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Identification of Novel and Known Oocyte-Specific Genes Using Complementary DNA Subtraction and Microarray Analysis in Three Different Species[1]

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ABSTRACT

The main objective of the present study was to identify novel oocyte-specific genes in three different species: bovine, mouse, and *Xenopus laevis*. To achieve this goal, two powerful technologies were combined: a polymerase chain reaction (PCR)-based cDNA subtraction, and cDNA microarrays. Three subtractive libraries consisting of 3456 clones were established and enriched for oocyte-specific transcripts. Sequencing analysis of the positive insert-containing clones resulted in the following classification: 53% of the clones corresponded to known cDNAs, 26% were classified as uncharacterized cDNAs, and a final 9% were classified as novel sequences. All these clones were used for cDNA microarray preparation. Results from these microarray analyses revealed that in addition to already known oocyte-specific genes, such as *GDF9, BMP15,* and *ZP,* known genes with unknown function in the oocyte were identified, such as a MLF1-interacting protein (*MLF1IP*), B-cell translocation gene 4 (*BTG4*), and phosphotyrosine-binding protein (*xPTB*). Furthermore, 15 novel oocyte-specific genes were validated by reverse transcription-PCR to confirm their preferential expression in the oocyte compared to somatic tissues. The results obtained in the present study confirmed that microarray analysis is a robust technique to identify true positives from the suppressive subtractive hybridization experiment. Furthermore, obtaining oocyte-specific genes from three species simultaneously allowed us to look at important genes that are conserved across species. Further characterization of these novel oocyte-specific genes will lead to a better understanding of the molecular mechanisms related to the unique functions found in the oocyte.

gamete biology, gene regulation, oocyte development

INTRODUCTION

During late follicular development, the oocyte, arrested at diplotene of the first meiotic prophase, produces a stockpile of transcripts and proteins that help to support development of the blastocyst. Therefore, the first cell cleavage events and the activation of zygotic gene expression must rely on the reservoir of mRNAs and proteins stored in the egg [1]. The point at which embryonic transcription begins and maternal mRNAs are replaced is referred to as the maternal-zygotic transition (MZT), which occurs in two phases, a minor and a major activation [2]. The major activation promotes a dramatic reprogramming of gene expression pattern, coupled with the generation of novel transcripts that are not expressed in oocytes. Depending on the species, the onset of MZT occurs at different stages [3, 4]. For example, MZT occurs at the 1- to 2-cell stage in mice [5], at the 8- to 16-cell stage in the bovine [6], and in 4000- to 8000-cell embryos (stage 8–8.5) in *Xenopus laevis* [7]. It is speculated that several hundred maternal genes participate in this transition, but so far, only a few have been identified [8]. Because whole-genome activation occurs only once in a lifetime, it is suspected that factors inducing this transition might be uniquely expressed in the oocyte slightly before the MZT events. Moreover, sequence information for only a limited number of these genes is currently known, meaning that our basic understanding of gene expression patterns driving preimplantation development is very restricted.

Previous studies, using different techniques, have uncovered oocyte-specific genes with important functions related to oocyte development and folliculogenesis. Well-known examples include *Mos* [9], *Zp3* [10], *Zp2* [11], *Zp1* [12], *Gdf9* [13], *Fig1a* [14], *Bmp15* [15], *H1foo* [16], and *Zar1* [17]. Recently, Zeng and Schultz [18] used the suppressive subtractive hybridization (SSH) technique to generate a subtracted mouse oocyte-specific library. In that study, 8-cell embryos were used in the subtraction to identify oocyte-specific transcripts. Using the serial analysis of gene expression (SAGE) technique, another attempt was made to identify human oocyte-specific genes [19]. Furthermore, the large amount of sequence information that has been placed into public databases during the last decade has allowed the use of in silico approaches to identify oocyte-specific transcripts [20, 21]. According to a recent transcriptome analysis of the mouse stem cells and early embryos, 119 oocyte-specific genes are still unknown [8].

Novel oocyte-specific genes also can be identified when their orthologues are well known and characterized in other species. A well-known example of a gene characterized by homology through another species is the mouse *Ybx2* gene

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(also known as *MSY2*), which is a member of the Y-box protein family [22]. Its *X. laevis* homologue, *FRGY2,* was the first to be identified and characterized [23, 24]. *Ybx2,* a highly conserved protein, has an expression that is highly restricted to the oocyte in the female mouse and plays an important role in translational control [25]. *H1foo,* an oocyte-specific linker histone, was first characterized from an ovary-selective cDNA library [16]. It was subsequently revealed that mouse *H1foo* shares a high level of homology between the oocyte-specific linker histone *B4* of *X. laevis* and the *cs-H1* linker histone of the sea urchin (*Parechinus miliaris*) [26].

The present study was undertaken to isolate and identify important novel genes that are specifically expressed in the oocyte. The use of SSH, a powerful method to identify novel genes by enriching low-abundance transcripts differentially expressed in the tester population [27], combined with cDNA microarray analysis, allowed us to identify new oocyte-specific genes. Furthermore, results obtained from a previous study in our laboratory showed that polymerase chain reaction (PCR)-based SSH combined with a SMART amplification is a successful technique to compare rare tissues, thus providing valuable information about the oocyte [28]. Microarray analyses allow the opportunity to select better candidates for validation with PCR and reverse transcription (RT)-PCR by screening more genes than is permitted by a dot-blot analysis. The specific objective of the present study was to identify genes preferentially expressed in the bovine, mouse, and *X. laevis* oocyte. The comparative analysis of species is becoming a wise approach to select important sequences or important genes conserved throughout evolution, giving us a better chance to focus on the most important factors. Furthermore, the full characterization of these genes will lead to a better understanding of the molecular mechanisms related to the unique functions found in the oocyte.

MATERIALS AND METHODS

Tissue Collection

Bovine ovaries and somatic tissues were collected at a slaughterhouse. Cumulus-oocyte complexes from 3- to 6-mm follicles were collected by aspiration. Germinal vesicle (GV)-stage oocytes were mechanically denuded, washed several times in PBS buffer to prevent cumulus cell contamination, and frozen in liquid nitrogen. Somatic tissue samples (muscle, kidney, liver, and spleen) were collected and immediately frozen in liquid nitrogen.

Fully grown GV-stage mouse oocytes and somatic tissues were obtained from 10 female BALB/c mice (age, 22–23 days) without superovulation treatment. Cumulus-oocyte complexes were collected in M2 medium, and GV-stage oocytes were mechanically denuded, thoroughly washed in PBS buffer, and transferred to liquid nitrogen for storage. Somatic tissue samples (muscle, kidney, liver, and spleen) were collected and immediately frozen in liquid nitrogen.

Xenopus laevis oocytes and somatic tissues were obtained from an adult female anesthetized in 0.1% methane sulfonate salt of 3-aminobenzoic acid ethyl ester (MS222; Sigma) for 20 min. A piece of ovary was isolated, and oocytes were defolliculated for 1 h at 18°C in OR2 saline containing 0.15% collagenase (Sigma). Oocytes of stage 4–5 (diameter, ~1 mm) were collected in OR2 medium at 18°C, washed in PBS buffer, and frozen in liquid nitrogen. Somatic tissue samples (muscle, kidney, liver, and spleen) were collected and immediately frozen in liquid nitrogen. Animals were cared for according to the recommended code of practice [29] and were killed by an acceptable method approved by the local animal care committee following the guideline of the Canadian Council on Animal Care [29].

RNA Extraction

For each species, total RNA was extracted from 200 mg of each somatic tissue (muscle, kidney, liver, and spleen) using Trizol reagent (Invitrogen, Burlington, ON, Canada) and from approximately 1000 oocytes using an Absolutely RNA Microprep Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The extracted RNA was dissolved in water and quantified spectrophotometrically at 260 nm. Purity of RNA was determined from the 260:280-nm ratios. Pools of somatic tissues were made by pooling equal amount of total RNA from each somatic tissue and were adjusted to a final concentration of 0.5 μg/μl. A sodium acetate precipitation was performed on total RNA from GV-stage oocytes to obtain a final concentration of 0.5 μg/μl. Total RNA (1 μg) from pool of somatic tissues and oocytes were treated with DNase I (Invitrogen) to remove contaminating genomic DNA.

Suppressive Subtractive Hybridization

Pools of total RNA (1 μg) from bovine oocytes and somatic tissues were used for cDNA production. The mRNAs were reverse transcribed, and the cDNAs were amplified using the BD SMART PCR cDNA Synthesis Kit (BD Biosciences, Mississauga, ON, Canada) according to the manufacturer's instructions. The SSH was performed with the PCR Select cDNA Subtraction Kit (BD Biosciences) as described by the manufacturer. Briefly, the pools consisted of bovine oocytes for the tester and bovine somatic tissues for the driver. The PCR was performed in a ThermoHybaid Hybrid MultiBlock System (Bio-Rad, Mississauga, ON, Canada). The same procedure was repeated with mouse and *X. laevis* tissues. The subtracted PCR products generated by SSH were cloned into the TOPO Cloning 5-min PCR Cloning Kit (Invitrogen) and then transformed into DH5-α-T1 Max Efficiency cells (Invitrogen). For each library, 1152 clones were randomly selected for PCR amplification (3456 clones in total). Briefly, a bacterial colony was picked and resuspended in 200 μl of Luria broth with ampicillin (50 μg/ml) and grown for 6 h at 37°C. The PCR amplifications were carried out from 2 μl of bacterial suspension with the HotMaster Taq DNA Polymerase (Eppendorf, Mississauga, ON, Canada) according to the manufacturer's instructions. The remaining bacterial suspension was kept in 20% glycerol at −80°C. Three microliters from each PCR reaction were electrophoresed on a 2% agarose gel to identify single insert-containing clones.

Microarray Preparation and Hybridization

The PCR products of 3456 randomly selected clones were purified using Unifilter 384-well purification plates (Whatman, Clifton, NJ) according to the manufacturer's instructions. Purified PCR products were speedvac-evaporated (SPD SpeedVac ThermoSavant), resuspended in a mixture of equal parts 3× SSC (1× SSC: 0.15 M sodium chloride and 0.015 M sodium citrate with dimethyl sulfoxide), and spotted in four replicates on GAPS II glass slides (Corning, Corning, NY) using a VersArray Chip WriterPro robot (Bio-Rad). A SpotReport Alien cDNA Array Validation System (Stratagene) was printed as negative controls, and a fragment of the green fluorescent protein was used as an exogenous positive control. Slides were then cross-linked with ultraviolet rays according to the manufacturer's instructions. Quality-control experiments were performed after each set of slides with TOTO-1 dye (Molecular Probe, Burlington, ON, Canada) and Terminal Transferase dye (Roche Diagnostics, Laval, QC, Canada).

Forward- and reverse-subtracted PCR products from a specific library were used as probes to hybridize the glass slides containing the PCR-amplified cDNA insert from that same library. Probes were labeled with Alexa Fluor 555 and 647 reactive dye pack (Molecular Probes) using Amino Allyle dUTP (Ambion, Austin, TX) according to the suggested protocol from Molecular Probes. Glass slides were prehybridized with DIG buffer (Roche) supplemented with yeast tRNA (4 mg/ml; Invitrogen) and Cot-1 DNA (1 mg/ml; Invitrogen) for 1 h at 37°C. Slides were then hybridized overnight at 37°C with labeled purified probes added to fresh prehybridization solution. Hybridizations were performed in the ArrayBooster using the Advacard AC3C (The Gel Company, San Francisco, CA). Slides were then washed once with 1× SSC/0.2% SDS for 10 min at room temperature, 1× SCC/0.2% SDS for 10 min at 55°C, and with 0.1× SCC/0.2% SDS for 5 min at room temperature. To minimize effects resulting from differences in dye incorporation, the dyes during probe labeling were reversed for a duplicate hybridization, and this procedure was repeated twice. Thus, for each experiment, four slides were hybridized. Because four spots are present on the slide for each clone, a total of 16 data points were generated per clone. Slides were scanned using the VersArray ChipReader System (Bio-Rad) and were analyzed using the ChipReader and ArrayPro Analyzer software (Media Cybernetics, San Diego, CA).

TABLE 1. Sequence of gene-specific primers.

Clone no.[a]	Species	Primer sequence	Amplicon size
27	Bovine	up 5′-TGT CTC TTG ACC TGC TGC TC-3′ low 5′-TGC TCT GCA GTT GGA GAA TG-3′	238
244	Bovine	up 5′-CCC ACC AAC CAG AAA TTA CC-3′ low 5′-CAA CAG CTT CAG TTT CAC TTT AGG-3′	203
675	Bovine	up 5′-AAG GCA AGA CGA TGA TGA CC-3′ low 5′-GGG TCA AAT CAG ATC CAA GG-3′	221
705	Bovine	up 5′-TAG GAC TAC GCC CAT TCA CC-3′ low 5′-GAT GCT GTA GGC TCA AAC TGC-3′	204
891	Bovine	up 5′-CCT TCA CTA AAG GAT CTC TCA AAC-3′ low 5′-CAT GGA AAG TCA TCT AGT CTA TGC-3′	159
1404	Mouse	up 5′-TTG GGT GGG TTT GCT TTA AC-3′ low 5′-GGT CTT GGA CAC AGT GAC AGG-3′	165
2198	Mouse	up 5′-CGG GTC CAT AGA TGG TTT AGG-3′ low 5′-GGC TTT GAA CCA AGT TGT GG-3′	174
2269	Mouse	up 5′-GAG CTA TAA GGC AGC CCA AG-3′ low 5′-AGA TGT GTC CCA AGC CTG TC-3′	163
2285	Mouse	up 5′-GCC TGA AAT TGC CAG TAA GG-3′ low 5′-GGC GTT TCC TTT GAC AGT TG-3′	193
2341	Mouse	up 5′-CGT TCA GAG GCT AGG ATT GG-3′ low 5′-CAT GTT GGA GAT CTC AAG ACT TTA C-3′	168
2588	Xenopus	up 5′-AGG TGC AGC TAA CTG GAT GG-3′ low 5′-GGG CAA CAG TAG CAT CAA TTC-3′	150
2633	Xenopus	up 5′-ATG CCA ACA CAC AAA CAT GC-3′ low 5′-TGC TCC AGG AAG AGA GAT GG-3′	219
2655	Xenopus	up 5′-TTA AGG GCT GTC CTC AGT GC-3′ low 5′-GAG GCA CTC CGT CTT CTT TG-3′	247
2853	Xenopus	up 5′-TCA GGG ATT TGC TGA CAA TG-3′ low 5′-TTT GCA ATA GGG CTG GAG AG-3′	233
3379	Xenopus	up 5′-AAT GCT GCA TGT GAT GTT CAT-3′ low 5′-TCA GAG TTG TGC TGC TTT GAC-3′	166
GAPDH Bt	Bovine	up 5′-CCA ACG TGT CTG TTG TGG ATC TGA-3′ low 5′-GAG CTT GAC AAA GTG GTC GTT GAG-3′	226
GAPDH Mm	Mouse	up 5′-ATG TCG TGG AGT CTA CTG GTG TC-3′ low 5′-CAT ACT TGG CAG GTT TCT CCA G-3′	486
GAPDH Xl	Xenopus	up 5′-TGT AGT TGG CGT GAA CCA TGA G-3′ low 5′-CAG CAT CQAA AGA TGG AGG AGT G-3′	500

[a] Bt, *Bos taurus*; Mm, *Mus musculus*; Xl, *Xenopus laevis.*

Microarray Data Analysis

Microarray data analysis was performed using Significance Analysis for Microarray (SAM), a free software developed at Stanford University (http://www-stat.stanford.edu/~tibs/SAM/). First, a pretreatment of the raw data was performed using a program written on the R freeware (http://cran.r-project.org/) to eliminate uninformative data according to a calculated threshold, which determines the background as $t = m + 2 \times$ SD, where t is the calculated threshold, m is the mean of the negative-control raw data, and SD is the standard deviation of those same negative-control raw data. Second, the bias introduced by the dye differences in the dye-swap experiments were corrected by a coefficient calculated with the positive controls (i.e., green fluorescent protein), which was added to probes in equal amounts before labeling. A mean ratio (oocyte to somatic tissues intensity values) of the informative normalized data were then calculated for the replicates to obtain one value per clone. Finally, ratios were log2-transformed and submitted to SAM analysis to get the list of genes that are preferentially expressed in the oocyte compared to somatic tissues.

DNA Sequencing and Analysis

The DNA sequencing was performed using an automated ABI 3730 DNA Sequencer (PE Applied BioSystems, Foster City, CA). Sequencing reactions were carried out with the ABI Prism BigDye Terminator Cycle Sequencing Ready Reactions Kits (PE Applied BioSystems) and nested PCR primer 1 (5′-TCGAGCGGCCGCCCGGGCAGGT-3′) or nested primer 2R (5′-AGCGTGGTCGCGGCCGAGGT-3′; both from BD Biosciences). Sequence traces were visualized with the online freeware Chromas 1.45 (http://www.technelysium.com.au/chromas.html) and uploaded in a cDNA Library Manager program (Genome Canada Bioinformatics). The cDNA Library Manager automates and facilitates the sequence-traces files analysis and the clone identification. Briefly, sequence traces are uploaded into the cDNA Library Manager, trimmed (Phred software, http://www.phrap.org/phredphrapconsed.html), and compared against a locally installed GenBank database (http://www.ncbi.nlm.gov/BLAST/). Finally, the BLAST results are compiled into a report chart. The cDNA Library Manager also allows library intra-BLAST alignment to identify and cluster redundant clones.

Selection of Clones and Gene Expression Analysis

The selection of clones for further analysis was based on the microarray results. As previously mentioned, the analysis was performed by SAM using the ratio of the signal intensity with oocytes to that with somatic tissues. This SAM analysis generated a list of clones with significant preferential expression in the bovine, mouse, and *X. laevis* oocytes, respectively. The differential expression pattern of the top-five uncharacterized candidates from each species was subsequently confirmed by PCR and RT-PCR. Gene-specific primers were designed based on the sequence of the cloned cDNA fragments (Table 1). The differential expression of these 15 uncharacterized genes was first confirmed by PCR using the nonsubtracted cDNA testers and drivers (10-fold dilution of the secondary PCR product). The PCR amplifications were performed in a 30-μl reaction volume using the Hotmaster polymerase (Eppendorf) and gene-specific primers (Table 1). Cycling conditions were 2 min at 94°C, followed by 20 cycles of 30 sec at 94°C, 30 sec at 55°C, and 30 sec at 68°C. The *GAPD* amplifications were carried out as a positive control of amplification to assure the quality and quantity of cDNAs used for this experiment and to see the potential effect of a distortion created by the SMART procedure (see Fig. 2). The amplified PCR fragments were electrophoresed on a 2% agarose gel and stained with ethidium bromide. The experiment was conducted at least three times for each gene. This was followed by RT-PCR analysis using equal amounts of total RNA isolated from oocytes and a pool of somatic tissues. The cDNA was generated using an oligo (dT) primer and the Omniscript reverse transcriptase (Invitrogen) according to the manufacturer's instructions. The RT-PCR was performed using 30 cycles, with the conditions described above.

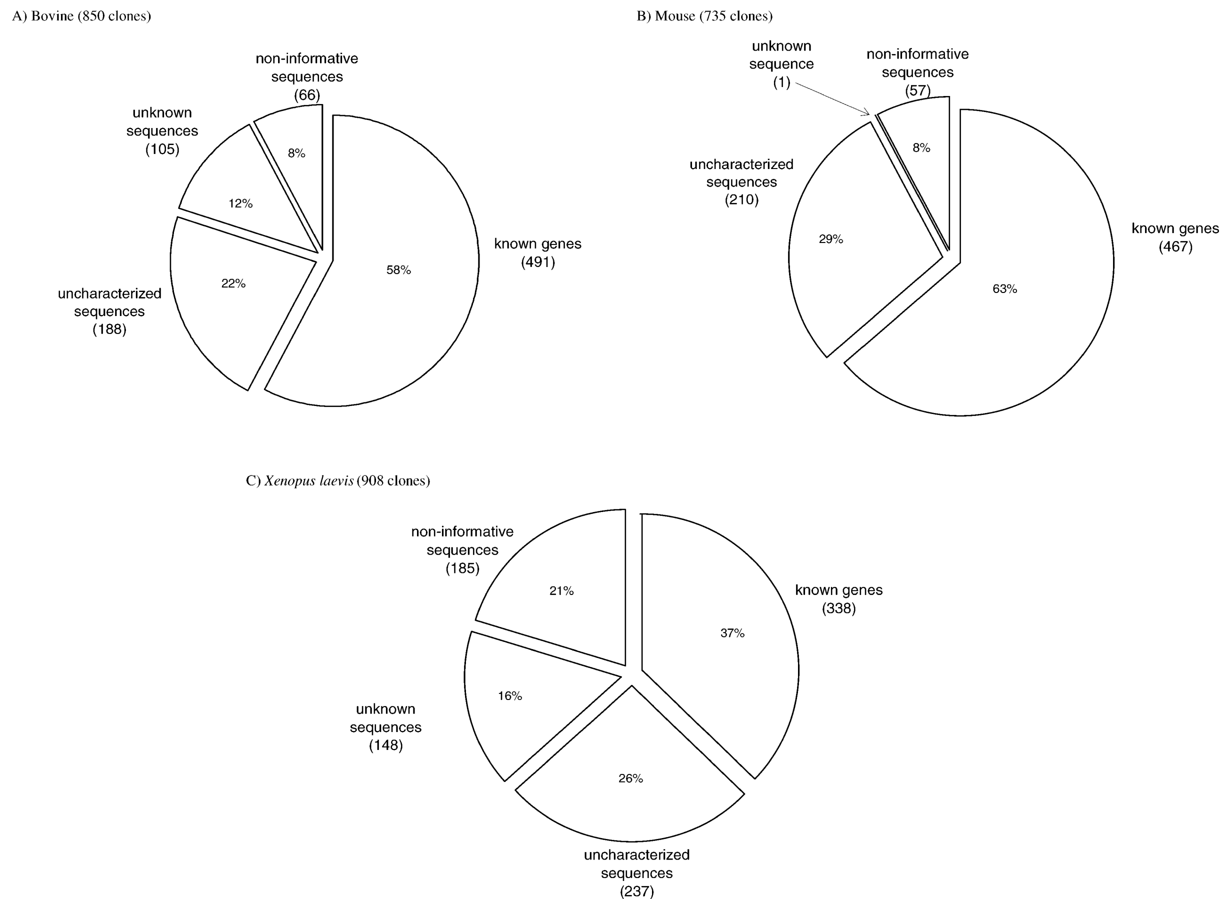

FIG. 1. Pie diagram of cDNA sequences obtained in the subtracted oocyte libraries for (**A**) bovine, (**B**) mouse, and (**C**) *Xenopus laevis*. The bovine, mouse, and *X. laevis* libraries had 850, 735, and 908 insert-containing clones, respectively, subjected to sequencing. Known genes, sequences with significant identity to known genes ($e < 10^{-5}$); uncharacterized sequences, sequences with significant identity to BAC clones, cDNAs, or EST ($e < 10^{-5}$); unknown sequences, sequences not found in the GenBank database; noninformative sequences, sequences with no sequencing results after two independent sequencing reactions.

RESULTS

Suppressive Subtractive Hybridization

Total RNA (1 μg) from oocytes and pools of somatic tissues was synthesized into double-strand cDNA and amplified using BD SMART PCR cDNA synthesis kit. The optimal cycle number of PCR reaction was used to assure that minimal distortion was created. Comparison of *GAPD* amplifications performed on amplified cDNA and nonamplified cDNA confirmed that no major distortion had occurred during the amplification process. The subtraction efficiency was estimated using the housekeeping gene *GAPD*. In all libraries, the amount of *GAPD* transcript was reduced dramatically after subtraction. After cloning and transforming the subtracted cDNA, more than 1000 clones from each subtracted library were amplified by PCR. This procedure revealed that 850 clones from the bovine library, 735 clones from the mouse library, and 908 clones from the *X. laevis* library were positive insert-containing clones. The insert size of the clones ranged from 0.2 to 2.5 kilobases, and the average fragment insert size was approximately 600 base pairs, which is in agreement with that statistically predicted by *Rsa*I digestion. All the 2493 cDNA inserts were analyzed by DNA sequencing, and 88% gave good sequencing results (Fig. 1). Analysis of these sequences with the GenBank database (nr and EST) resulted in the identification of 1296 known cDNA and 635 uncharacterized cDNAs (BAC or EST clones), 254 of which were novel sequences (Fig. 1). Clones that were classified as novel genes were compared against one another to detect multiple occurrences. Detailed information for each library is available in Figure 1. With the information available through the GenBank database, we estimated that for the cDNA corresponding to known genes in our subtracted libraries, 59% of the cDNA contained untranslated region (UTR) sequences (5′ or 3′). However, the majority of these clones had a 3′-UTR region (45%), and a smaller number had a 5′-UTR region (14%).

Detection of several genes that were already known to be expressed preferentially in the oocyte, such as the oocyte-specific zona pellucida genes (*ZP*); growth differentiation factor 9 (*GDF9*); bone morphogenetic protein 15 (*BMP15*); H1 histone family, member O, oocyte specific (*H1foo*); oocyte secretory protein 1 (*Osp1*); cytoplasmically polyadenylated oocyte-specific maternal transcript (*Om2b*); oocyte maturation, alpha (*Omt2a*); and cyclin B1 (*CCNB1*) in the oocyte-subtracted library increased confidence in the quality of the subtraction procedure. Interestingly, many novel genes also were detected in all three libraries.

Microarray Analysis

To confirm the differential expression in the three oocyte-subtracted libraries, microarray analysis were performed with forward and reverse PCR-subtracted products. Hybridizations were always performed with probes and slides of the same species; thus, probes were hybridized on

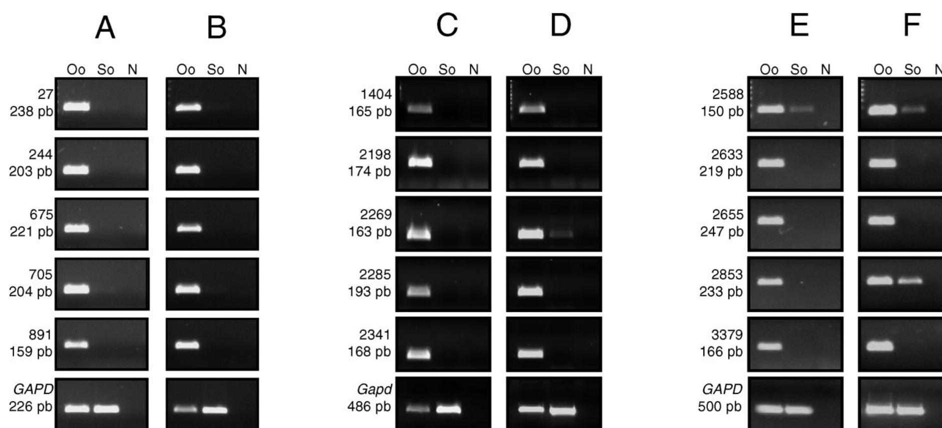

FIG. 2. Differential expression of 15 uncharacterized clones. **A**) PCR amplifications of the top-five uncharacterized bovine clones using the bovine nonsubtracted cDNA testers and drivers (10-fold dilution of the secondary PCR product) and gene-specific primers. **B**) Subsequent RT-PCR analysis of these five clones using equal amount of total RNA isolated from bovine oocytes and a pool of bovine somatic tissues and gene-specific primers. **C** and **D**) The same experiments were conducted for the mouse top-five uncharacterized clones. **E** and **F**) The same experiments were conducted for the *Xenopus laevis* top-five uncharacterized clones. For each species, an amplification of *GAPD* was carried out as a positive control for both samples. These experiments were conducted in triplicates. N, Negative; Oo, oocyte; So, somatic tissues.

a slide of the corresponding species. Microarray analysis performed by SAM revealed that in total, 27% of the clones had significant preferential expression in the oocytes. When the libraries were analyzed individually, these results were 22% for the bovine oocyte-specific library, 32% for the mouse oocyte-specific library, and 27% for the *X. laevis* oocyte-specific libraries. From that list of clones preferentially expressed in the oocyte generated by SAM, the top-10 candidates from each species are presented in Tables 2–4. Clones that are significantly preferentially expressed in the oocytes and have a significant BLAST hits within the same species against the current GenBank databases ($e < 10^{-5}$) have been submitted to the GenBank EST database.

PCR and RT-PCR Analyses

To confirm the results obtained with the microarray analysis, 15 candidates were selected for further analysis by PCR and RT-PCR. The selection of these candidates was based on the microarray results analyzed by SAM (intensity ratios of oocyte to somatic tissues). The top-five uncharacterized genes preferentially expressed in the oocyte from each library were chosen for quantification (Tables 2–4). First, PCR analysis was performed using the nonsubtracted cDNA testers and drivers samples that were amplified with the SMART technique and subjected to PCR amplifications during the SSH experiment. Second, RT-PCR analysis was performed using equal amount of total RNA from oocytes and from a pool of somatic tissues. The results confirmed that all the genes analyzed were preferentially expressed in the oocyte compared to somatic tissues (Fig. 2). For the bovine oocyte-specific library, two of five clones (clones 27 and 705) appeared to be expressed in the somatic tissues at a much lower level than in the oocyte (Fig. 2, A and B). As for the other three clones (clones 244, 675, and 891), they were detected only in the oocyte samples (Fig. 2, A and B). For the mouse oocyte-specific library, four clones (clones 1404, 2198, 2285, and 2341) gave a positive signal only in the oocytes, and clone 2269 also had a very low level of expression in the somatic tissues (Fig. 2, C and D). Finally, for the *X. laevis* oocyte-specific library, two clones (clones 2588 and 2853) were expressed in the somatic tissues with a lower level of expression compared to that in the oocyte (Fig. 2, E and F). As for the three remaining clones in the *X. laevis* library (clones 2633, 2655, and 3379), they were detected only in the oocyte (Fig. 2, E and F).

DISCUSSION

We report the construction of three subtracted cDNA libraries from bovine, mouse, and *X. laevis* oocytes. Using the SSH technique, we suppressed common cDNAs that the oocyte shares with somatic cells to obtain enriched oocyte-specific libraries. Moreover, we know that functionally important genes are highly conserved through evolution; therefore, this procedure was performed simultaneously on three different species. The isolation and characterization of novel oocyte-specific genes will help us to uncover the molecular mechanisms related to the unique functions of the oocyte.

More than 1000 clones were isolated and amplified by PCR for each library to ensure that screening of the oocyte-specific libraries was as complete as possible and that few new sequences would be discovered after this screening. Based on the study by Hennebold et al. [30], the discovery of new independent clones is very limited when an excess of 800 clones are sequenced. Additionally, no well-known housekeeping gene was detected in the subtracted oocyte-specific libraries, increasing our confidence in the validity of the clones isolated from these libraries.

Microarray analysis allowed us to identify clones from the subtracted cDNA libraries that were truly differentially expressed between the oocyte and the somatic tissues. The SAM analysis was based on the ratio of oocyte to somatic tissue expression level. Results showed that the percentage of clones in the oocytes with significant preferential expression is fairly similar in all three oocyte-specific libraries (22, 32, and 27% for the bovine, mouse, and *X. laevis*, respectively), which is relatively small considering that the slides were spotted with our oocyte-specific cDNA libraries. However, we took a stringent approach, because our main objective was to identify novel oocyte-specific genes. A list of the top-10 clones was elaborated for each species, including the first five known genes and the first five uncharacterized cDNA (Tables 2–4).

For the bovine oocyte-specific library (Table 2), the top-five known genes include growth differentiation factor 9 (*GDF9*), MLF1-interacting protein (*MLF1IP*), polyadenyl-

TABLE 2. Top 10 bovine clones preferentially expressed in the oocyte based on microarray analysis.

Clone no.	Frequency[a]	Blast identity[b,c]	GenBank accession no.[b]	% Identity[b]	e value[b]
Known genes					
731	3	Hs similar to Protein tyrosine phosphatase, receptor type Q	XM_291991	89	e-63
769	17	Bt Growth differentiation factor 9	AF307092	99	0.0
917	26	Hs B-cell translocation gene 4	NM_017589	89	0.0
1013	26	Hs Polyadenylate binding protein-interacting protein 1	NM_183323	93	0.0
1153	6	Hs MLF1 interacting protein	BC031520	84	e-93
Uncharacterized genes					
27[d,e]	1	27_bovine oocyte cDNA subtracted library	CX123768	100	0
244[d,e]	3	244_bovine oocyte cDNA subtracted library	CX123769	100	0
675[d,e]	1	675_bovine oocyte cDNA subtracted library	CX123770	100	0
705[d,e]	1	705_bovine oocyte cDNA subtracted library	CX123771	100	0
891[d,e]	1	891_bovine oocyte cDNA subtracted library	CX123772	100	0

[a] Number of clones in the bovine oocyte-specific library corresponding to that gene.
[b] Based on the BLAST searches of the GenBank database.
[c] Bt, *Bos taurus*; Hs, *Homo sapiens.*
[d] DNA sequence submitted to the GenBank database (EST).
[e] Clone validated by PCR and RT-PCR.

ate-binding protein-interacting protein 1 (*PAIP1*), B-cell translocation gene 4 (*BTG4*), and protein tyrosine phosphatase, receptor type, Q (*PTPRQ*). The first gene found in our bovine top-10 list is *GDF9*. This gene is part of a short list of well-known oocyte-specific genes and is required for ovarian folliculogenesis [13]. The second gene, *MLF1IP*, recently was characterized [31]. It specifically associates with myeloid leukemia factor 1 (*MLF1*), a protein that forms a fusion gene with nucleoplasmin 2 (*NPM2*). *Npm2* also was found in our mouse oocyte-specific library (see description below). A nuclear function for *MLF1IP* is supported by the presence of two nuclear receptor-binding motifs, and preliminary data reported by Hanissian et al. [31] demonstrates association of *MLF1IP* with proteins involved in cell division, suggesting a role in cell division. In that same study, Northern blot analysis demonstrated high-level *MLF1IP* expression in spermatogenic cell layers, suggesting a role in spermatogenesis. Additionally, *MLF1IP* was detected in the mouse ovary by Western blot analysis, but no further analysis was performed. *PAIP1* is involved in translational initiation and protein biosynthesis through its interaction with *EIF4A* [32]. Although expressed in different tissues, it also is expressed in primary mouse oocyte (Unigene no. Mm.132584). In the bovine embryo, *PAIP1* mRNA expression levels are at their highest in the GV-stage oocyte, followed by a constant reduction during oocyte maturation through to the blastocyst stage, at which its expression is almost nonexistent (unpublished data). *BTG4,* also named *PC3B,* is endowed with marked antiproliferative activity, being able to induce G_1 arrest, and is highly expressed in the testis, oocytes, and preimplantation embryos [33]. The last known gene in the bovine top-five list is *PTPRQ*, a phosphatidylinositol phosphatase that can regulate cell survival and proliferation [34]. To our knowledge, the present study is the first to report the expression of *PTPRQ* in the oocyte.

For the mouse oocyte-specific library (Table 3), the top-five known genes include bone morphogenetic protein 15 (*Bmp15*), zona pellucida glycoprotein 2 (*Zp2*), Spindlin (*Spin*), Oogenesin 1 (*Oog1*), and nucleoplasmin 2 (*Npm2*). All the genes in the mouse top-five list are known to be preferentially expressed in the oocyte and play a major role in the oocyte. The Bmp15, which is specifically expressed in the oocyte, is involved in oocyte maturation and follicular development as a homodimer or by forming heterodimers with a related protein, *Gdf9* [15]. *Zp2* is a structural component of the zona pellucida, an extracellular matrix that surrounds the oocyte and early embryo, and it functions as a secondary binding site for the penetration of acrosome-reacted spermatozoa [11]. *Spin* is an abundant ma-

TABLE 3. Top 10 mouse clones preferentially expressed in the oocyte based on microarray analysis.

Clone no.	Frequency[a]	Blast identity[b,c]	GenBank accession no.[b]	% Identity[b]	e value[b]
Known genes					
1302	15	Mm Oogenesin 1	AB050008	99	0.0
1360	14	Mm Bone morphogenetic protein 15	BC055363	97	0.0
1378	9	Mm Spindlin	BC016517	100	0.0
2119	7	Mm Nucleophosmin/nucleoplasmin 2	NM_181345	98	0.0
2271	9	Mm Zona pellucida glycoprotein 2	NM_011775	99	0.0
Uncharacterized genes					
1404[d]	1	Mm 2 days pregnant adult female ovary, E330034G19Rik	AK087874	100	0.0
2198[d]	2	Mm 2 days pregnant adult female ovary, hypothetical protein E330017A01	AK087761	99	0.0
2269[d]	1	Mm expressed sequence C87414	BC052888	100	0.0
2285[d]	1	Mm similar to Nur77 downstream protein 1 (LOC381251)	XM_355193	99	0.0
2341[d]	3	Mm adult male testis, 4921520L01Rik	AK014932	99	e-180

[a] Number of clones in the mouse oocyte-specific library corresponding to that gene.
[b] Based on the BLAST searches of the GenBank database.
[c] Mm, *Mus musculus.*
[d] Clone validated by PCR and RT-PCR.

TABLE 4. Top 10 xenopus clones preferentially expressed in the oocyte based on microarray analysis.

Clone no.	Frequency[a]	Blast identity[b,c]	GenBank accession no.[b]	% Identity[b]	e value[b]
Known genes					
3002	4	Xl Protein arginine methyltransferase1	AB085173	99	0.0
3012	5	Xl Mitotic phosphoprotein 67	AF419153	99	0.0
3121	3	Xl 13S condensin XCAP-D2 subunit	AF067969	99	0.0
3135	3	Xl Phosphotyrosine binding protein	AY183756	95	0.0
3149	2	Xl Importin alpha 1a	L36339	99	0.0
Uncharacterized genes					
2588[d]	1	Xl de92b11.x1 Wellcome CRC pRN3 St19 26	BF231739	98	e-175
2633[d]	1	Xl NIBB Mochii clone XL171a05 3′	BJ631866	99	0.0
2655[d]	1	Xl clone S10-21-F5	AF549916	98	0.0
2853[d]	2	Xl NICHD_XGC_OO1 clone IMAGp998K1111210	BX843422	99	0.0
3379[d]	1	Xl AGENCOURT_10748348 Wellcome CRC pSK egg clone: 6326625 5′	CA974130	92	e-153

[a] Number of clones in the xenopus oocyte-specific library corresponding to that gene.
[b] Based on the BLAST searches of the GenBank database.
[c] Xl, *Xenopus laevis.*
[d] Clone validated by PCR and RT-PCR.

ternal transcript in the unfertilized egg and in the 2-cell, but not the 8-cell, embryo [35]. It was found to associate with the meiotic spindle, and it plays a role in cell-cycle regulation during the transition from gamete to embryo [35]. *Oog1* is expressed throughout oogenesis and early embryogenesis in the mouse [36]. This protein localizes in nuclei at the late 1-cell and early 2-cell stages, suggesting that it has some roles in zygotic transcription of early preimplantation embryos as well as in folliculogenesis and oogenesis in the mouse [36]. *Npm2* is a very well-known gene that is expressed in *X. laevis* eggs involved in the remodeling of sperm chromatin at fertilization [37]. A mouse orthologue also has been characterized and is known as a maternal effect gene critical for nuclear and nucleolar organization as well as embryonic development [38]. It also is reported to be expressed in the mouse ovary, pituitary gland, and brain (Unigene no. Mm.347749).

For the *X. laevis* oocyte-specific library (Table 4), the top-five known genes include phosphotyrosine-binding protein (*xPTB*), protein arginine methyltransferase 1 (*x-PRMT1*), 13S condensin XCAP-D2 subunit, mitotic phosphoprotein 67 (*MP67*), and importin α 1a (*IMA1a*). The first gene, *xPTB,* recently has been isolated from a *X. laevis* cDNA library derived from oocyte vegetal cortex [39]. This novel gene contains a phosphotyrosine-binding domain and also is expressed in the liver diverticulum region of tailbud-stage embryos [39]. The *xPTB* has human and mouse orthologues that play a critical and specific role in low-density lipoprotein-receptor endocytosis in the liver [40]. Although no precise function is known in the oocyte for *xPTB,* its mRNA expression also has been found in the 2-cell mouse embryo (Unigene no. Mm.27486). Protein arginine methyltransferase 1 (*xPRMT1*), a gene conserved in eukaryote (HomoloGene no. #21477), is an *xCIRP2*-binding protein. The methylation of *xCIRP2* by *xPRMT1* results in the accumulation of *xCIRP2* in the cytoplasm [41]. Aoki et al. [42] found that *xCIRP2,* which is highly expressed in *X. laevis* oocytes, is associated with ribosomes, suggesting that it participates in translational regulation in oocytes. The 13S condensin *XCAP-D2* subunit, which also is known as *PEG7,* is an *X. laevis* protein required for mitotic chromosome condensation in egg extracts [43]. *PEG7* is a maternal mRNA with an expression that is strongly increased during oocyte maturation, and the tissue and cell expression pattern of *PEG7* indicates that this protein is mainly detected in cultured cells and in germ cells [43]. Another gene in the *X. laevis* library is *MP67,* a member of the large family of mitotic phosphoproteins found in *X. laevis* oocytes [44]. These proteins play an important role in cell-cycle progression through phosphorylation. Two of the most important phosphoproteins involved in the G_2/M transition are *WEE1* and *CDC25* [45, 46]. *IMA1a*, a cytosolic protein from *X. laevis* eggs, is essential for selective protein import into the cell nucleus by promoting signal-dependent binding of proteins to the nuclear envelope [47].

This concludes our lists of top-five known genes for our three species. Before proceeding with the lists of top-five novel genes, it should be mentioned that well-known oocyte-specific genes, such as *BMP15, ZP, GDF9,* and *H1FOO,* were all found in at least two of our subtracted libraries and always were preferentially expressed in the oocyte after the microarray analysis. However, they were not always found in the top-five list for all three species. For example, *GDF9* is found in the bovine top-five list and *Bmp15* is found in the mouse top-five list, but both of them are preferentially expressed in the oocyte when compared to the somatic tissue in both species according to the SAM microarray analysis.

Both PCR and RT-PCR were performed on all of the top-five uncharacterized genes from the three libraries. These analyses confirmed that all these clones are preferentially expressed in the oocyte compared to somatic tissues (Fig. 2). Additionally, a multiple sequence alignment with clustalW was performed on these 15 uncharacterized genes to confirm that these 15 clones are unique genes.

For the bovine oocyte-specific library, our top-five uncharacterized genes validated by PCR and RT-PCR consisted of clones 27, 244, 675, 705, and 891 (Table 2 and Fig. 2, A and B). Because all these clones were novel sequences (no match in the GenBank database), their DNA sequences were submitted to the GenBank database (accession nos. are listed in Table 2).

With the information available through the National Center for Biotechnology Information database, we found that all five uncharacterized mouse clones that were selected for validation by PCR and RT-PCR had been found previously to be expressed in fertilized or unfertilized eggs (Table 3 and Fig. 2, C and D). Only clone 2198 was reported in the literature (Unigene no. Mm.26145) to be expressed in a somatic tissue (spleen), but in our PCR and RT-PCR validations, no expression was found in somatic tissues (Fig. 2, C and D). This clone is a ferritin-like struc-

ture containing protein that is presumably involved in inorganic ion transport and metabolism. While this work was being prepared for publication, a study [48] identified and characterized a group of new oocyte-specific genes members of the human *NACHT* leucine-rich repeat- and PYD-containing (*NALP*) gene family. Clone 2341 from our mouse oocyte-specific subtracted library is a member of this family and is named *Nalp iota* [48]. Northern blot analysis revealed that *Nalp iota* is specifically expressed in the oocyte and testis, and RNA interference revealed that the expression of *Nalp iota* gene is required for the normal development of mouse preimplantation embryos, a role that is attributed to *Mater* (also known as *Nalp5*), the founding member of this gene family [48, 49].

For the *X. laevis* oocyte-specific library, our top-five uncharacterized genes validated by PCR and RT-PCR consisted of clones 2588, 2633, 2655, 2853, and 3379 (Table 4 and Fig. 2, E and F). BLAST analysis revealed that these clones correspond to known cDNA coming from different EST libraries. These clones have been reported previously to be expressed in the oocyte with the exception of clone 2588 (Wellcome CRC pRN3 St19 26), which has been isolated from a pooled-embryos library. Based on our validation results, all theses clones are preferentially expressed in the *X. laevis* oocyte (Fig. 2, E and F).

Finally, the present study has revealed that combining SSH and microarray analysis is an excellent approach to isolate differentially expressed genes. The strategy used in the present study allowed us to make a good selection of clones preferentially expressed in oocyte, and most importantly, it allowed us to generate a concise list of novel genes that presumably are key factors in the oocyte. This is in contrast to results obtained in previous studies using different approaches in which extensive lists of clones are produced with no indication as to which ones may be truly important factors [8, 50]. Moreover, the simultaneous approach with three different species used in the present study gives us a better chance to focus on the most important genes. The establishment and validation of these three high-quality oocyte-specific libraries provide a solid foundation for future studies considering the huge potential of making comparisons between species to find genes conserved throughout evolution.

The present study successfully generated a list of candidate novel oocyte-specific genes. Although these candidates were validated by PCR and RT-PCR, further analysis is needed on a larger variety of somatic tissues before they can be identified has oocyte-specific genes. To our knowledge, this is the first study to combine SSH and microarray in three different species and identify novel oocyte-specific genes. Results obtained from this powerful combination will allow us to make rapid breakthroughs in our understanding of the unique molecular mechanisms in the oocyte and will be useful in other areas, such as embryonic stem cell, cloning, and cancer research, where the potency and immortality of cells and nuclear reprogramming are of interest.

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