 mice.

Sex determination of E11.5 gonads The sex of the E11.5 gonads could not be distinguished by morphology and was therefore determined by the polymerase chain reaction (PCR) by using primers specific for the Sry gene. DNA was isolated from a limb bud from each E11.5 embryo by the PureGene kit (Gentra Systems), and PCR was conducted under cycling conditions of 95°C for 5 min, then 40 cycles of 95°C for 20 s, annealing at 60°C for 20 s, and 72°C for 20 s, followed by 72°C for 5 min. The Sry sense sequence was 5′-CTGCAGTTGCCTCAACAAAA-3′, and the antisense was 5′-TGGTTATGGAACTGCTG CTG-3′. For PCR, the PCR Reagent System (Invitrogen) was used according to the manufacturer's instructions with the modification that the supplied *Taq* DNA polymerase was replaced with DyNAzyme II (Finnzymes) at a final concentration of 0.4 U/μl in a total reaction volume of 20 μl.

Tissue isolation and RNA preparation for microarray analysis The E11.5 and E13.5 gonads were isolated and separated from mesonephroi in cold phosphate-buffered saline under a light microscope, transferred to RNAlater (Ambion), and immediately frozen in liquid N_2. The embryonic stage was confirmed by examination of the limb bud (Rugh 1968). RNA was isolated by using the Stratagene Micro RNA isolation kit according to manufacturer's instructions, with the following modifications. All reactions were adjusted to a lysis buffer volume of 100 μl, and Eppendorf phase lock gel was used for phase separation during phenol-chloroform extractions. Glycogen was added to the RNA precipitation in isopropanol, which was allowed to stand for 10 min on ice and spun at 13g for 30 min. The pellet was washed twice in ethanol and hydrolyzed in 10 μl diethylpyrocarbonate-treated water (DEPC-H_2O). RNA was pooled from approximately 11 pairs of female gonads for each sample at E11.5 and four pairs of female gonads for each sample at E13.5. RNA quantity was measured by using the Ribo Green RNA quantification kit (Molecular Probes, Europe), and RNA quality was evaluated by using an Agilent 2100 Bioanalyzer.

aRNA amplification and labeling Intact total RNA (1–2 μg) was amplified per sample following the aRNA amplification protocol of Van Gelder et al. (1990) by using the MessageAmp kit (Ambion). During in vitro translation, aRNA was labeled by incorporation of biotin-labeled nucleotides (biotin 11-CTP, Perkin Elmer, DK; biotin 16-UTP, Roche). The reaction mixture was incubated for 12 h in water bath, and labeled aRNA was purified by using the RNeasy kit (Qiagen). The quantity of aRNA after amplification ranged from 12 μg/sample to 15 μg/sample as determined by absorbance at 260 nm, corresponding to an amplification rate of 600–fold to 1500-fold (given an mRNA content of 1% of total RNA).

Fragmentation, hybridization, and scanning Fragmentation, hybridization, and scanning of the microarrays were performed according to the manufacturer's protocol (GeneChip Expression Analysis Technical Manual; Affymetrix, Santa Clara, Calif.). Briefly, 12–15 μg/sample labeled aRNA was fragmented in fragmentation buffer (200 mM TRIS acetate pH 8.1, 500 mM KOAc, 150 mM MgOAc) at 94°C for 35 min. Fragmented aRNA from each sample (E11.5 or E13.5 ovaries) was hybridized to Affymetrix Mouse Expression Set 430A in triplicate. Hybridization was performed according to the Affymetrix protocol modified to a total volume of 250 μl. The arrays were incubated with the hybridization cocktail for 16 h at 45°C with rotation in a hybridization oven. After hybridization, the arrays were filled with 200 μl non-stringent wash buffer and kept at 4°C until further processing. The arrays were washed and stained in an Affymetrix fluidics station according to the fluidic script for antibody amplification stain for eukaryotic targets. Immediately after hybridization, the arrays were scanned twice in an argon-ion scanner controlled by Affymetrix software (Affymetrix Microarray Suite).

Microarray data analysis Data from the microarray scanning was analyzed by using the affyR package available at http://www.cbs.dtu.dk. The expression index was calculated for each gene by using the Li-Wong Model-based expression index (Li and Wong 2004) based on the number of perfect matches, whereas mismatched probes were omitted. Array hybridization quality was evaluated by an M versus A plot depicting log(A/B) versus log((A+B)/2) where A and B were the expression index of genes on the two arrays compared in the plot. Next, the data was normalized with the non-linear qspline (Workman et al. 2002) method (Fig. 1). Hierarchical clustering based on Euclidian distance of the expression of all genes on the chips and Principal Component analysis were both employed to determine whether the arrays with E11.5 ovaries grouped separately from the arrays with E13.5 ovaries. Differentially expressed genes were identified by applying the Welch *t*-test (assuming unequal

Fig. 1 M versus A plots used for visualization and normalization of inherent variation between individual microarrays. Scatter plots of log intensity ratios M=log(A/B) on the *y*-axis versus average log intensities A=log(A+B)/2 on the *x*-axis for all 15 possible array-to-array comparisons after normalization (*upper triangle*), where A and B are expression indexes of genes on the two microarrays compared in each plot. The *diagonal* indicates the arrays compared. In total, six hybridizations were made, as E11 1/2 and E13 1/2 ovaries were run in triplicates. The *horizontal line* through zero in each plot indicates a perfect match, whereas the other line shows the data trend. The *lower triangle* shows the variance of the ratios between the two arrays compared

variance of the two populations) by using R statistics (http://www.r-project.org), and *P*-value cutoffs were adjusted for multiple comparisons.

Screening for gonad-specific or predominantly gonad-expressed transcripts Total RNA was isolated from fetal liver, kidney, heart, and brain at embryonic age 13.5 and from adult heart, kidney, liver, and brain by using Trizol Reagent (Invitrogen). Prior to cDNA synthesis, 2.0 μg total RNA of each tissue was subjected to DNase I treatment by using the Dnase I Amplification Grade kit (Invitrogen), and cDNAs were prepared with the Super-Script III First-Strand Synthesis System for reverse transcription/PCR (RT-PCR; Invitrogen) according to the supplier's protocol in a final reaction volume of 60 μl. The final cDNA mixes were prepared by mixing 1 μl of each of the four fetal tissues or 1 μl of each of the four adult tissues. A 1-μl sample of either the fetal or adult cDNA mixes were subjected to PCR in a total volume of 20 μl. PCR and cycle conditions were as described for sex determination. All products were examined by gel electrophoresis.

RT-PCR expression analysis of selected transcripts in fetal and adult tissues Total RNA was isolated from fetal liver, kidney, heart, brain, and lung at the age of E13.5 and from adult (3 month) ovary, liver, kidney, adrenal gland, spleen, brain, heart, and testis by using Trizol Reagent (Invitrogen). The DNase treatment and cDNA synthesis were described as above. The PCR mixture and the PCR cycle conditions were as described for sex determination. The annealing temperatures and primer sequences are given in Table 1. The resultant cDNA preparations were compared in 23 cycles of PCR with beta-actin-specific primers as a control for integrity and concentration adjustment. The PCR products were examined by gel electrophoresis.

Real-time quantitative RT-PCR analysis of SytIX expression in developing gonads and brains Synaptotagmin9 (SytIX) expression was quantified by using the Taqman system on a DNA Engine Opticon 2 (MJ Reached). The probe and SytIX-specific primers were designed with Primer Express Software v2.0 (Applied Biosystems). The probe, 5′- TTTCCAAATTGGATGTGGTGAATCTGAA GG-3′, was 5′- and 3′-end-labeled with a reporter (FAM, 6-carboxy-fluorescein) and a quencher dye (TAMRA, 6-carboxy-tetramethylrhodamine). The SytIX primer sequences were 5′-CCACAGCA-ATAACTGGCCCT-3′ and 5′-TCATCTATTTTCTTCAGGTGGCC-3′. The actual expression level in a particular sample was determined by the comparative threshold cycle (C_T) method by using the Opticon Monitor 2 program (MJ Research; Bernard and Wittwer 2002). Beta-actin was used as an endogenous reference gene to calculate a normalized target gene value. The beta-actin probe sequence was 5′-CCAGGGTGT GATGGTGGGAATGG-3′, and the primer sequences were 5′-ATCGTGGGCCGCCCT-3′ and 5′-AGTCTTCCTGAG GATACACCCAC-3′. Each sample was run in triplicate, and both the target and reference gene were measured in each run. Real-time PCR was carried out in a final volume of 30 μl containing 15 μl 2×TaqMan Universal PCR Master Mix (Applied Biosystems), 1 μl 200 nmol/μl probe, 1 μl 333 nmol/μl each gene-specific primer, 1 μl cDNA, 11 μl water. Total RNA isolation, DNase treatment, and cDNA synthesis were as described above with the only exception being that the resultant cDNA was further diluted six-fold in DEPC-H_2O before PCR. The cycle conditions were 95°C for 15 s followed by 60°C for 1 min. This two-step PCR cycle was repeated 39 times. Fetal brains and E11.5 gonads were sex-determined as described above.

Real-time quantitative RT-PCR analysis of Get-1 expression in fetal gonads Gonad-expressed transcript 1 (Get-1) expression was quantified by using a LightCyclerTM

Table 1 Primer sequences used for RT-PCR analysis

Gene ID	LocusLink number or accession number[a]	Primer sequences	Annealing temperature (°C)	PCR product size (bp)
Ric-8	101489	s 5′-gtaacagcaatggggtgctt-3′ as 5′-accaatggcaggcattactc-3′	60	154
cDNA	BC018510[a]	s 5′-gccactttcccattcttga-3′ as 5′-tttaggcaaaacctgcaagc-3′	55	212
Mbp	17196	s 5′-gaaggcaggtgatggttgat-3′ as 5′-agtcacactggagggcaaac-3′	60	198
Syt9	60510	s 5′-aaggacacagtcggacaacc-3′ as 5′-tgacagtgagggaggagctt-3′	60	213
Osp94	72831	s 5′-tgcttttgttggtagcatgg-3′ as 5′-acgtctccctaccctggatt-3′	60	239
Rbpms2	71973	s 5′-cctgaacttcgaaaccaacc-3′ as 5′-cggaaaccccttttcttctt-3′	50	178
cDNA	4930413O22[a]	s 5′-tgatccaccgtacatggaga-3′ as 5′-tgcttgtgtaatgccctgag-3′	55	247
cDNA	4921526G09[a]	s 5′-catcagggccattaagaaa-3′ as 5′-gccaatctgagcaggagaac-3′	55	243
Nr3c1	14815	s 5′-ggaaggtctgaagagccaag-3′ as 5′-tcatgcatggagtccaaaag-3′	55	150
cDNA	2310061I04[a]	s 5′-ttatgtccctgaccctttgc-3′ as 5′-gcctgtagaaacgcaggaag-3′	60	162
B3galt3	26879	s 5′-tgggtcgtttaaaggtgacag-3′ as 5′-cacactgtattctggctgtgg-3′	60	164
B-ind1	57874	s 5′-tggcacatctgtcctgtgtt-3′ as 5′-ttcagctgcaagagcttcaa-3′	50	212
Ankrd17	81702	s 5′-aattttgccacggttctgtc-3′ as 5′-cccacccttcttccttttc-3′	55	188
Beta-actin	X00351[a]	s 5′-aagtgtgacgttgacatccg-3′ as 5′-gatccacatctgctggaagg-3′	55	250

Instrument (Roche Diagnostics, Germany) with Light-Cycler FastStart DNA MasterPLUS SYBR Green I (Roche Applied Science, Germany). The primers were designed with Primer Express Software v2.0 (Applied Biosystems). The Get-1 primer sequences were 5′-GCCACTTTTCC CATTCTTGA-3′ and 5′-TTTAGGCAAAACCTGCAAG C-3′. Normalization and calculation of the expression level were as described above. The samples were run in quadruplets, and both target and reference genes were measured in the run. Real-time PCR was carried out in a final volume of 10 µl containing 2 µl 5× FastStart DNA MasterPLUS SYBR Green I mix (Roche), 0.5 µl 10 pmol/µl sense and antisense primer, 1 µl cDNA, 6 µl water. The cDNA was synthesized as described above and further diluted four-fold in DEPC-H_2O prior to the PCR. The cycling conditions were: denaturation at 95°C for 10 min followed by 40 cycles of 95°C for 10 s, annealing at 58°C for 5 s, and extension at 72°C for 15 s. The temperature transition rate was set at 20°C/s. Fluorescent product was measured by a single acquisition mode at 72°C. For distinguishing specific from nonspecific products and primer-dimers, a melting curve was obtained after amplification by holding the temperature at 65°C for 15 s followed by a gradual increase in temperature to 95°C at a rate of 0.1°C/s, with the signal-acquisition mode set at continuous.

In situ hybridization analysis In situ hybridization analysis was performed according to Moller et al. (1997) and Olesen et al. (2004) with S^{35}-labeled oligonucleotides as probes. For Get-1 hybridization, a mixture of the following three antisense probes was used: 5′-CATTAGAG GATGTCCCACAATGCCTGATCTAACAAACT-3′, 5′-C TCAAGTCTAAGAGATTAACTCCATGGCTAAAATAG C-3′, and 5′-TGTATTCCAGAAGAGTACTCCACTTGA AAACTATGCTG-3′. The Get-1 sense probe was 5′-AG TTTGTTAGATCAGGCATTGTGGGACATCCTCTAAT G-3′. The SytIX antisense sequence was 5′-CCACCTAG CAACACCCAGATGACTCCAGTGAAGAAAAT-3′, and the sense sequence was 5′-ATTTTCTTCACTGGAGT CATCTGGGTGTTGCTAGGTGG-3′.

Results

Comparison of transcriptional activity in E11.5 and E13.5 ovaries

In this study, we set out to identify novel genes related to early meiosis and early ovarian development. For this purpose, we used DNA microarray methology to compare the transcriptional activity in pre-meiotic ovaries aged E11.5 and E13.5 ovaries with germ cells in the transition stages of the first meiotic prophase. At E13.5, about 35% of the oogonia have entered meiotic prophase I (Peters 1970; Di Carlo et al. 2000).

The M versus A (MVA) plot showed that all six hybridizations were acceptable, as no arrays showed a constant curvature in comparison with all other arrays. However, the plot showed the need for non-linear normal-ization, as the data trend line deviated from a straight line. After normalization, total signals were similar on the compared arrays as seen by the straight line in the MVA plots (Fig. 1). A hierarchical clustering analysis showed that replica arrays from the same embryonic ages clustered together as expected. The principal component analysis confirmed this result (data not shown).

The microarray expression analysis resulted in a total of 182 genes that were significantly differentially expressed in E13.5 ovaries compared with E11.5 ovaries at a threshold *P*-value of 4.4×10^{-4} (Figs. 2, 3) equivalent to a Bonferroni cutoff value of 10 false-positive predictions of differential regulation with the same *P*-value at the bottom rank gene. We found that 130 genes were upregulated, and 52 were downregulated in the E13.5 ovaries. Moreover, 55% of the upregulated genes were upregulated by two-fold or more, whereas only 12% of the downregulated genes were downregulated by two-fold or more. Overall, the DNA microarray detected 134 published and 48 previously uncharacterized genes that were differentially expressed in E11.5 and E13.5 ovaries.

A comparison of our micro-array data with the literature showed that the up- or downregulation of at least 13 genes was in agreement with previously published expression patterns in fetal ovaries (Fig. 4). These genes will be further discussed below.

RT-PCR screening for gonad-specific genes

In our experimental set-up, two embryonic stages of ovaries were compared. In addition to detecting genes related to meiosis and organ-specific development, the array probably detected genes involved with ubiquitous developmental processes such as mitosis and apoptosis. We therefore reasoned that it would be of particular interest to focus on genes that were gonad-specific or predominantly expressed in the gonads and not ubiquitously expressed. We thus used a fast RT-PCR screening strategy with mixtures of cDNA populations from four different fetal tissues and four different adult tissues (Fig. 5). The principle of this strategy was as follows. If a transcript was gonad-specific or predominantly expressed in the gonads during adulthood, no product or only a weak product would be produced in the adult cDNA mix because of the dilution factor. Likewise with the fetal cDNA mix in fetal life. For comparison, undiluted adult testis cDNA was included in the analysis. We examined 45 of the 182 genes by this procedure (data not shown). The genes were randomly chosen from the group of upregulated genes. Thirteen genes produced a band-pattern consistent with the pattern expected for a gonad-specific transcript or a predominantly gonad-expressed transcript. All 13 genes were subjected to further semiquantitative RT-PCR expression analysis with a panel of five fetal tissues (aged E13.5) and eight adult tissues (Fig. 6). This analysis showed that one transcript was gonad-specific, six were differentially expressed, and the remaining six were ubiquitously expressed in adult tissues. In fetal life, two genes, viz., cDNA

212

Fig. 2 Genes upregulated in E13.5 ovaries compared with E11.5 ovaries. All genes pass a threshold P-value of 4.4×10^{-4}. The genes are listed after the magnitude of fold-change. The gene symbol is followed by the NCBI LocusLink number (http://www.ncbi.nlm.nih.gov/ entrez/query.fcgi?db=gene& cmd=Retrieve&dopt=Graphics& list_uids=). Uncharacterized genes are denoted cDNA or IMAGE clone, followed by their GeneBank accession number
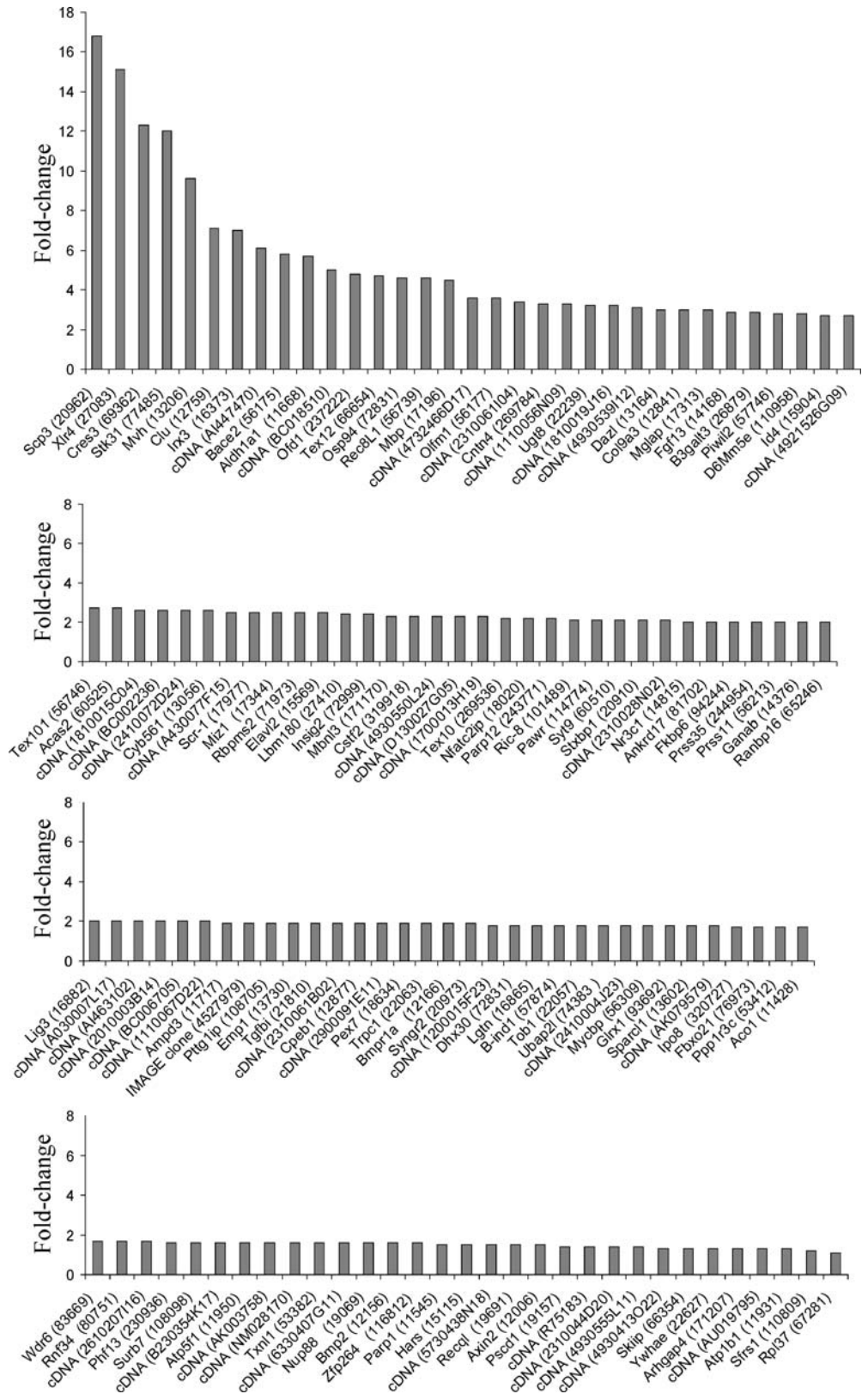
Fig. 3 Genes downregulated in E13.5 ovaries compared with E11.5 ovaries. All genes pass a threshold *P*-value of 4.4×10^{-4}. The genes are listed after the magnitude of fold-change. The gene-symbol is followed by the NCBI LocusLink number (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene&cmd=Retrieve&dopt=Graphics&list_uids=). Uncharaterized genes are denoted cDNA or IMAGE clone, followed by their GeneBank accession number
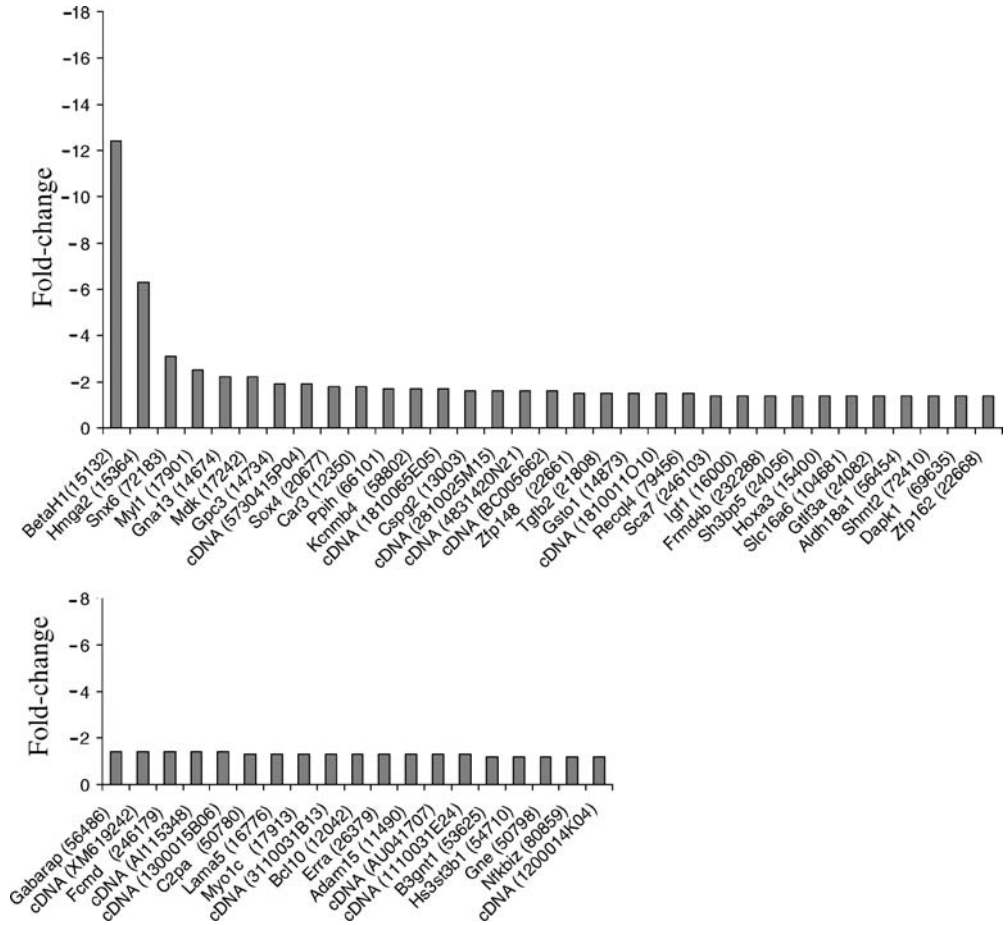
BC018510 and SytIX, were differentially expressed. The remaining 11 genes were expressed in all fetal tissues examined. As cDNA BC018510 was also the only gonad-specific transcript, and as SytIX was the only predominantly gonad-expressed transcript in adulthood, we selected these two genes for further validation and expression analysis.

Expression analysis of SytIX in developing gonads and fetal brain

Because of the inconsistent nomenclature of the Synaptotagmin gene family, we emphasize that the mouse SytIX sequence described here is identical to the sequence that, together with SytIII, SytVI, and SytX, form a distinct subclass of Synaptotagmins. The sequence is referred to as

Fig. 4 Examples of published genes that validate the experimental set-up. The genes are classified according to their known function in meiosis/germ cell differentiation, ovarian/gonadal development, or embryogenesis. For each gene, both the E11.5 (*open bars*) and E13.5 (*filled bars*) expression values are indicated
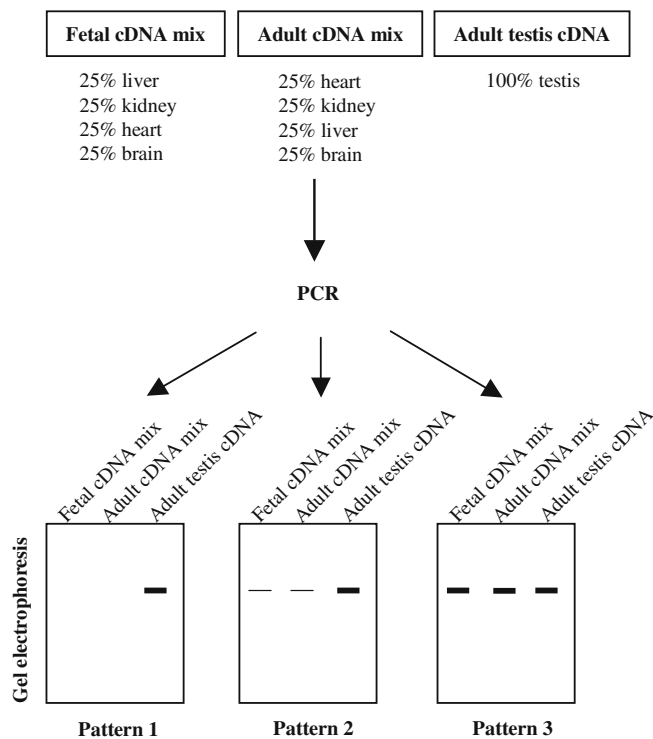
214
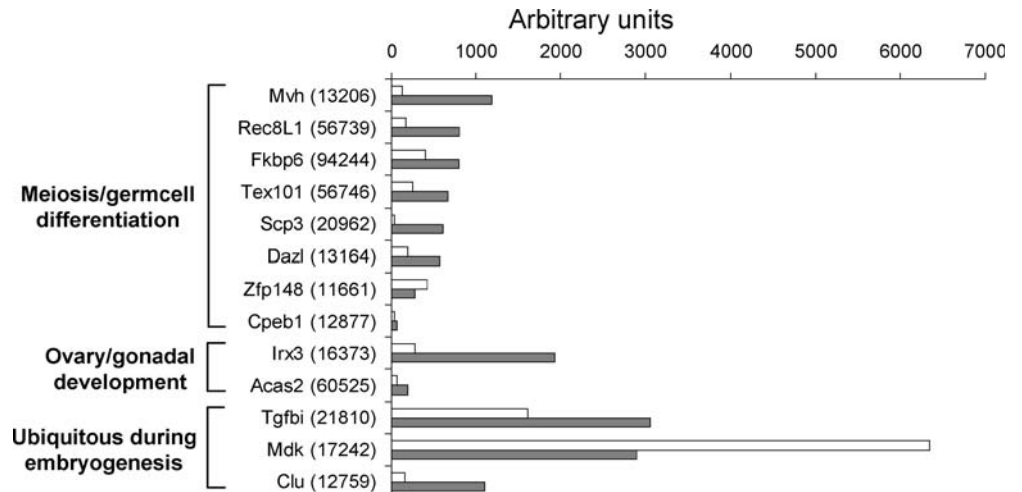
Fig. 5 Principles of the screening strategy with mixed cDNA populations. The procedure was used to obtain a preliminary indication of whether a transcript was predominantly confined to gonads or ubiquitously expressed in fetal and adult tissues. The three banding patterns shown illustrate the pattern expected from a gonad-specific transcript (*Pattern 1*), from a predominantly gonad-expressed transcript (*Pattern 2*), or form a ubiquitously expressed transcript (*Pattern 3*). Other band patterns and band intensities may however occur
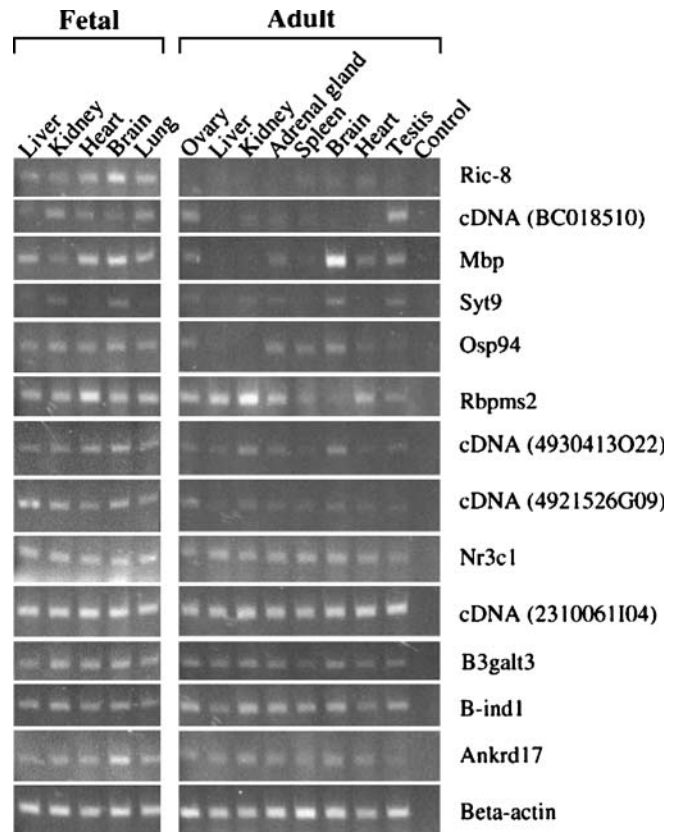

Fig. 6 RT-PCR expression analysis of 13 selected genes in the indicated fetal (E13.5) and adult tissues. Beta-actin was used as a control for the concentration of the cDNA populations (*Control* PCR mix without template)

SytIX by Craxton (2004) but as SytV by Fukuda et al. (1999). We have adopted the same nomenclature as that used by GenBank, i.e., SytIX.

SytIX expression was quantified in developing gonads using real-time PCR (Fig. 7a). The analysis confirmed the observed upregulation in E13.5 ovaries. The microarray revealed a 2.1-fold upregulation, whereas the PCR-based method showed a 2.3-fold upregulation. Overall, SytIX was detected in gonads at all developmental stages examined. However, the expression level oscillated in a sexually dimorphic manner. In the male, the expression appeared constant during fetal life. In the female, however, expression was transitionally upregulated in E13.5 ovaries by two-fold relative to the E13.5 testis and E11.5 ovaries and testis. At early prepuberty in the male (P7), expression started to increase and peaked around P10. Thereafter, expression declined in the mature testis and reached a level about ten-fold lower this peak level. In contrast, the expression in juvenile ovary gradually declined and reached a barely detectable level in the adult. Some weak temporally upregulation was, however, observed in P10 ovaries.

SytIX expression was also quantified in fetal brain of both sexes, as the semiquantitative expression analysis showed some expression in fetal kidney and brain. In contrast to the gonads, SytIX was not expressed in a sexual dimorphic fashion in the fetal brains (Fig. 7b). The expression level in E16.5 and newborn brains was equal to the level observed in fetal gonads, except E13.5 ovaries. In addition, SytIX expression was upregulated in both male and female brains in E16.5 and newborns. Expression in E11.5 and E13.5 brains was about half the level seen in E16.5 and newborns.

Analysis of Get-1 expression in fetal and adult gonads

cDNA BC018510 is a previously uncharacterized transcript. According to our new data, the transcript is gonad-specific in adulthood. Some expression has also been detected E13.5 kidney, heart, brain, and lung (Fig. 6). We have named this novel transcript Get-1. The Get-1 messenger is 1032 bases in length and encodes a polypeptide of 11.1 kDa (Fig. 8). Neither the nucleotide sequence nor the encoded polypeptide has homology to any known genes or proteins.

The five-fold upregulation of Get-1 expression seen in E13.5 ovaries was largely confirmed by quantitative real-time PCR (Fig. 9). This RT-PCR technique demonstrated a
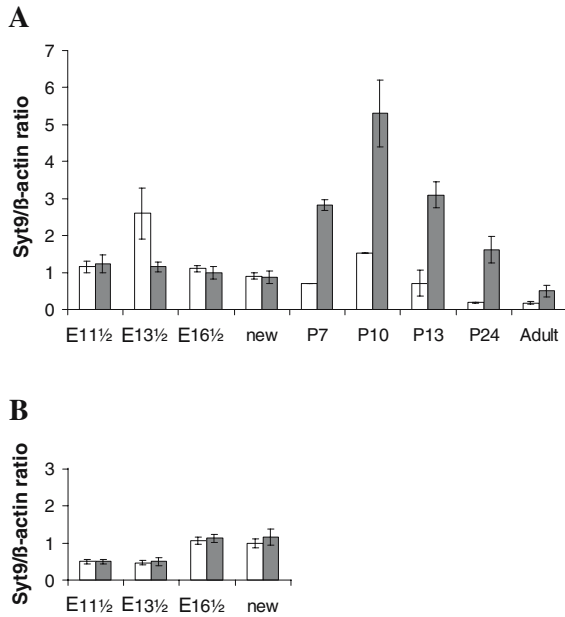
A

B

Fig. 7 Quantitative PCR expression analysis of SytIX (*βactin* beta actin). **a** Quantification of SytIX expression in mouse ovaries (*open bars*) and testis (*filled bars*) at the indicated ages (*E11 1/2* E11.5, *E13 1/2* E 13.5, *E16 1/2* E16.5, *new* newborn). **b** Quantification of SytIX expression in brains of female (*open bars*) and male (*filled bars*) mice at the indicated ages. For each tissue, the 95% confidence interval is indicated

Fig. 9 Quantitative PCR expression analysis (*βactin* beta actin) of Get-1 (cDNA BC018510) at the indicated ages (*E11 1/2* E11.5, *E13 1/2* E 13.5) of ovaries (*open bars*) and testis (*filled bars*)

three-fold upregulation in E13.5 ovaries. The analysis also showed that the expression in E11.5 and E13.5 testis did not change, and that the testicular expression level was about three-fold to 14-fold lower than the level seen in fetal ovaries.

Get-1 expression in the gonads was further confirmed by in situ hybridization analysis (Figs. 10, 11). Expression in the adult ovary was too weak to be detected by this method.

In the fetal testis, a weak but specific signal was observed in the pre-spermatogonia. No signal was present in the Sertoli cells. In the fetal ovary, a moderate signal was detected specifically in large round dark cells that appeared in clusters in the cortex. These cells were presumably meiotic germ cells. In the mature testis, the hybridization signal was specifically confined to meiotic germ cells. No expression was observed in Sertoli cells, Leydig cells, spermatogonia, or spermatocytes, or at the second meiotic division. During prophase I, Get-1 was expressed in pre-leptonema and leptonema. However, at zygonema to the early pachytene stage, this expression disappeared. Around mid-pachynema, the spermatocytes resumed expressing Get-1, and the mRNA level increased in the spermatocytes as they advanced through prophase I, peaking in the late-diplotene spermatocytes.

Discussion

We have compared the gene expression in E11.5 and E13.5 ovaries by using the microarray technique. The structure of

Fig. 8 Nucleotide and deduced amino acid sequence of murine Get-1 (accession no. BC018510; Benson et al. 2000). The amino acid positions are shown in *bold*. A putative polyadenylation signal (AATAAA) is also indicated in *bold*

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1                                                GTGTTTCTCTAACA
15        CTGTGCCTTTTACTATCCCATGTTGCAGTTTGTGAACCCTTTGTTTGGGGACAGTCTTAC
75/1      ATGTATTGTACTCGTTTTGGAATTTTCTGTGTTTCTAATTTGCATTCAGTATATACTTCT
           M  Y  C  T  R  F  G  I  F  C  V  S  N  L  H  S  V  Y  T  S
135/21    GTTACCTTTAGTTTTATATATACCCTACCATGGACCCATGTTGCCACTGTGTGTTCCTGT
           V  T  F  S  F  I  Y  T  L  P  W  T  H  V  A  T  V  C  S  C
195/41    GGTGGCTATGGAATTGCTGCTGTGTCTGAAGCCCTCAGATACAAGCAGCCACTTTTCCCA
           G  G  Y  G  I  A  A  V  S  E  A  L  R  Y  K  Q  P  L  F  P
255/61    TTCTTGACAATCAGCTATAAGCAAGACCAACCATTTGCTAAAGGTAGGTCATCTCGGATG
           F  L  T  I  S  Y  K  Q  D  Q  P  F  A  K  G  R  S  S  R  M
315/81    CATCTCTGTCATATCCAGCATTCAGAAGTTCTTTCCTGCTGTTTTCATTCTAAAGCAGGC
           H  L  C  H  I  Q  H  S  E  V  L  S  C  C  F  H  S  K  A  G
375/101   TATTGATTTGTATCCTTTGTCTTTGTTTTTGTTTGACTGGCAATCATTTGAGAGAAATAG
           Y  stop
435       CTTGCAGGTTTTGCCTAAACAGCACAGTTTGTTAGATCAGGCATTGTGGGACATCCTCTA
495       ATGCCAGCACTTGGAAGGCAAAGGCAATGCCAGCTTGGTCTACATAAAAAGTTCCAATCC
555       AGCCAGAGCTACATAATAACAGACTTTTTAAAAAATGGTTTTGTTAGACTTTGTGTACTA
615       TGAACATGAAAATGCCCAGGGTTTCATATAAAAAAATATTATTCAACCTGCTATTTTAGCC
675       ATGGAGTTAATCTCTTAGACTTGAGGCTTTAATTTGCTGATAATTTCTCTAAATACCTCC
735       TTTGCTAATTTCCTGGTTTCTATGAGAACATAACACTAAAGGCTCTAATAGAGGACGAGT
795       CCTATGGATACTTTGCACTGAAGTGAAGTGCAAGCCGTTCAGGAGAGACTTGAGATGTCT
855       AAGTCACTACCCAAAAGAAACAAACTAGGTAGCTAGTTACTGGGATGTTAAAAAGTGAAA
915       CTGCAGCATAGTTTTCAAGTGGAGTACTCTTCTGGAATACAGTGGAAGTTCTGAGATAAC
975       AGTTTCCCTTGGTATTTGAGATGACTCCATCTTCACA**AATAAA**GCTGTGTTACTTTTAAA
1035      AAAAAAAAAAAAAAA
```
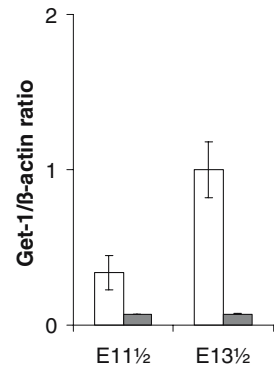
Fig. 10 In situ hybridization analysis of the Get-1 messenger in fetal gonads and mature ovary. Phase contrast. **a** Antisense, E16.5 testis (*Sp* spermatogonia, *Se* Sertoli cell). *Inset:* Higher magnification of the fetal testis tubulus showing a labeled spermatogonium. **b** Sense, E16.5 testis. **c** Antisense, E16.5 ovary. *Inset:* Higher magnification of the central part of the fetal ovary showing labeling of a group of large round dark cells. **d** Sense, E1612 ovary. **e** Antisense, mature ovary. **f** Sense, mature ovary. *Bars* 20 μm
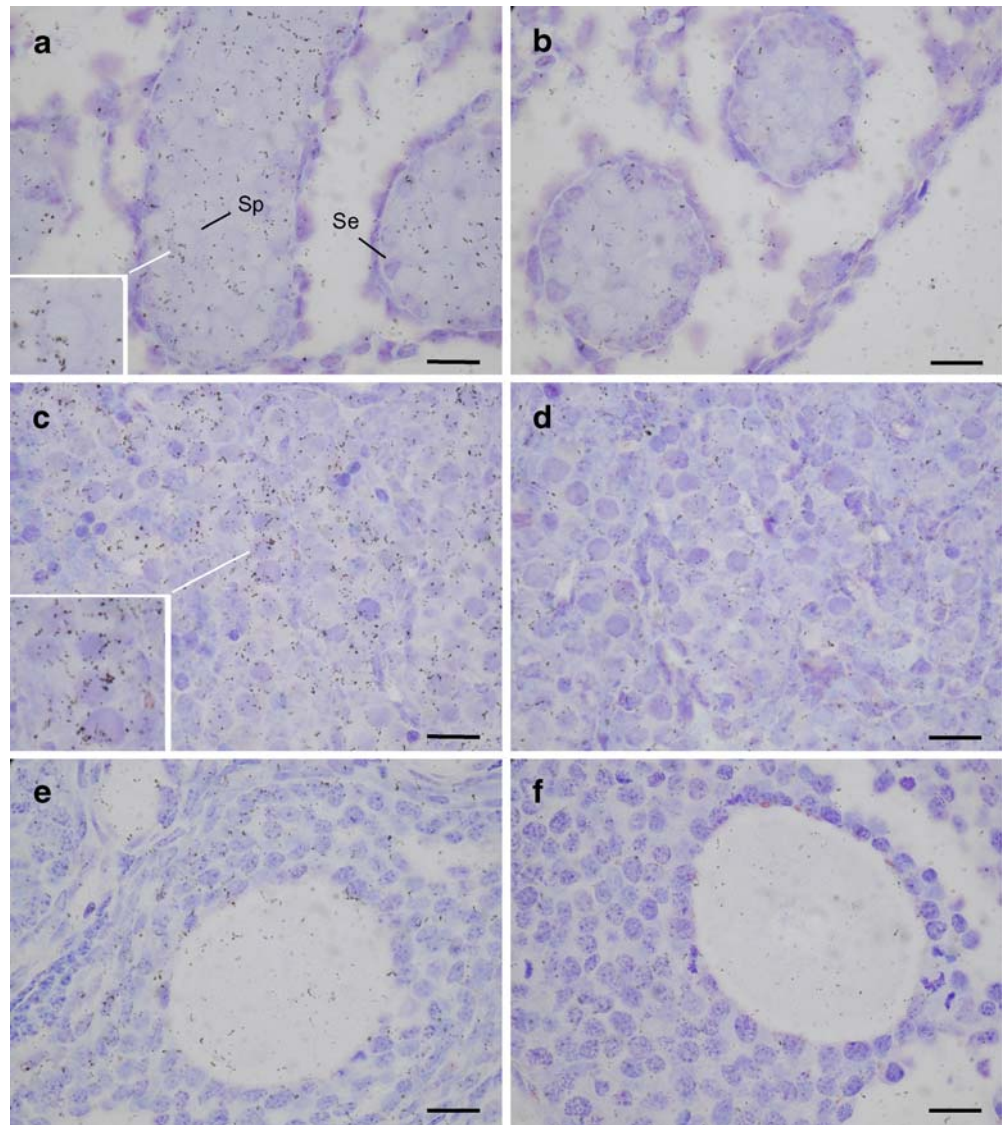
E11.5 and E13.5 ovaries is similar, and only meiotic initiation and ovarian differentiation separate the two stages physiologically (Byskov and Hoyer 1994). Furthermore, in E11.5 ovaries, all oogonia are still in interphase, and no germ cells have embarked on meiosis. In E13.5 ovaries, however, about 35% of oogonia have entered the first stages (leptonema and zygonema) of meiotic prophase I (Peters 1970; Di Carlo et al. 2000). The current microarray therefore specifically addresses the early transition stages of meiotic prophase I and the first events of ovarian development.

Because of this highly specific array design, we expected that a limited number of genes would be identified. The microarray analysis revealed that 182 genes were differentially expressed in E13.5 ovaries compared with E11.5 ovaries. This number is indeed small, as microarray studies usually identify thousands of differentially expressed genes. In a recent study, a comparison of the expression in newborn and adult ovaries has shown that more than 2000 genes are differentially expressed (Herrera et al.

2005). Clearly, the variations in cell population and tissue complexity are higher in comparisons of neonatal and adult ovary than of E11.5 and E13.5 ovaries. In adult versus newborn mouse ovary, complex processes such as folliculogenesis, steroid synthesis, and meiotic resumption take place only in the adult.

Several lines of evidence have established the utility of the present array. First, we have compared the list of differentially expressed genes with the literature in order to search for genes with a known expression pattern consistent with our findings. This search has revealed 13 published genes that are either upregulated or downregulated in the fetal ovaries in a temporal manner supporting our data (Fig. 4). Interestingly, most of the genes have a known function in meiotic prophase I or germ cell differentiation (Mvh, Rec8L1, Tex101, Fkbp6, Scp3, Dazl, Zfp148, Cpeb1), whereas two genes, Irx3 and Acas2, are likely to play a role in ovarian development. The remaining three genes, Tgfbi, Mdk, and Clu, are known to have a ubiquitous function during embryogenesis (Ahuja et al.
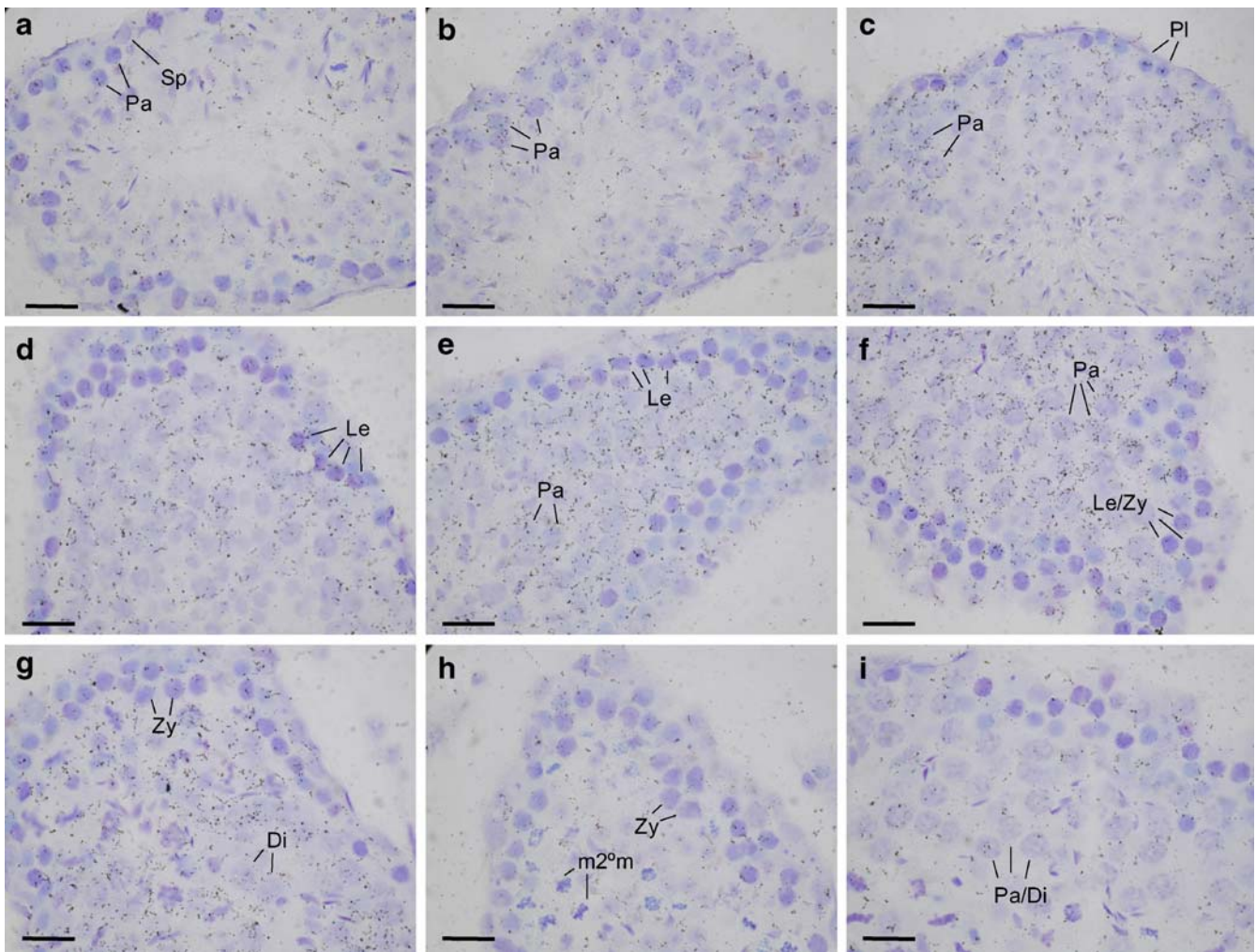
Fig. 11 In situ hybridization analysis of the Get-1 messenger at the various stages of the seminiferous tubule cycle in mature testis (*Pl* pre-leptotene spermatocyte, *Le* leptotene spermatocyte, *Zy* zygotene spermatocyte, *Pa* pachytene spermatocyte, *Di* diplotene spermatocyte, *Sp* spermatogonium, *m2°m* second meiotic cell division). Phase contrast. **a** Stage I. **b** Stages II–VI. **c** Stage VII. **d** Stage VIII. **e** Stage IX. **f** Stages X–XI. **g** Stages XI–XII. **h** Stage XII. **a–h** Antisense. **i** Sense. *Bars* 20 μm

1994; Takayama et al. 2005; Crackower et al. 2003; Di Carlo et al. 2000; Ferguson et al. 2003; French et al. 1993; Fujiwara et al. 1994; Gebauer and Richter 1996; Jorgensen and Gao 2005; Loikkanen et al. 2002; Prieto et al. 2004; Seligman and Page 1998; Takeuchi et al. 2003; Tay and Richter 2001). Of note, the expression and downregulation of Midkine in E13.5 ovaries has not previously been reported. However, Midkine is a widely expressed gene in mouse embryogenesis and peaks around E8–E11. Subsequently, expression decreases in the majority of embryonic tissues (Kadomatsu et al. 1988, 1990). The observed downregulation of Midkine expression in E13.5 ovaries is therefore in good agreement with the temporal expression pattern seen in most fetal tissues. The microarray data have further been confirmed by quantitative PCR analysis of two selected genes SytIX and Get-1 (Figs. 7, 9). Taken together, we conclude that the present microarray represents a valid and robust profile of genes differentially expressed in E11.5 and E13.5 ovaries.

During the search for known genes validating our data, certain additional genes have come to prominence and deserve special attention (Fig. 12). The first group of genes has a known function in meiotic prophase I and germ cell differentiation only in the testis and includes Aldh1a1, C2pa, Tex12, Stk31, Id4, Lig3, and Recql (Zhai et al. 2001; Linares et al. 2000; Wang et al. 1998; Mackey et al. 1997; Chaudhary et al. 2001; Sablitzky et al. 1998; Wang et al. 2001). The present microarray has documented their existence and upregulation in meiotic ovaries, and we suggest that these seven genes are likely to have a function in female meiosis and female germ cell differentiation. Another germ-cell-related gene, Piwil2, has indeed been reported in fetal ovaries around E12.5 and in spermatogonia and early stages of meiosis in testis (Kuramochi-Miyagawa et al. 2001, 2004). However, this is the first time that an upregulation of Piwil2 expression coinciding with meiotic initiation has been reported in embryonic ovaries. The next group of genes includes Mglab, Mbnl3, and Olfm1. These genes are characterized by having a re-

stricted expression pattern during fetal life, indicating distinct roles in the development of certain organs (Luo et al. 1995; Kanadia et al. 2003; Moreno and Bronner-Fraser 2002). Our novel observation that they are expressed and upregulated in embryonic ovaries suggests that their function in organogenesis extends to include the ovaries. Another interesting group of genes that are differentially expressed are the five vesicle-related genes Syngr2, Stxbp1, Myo1c, SytIX and Ric-8. These genes encode proteins that are believed to have various functions in vesicle transport, vesicle docking with the plasma membrane, and the priming of vesicles (Reynolds et al. 2004; Tonissoo et al. 2003; Fukuda et al. 1999; Bose et al. 2004; Macaulay et al. 2002; Hata and Sudhof 1995; Kedra et al. 1998). Some of them (Syngr2, SytIX, and Stxbp1) are members of larger gene families that have both neuronal and non-neural forms functioning in synaptic and non-synaptic vesicles. Ric-8 and SytIX have not previously been reported in non-neural tissue. To our knowledge, this is the first time that an apparent increase in vesicle activity has been associated with initiation/early meiosis. Currently, whether this phenomenon is related to a specific event in the meiotic process or to a general increase in the metabolism and intracellular activity caused by gross cellular and chromosomal changes occurring during prophase I is unknown. An interesting line of study would be to determine whether an increase in vesicle activity is also associated with the start of meiosis in the testis. Indeed, we have initiated such a study of SytIX (discussed below). The last genes prominent in our literature search are Parp1 and Parp12, which are both upregulated in E13.5 ovaries. The Parp-1 gene encodes a ubiquitous enzyme thought to have a critical role in genome stability and in repair of DNA damage caused by genotoxic exposure (Vidakovic et al. 2005). Meiotic and mitotic processes are known to share many central functions in the cell cycle machinery (Pelech et al. 1990). These two DNA repair components may represent novel examples of factors that are shared between mitosis and meiosis.

As shown above, the literature study has been useful in validating the expression data and has also facilitated the identification of new biological features of fetal ovaries. We have sought, however, to identify additional genes of interest in the array dataset by applying a fast RT-PCR screening to search for gonad-specific transcripts or transcripts predominantly expressed in the gonads. Following this procedure, our attention has been attracted to Get-1 and SytIX, of which the former is gonad-specific and the latter predominantly gonad-expressed (Fig. 6). SytIX belongs to a large family of 15 genes that are believed to function in the transport and docking of vesicles/membranes in the cell. Some of the forms are neuron-specific, whereas others are also expressed in various non-neural tissues (Marqueze et al. 2000). In neural tissue, the Syts are involved with the regulation of synaptic neurotransmitter release. The specific function of Syts in other organs is poorly understood. However, they may have a role in exocytosis (Fukuda et al. 2004). The present report is the first to demonstrate SytIX expression in non-neural tissue. Our detailed analysis of SytIX expression in developing gonads has revealed a transitional upregulation that is correlated with early meiotic prophase I in the female. In the male, a correlation with early meiosis is also evident, but apparently only during the first meiotic wave that occurs between P7 and P30 (Bellve et al. 1977). We therefore propose that SytIX plays a role during meiotic initiation and/or the early transition stages of the first meiotic prophase in both the male and female. We have also found the SytIX messenger in mature testis and ovary. However, the expression is about 1/11 and 1/14 of the level observed in P10 testis and E13.5 ovaries, respectively, and the biological importance, if any, of such low levels should be considered. Interestingly, another member of the Syt family, Syt8, has been implicated in the reproductive system. The Syt8 gene-product is present on the acrosomal crescent and is lost following the acrosome reaction. Syt8 is therefore believed to function in acrosomal exocytosis (Hutt et al. 2002). Thus, other members of the large Synaptotagmin gene family may have important roles in reproductive organs.

Fig. 12 Examples of published genes in the microarray dataset that are of particular interest for the current study. For each gene, both the E11.5 (*open bars*) and E13.5 (*filled bars*) expression values are indicated
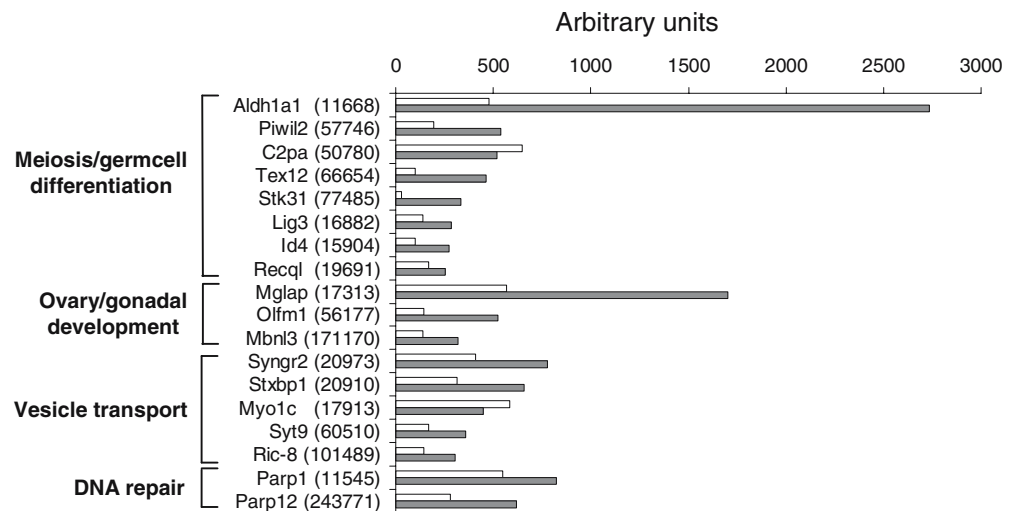
Get-1 is a formerly uncharacterized transcript that, according to our data, is gonad-specific in adulthood. In fetal life, however, Get-1 is not gonad-specific, and expression has been observed in kidney, heart, brain, and lung at E13.5. The three-fold to five-fold upregulation in E13.5 ovaries suggests a function in early meiotic prophase I. In situ hybridization analysis in E13.5 ovaries and adult testis has confirmed that Get-1 is indeed expressed in meiotic germ cells in both ovaries and testis (Figs. 10, 11). A determination of the meiotic stages expressing Get-1 in the ovary has not been possible because of the less than optimal morphology following in situ hybridization. In adult testis, Get-1 appears to be expressed at all stages of prophase I, except zygonema and early/mid pachynema. The reason for this temporary pause in expression remains unexplained. Taking the data collectively, we propose that Get-1 is a novel messenger with a function in the meiotic process in both the male and female. Some Get-1 expression has also been observed in spermatogonia in the fetal testis. Apparently, this pre-meiotic expression in the germ cells is lost in the spermatogonia in the adults (Figs. 10, 11). Our in situ hybridization analysis does not include gonads younger than E13.5, and it therefore remains unclear whether Get-1 is also expressed in female germ cells before meiosis. Quantitative PCR analysis has shown, however, that Get-1 is expressed at levels about five-fold higher in E11.5 ovaries than in E11.5 and E13.5 testis (Fig. 9). We therefore cannot exclude that Get-1 is indeed expressed in oogonia.

In conclusion, we have identified at least 182 genes that are differentially expressed in E11.5 and E13.5 ovaries. Moreover, in 7% of the genes, published studies have confirmed the observed expression pattern in fetal ovaries, and in 10% of the genes, important new information concerning the physiology of the embryonic ovaries and meiosis has been elucidated by comparisons with the literature (Figs. 4, 12). Combined with the detailed analysis of SytIX and Get-1 expression showing that they probably have a function in male and female meiosis, our data indicate that additional genes in the dataset are most probably related to early meiosis or ovarian/gonadal development. Finally, in the light of the growing number of microarray studies on whole fetal ovaries, it would be interesting in the future to focus on the expression in enriched populations of germ cells. In particular, it would be interesting to compare the expression in purified oogonia with isolated oocytes in the leptotene and zygotene stages. Such studies would undoubtedly extend our current knowledge of meiotic initiation and meiotic gene activity in general.

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