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ARTICLE

Transcriptome analysis of bull spermatozoa: implications for male fertility

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The orchestration of gene expression during the formation of gametes and embryos has been a source of wonder for Nelida Rodriguez-Osorio. She became interested in animal reproduction, gametogenesis and embryogenesis as a veterinary student and has focused on differential gene expression during early embryo development and the particular genes that confer developmental potential to eggs and sperm. Currently, Nelida is an associate professor at the School of Veterinary Medicine of the University of Antioquia in Colombia. Her research interests include using functional genomics to improve oocyte maturation and early embryo development in domestic animals.

Abstract Spermatozoa deliver more than the paternal genome into the oocyte; they also carry remnant messenger RNA from spermatogenesis. The RNA profiles of spermatozoa from high-fertility and a low-fertility Holstein bulls were analysed using Affymetrix bovine genechips. A total of 415 transcripts out of approximately 24,000 were differentially detected in spermatozoa collected from both bulls (fold change $\geq$2.0; $P < 0.01$). These transcripts were associated with different cellular functions and biological processes. Spermatozoa from high-fertility bulls contained higher concentrations of transcripts for membrane and extracellular space protein locations, while spermatozoa from the low-fertility bulls were deficient of transcripts for transcriptional and translational factors. Quantitative real-time PCR was used on three low-fertility and four high-fertility bulls to validate the microarray data. Two highly represented transcripts in the microarray analysis (protamine 1 and casein beta 2) were validated, as well as a third transcript (thrombospondin receptor *CD36* molecule) that showed a lower concentration in low-fertility bulls. This study presents the global analysis of spermatozoa originating from bulls with opposite fertility. These results provide some specific transcripts in spermatozoa that could be associated with bull fertility.

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KEYWORDS: bovine, fertility, gene expression, male, microarray, spermatozoa

Introduction

Reproduction and fertility are central to the survival of species. Infertility is a common disorder affecting not only humans, but also animals. Male infertility is characterized by a diminished or absent capacity to produce spermatozoa capable of fertilizing the oocyte and supporting embryonic and fetal development until delivery of living offspring.

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While infertility represents a psychological shock for the 10—15% of human couples that experience it, subfertility represents a great economic loss for livestock breeders (Bhasin, 2007; Ferlin et al., 2006; Lopez-Gatius, 2003; Lucy, 2001). Male infertility accounts for up to two-thirds of the cases of infertility and constitutes a complex disease, usually with genetic origins such as chromosomal aberrations and single gene mutations (Cram et al., 2001; De Kretser and Baker, 1999; Ferlin et al., 2007; Krausz and Giachini, 2007).

In cattle, extensive fertility data and progeny records have revealed the inability of a subset of high-merit bulls to produce successful full-term pregnancies, despite the acceptable sperm motility and morphology levels (compensable traits) of their spermatozoa (Chenoweth, 2007; Parkinson, 2004). This observation underlines the molecular and cellular defects (uncompensable traits) affecting the ability of spermatozoa to fertilize and contribute to normal embryo development (De Jonge, 1999; Dejarnette, 2005; Lewis, 2007; Zini and Libman, 2006). Unlike compensable traits, which can be overcome by increasing the quantity of spermatozoa per insemination, uncompensable traits are severe and related to molecular defects in the spermatozoa and cannot be overcome by increasing the quantity of spermatozoa per insemination (Chenoweth, 2005; Dejarnette, 2005; Okabe et al., 1998; Zheng et al., 2007; Zini and Libman, 2006).

The mature spermatozoon fully capable of fertilizing the oocyte is produced through a long and complex process which includes serial mitotic and meiotic divisions of diploid sperm stem cells (to give rise to haploid round spermatids), dramatic morphological remodelling (extensive chromatin condensation, reduction in nuclear and cytoplasmic volume, formation of an acrosome system and tail production) and capacitation (Parks et al., 2003; Sassone-Corsi, 2002). During this process, mutations might lead to morphological and functional abnormalities found in mature spermatozoa (Carrell et al., 2007; Erenpreiss et al., 2006; Iritani and Niwa, 1977; Parrish et al., 1989). Natural selection prevents or minimizes the transmission of such mutations causing infertility to the next generation (De Jonge, 1999; Gosden and Nagano, 2002).

Spermatozoa deliver more than the paternal genome into the oocyte. The newly transcribed and remnant sperm RNA from spermatogenesis are totally or partially transmitted to the oocyte (Eddy, 2002; Gur and Breitbart, 2006; Miller and Ostermeier, 2006a,b; Ostermeier et al., 2002, 2004). Diverse mRNA have been found in human, rodent and bovine mature spermatozoa (Dadoune et al., 2005; Gilbert et al., 2007; Miller and Ostermeier, 2006a). Although the roles of these transcripts during embryonic and fetal development are still unclear, analysis of their profiles could serve as a diagnostic tool to assess male infertility (Bissonnette et al., 2009; Garrido et al., 2009; Lalancette et al., 2009) or could have prognostic value for fertilization and embryo development (Boerke et al., 2007; Ostermeier et al., 2004; Sassone-Corsi, 2002).

Microarray technology offers insights into the molecular mechanisms of spermatogenesis and the genetic aetiology of male infertility. This high-throughput technology has been successfully used for global gene expression profiling in human, mouse and bovine spermatozoa (Gilbert et al., 2007; He et al., 2006; Ostermeier et al., 2002). Here, the bovine oligonucleotide microarray (Affymetrix Bovine GeneChip) was used to profile the transcript 'fingerprints' of spermatozoa collected from high- and low-fertility bulls.

Materials and methods

Selection of male donors of semen

Bull fertility was predicted from a pool of 934 bulls with an average of 1124 breedings, ranging from 300 to 18,442, using the analysis methods developed by Zwald et al. (2004, 2005), which take into account the breeding event as well as environmental and herd management factors that influence fertility performance of sires (i.e. effects of herd/year/month, parity, cow, days in milk, sire proven status). The fertility prediction of each bull was obtained using the Probit.F90 software (Chang et al., 2004) and expressed as the percentage deviation of its conception rate from the average conception of all bulls. Those with at least two standard deviation (SD) values above or below the average were classified a high or low fertility, indicating a four SD distance between both groups. This program allowed the retrieval of bulls from cohorts averaging 1113 ± 65.8 and 1507 ± 582.6 breeding numbers for low- and high-fertility bulls, respectively. Cut-offs for fertility status were established at $-10.8 \pm 4.1\%$ for low and $+5.1 \pm 0.8\%$ for high-fertility bulls and those within these fertility status ranges were selected for the present study.

Spermatozoal RNA isolation

Frozen straws containing semen of selected five high-fertility or five low-fertility bulls were obtained from (Alta Genetics, Watertown, WI, USA). Straws were thawed and highly motile spermatozoa were purified using a discontinuous Percoll gradient (90—45%). Spermatozoa were further washed three times with cold phosphate-buffered saline and pelleted (about 25×10^6 spermatozoa) by centrifugation at 16,000*g* at 4°C for 10 min.

Total RNA was extracted using the TRIzol reagent as recommended by the manufacturer, with minor changes (Invitrogen, Carlsbad, CA, USA). Briefly, sperm pellets were resuspended in TRIzol solution with 3 μl of glycogen (20 μg/μl: Invitrogen) and ground at high speed for 30 s. Samples were incubated at 65°C for 15 min, followed by chloroform separation of RNA and precipitation from the aqueous phase with isopropanol. RNA pellets were washed twice with 70% ethanol, dried on a heat-block at 37°C and dissolved in diethylpyrocarbonate water. After a treatment with DNase I following the manufacturer's instructions (Invitrogen), aliquots of RNA were used for a non-real-time PCR reaction using intron-spanning bovine protamine 1 (*PRM1*) primers (NM_174156.2, M18395.1; forward: 5′-AGATGTCGCAGACGAAGGAG-3′ and reverse: 5′-AGTGCGGTGGTC TTGCTACT-3′), followed by gel electrophoreses to check for genomic DNA contamination. When necessary, these three steps were repeated until samples were devoid of any genomic DNA contamination. The total RNA quantity and purity were determined using the ND-1000 spectrophotometer (NanoDrop Technologies, Palo Alto, CA, USA) and

2100 Expert BioAnalyzer (Agilent Technologies, Palo Alto, CA, USA). The total rat embryo RNA (Ambion, Austin, Texas, USA) and deionized water were used as positive and negative controls, respectively, and were run together with the sperm samples on the bioanalyser. Three independent RNA isolations were performed for each bull with a total of 30 (3×10 bulls) RNA samples constituted for experiments (DNA microarray and quantitative real-time PCR).

DNA microarray experiments

A total of three sperm RNA samples obtained from a single low-fertility bull and three sperm RNA samples from a single high-fertility bull were used for DNA microarrays. All sample concentrations were normalized and hybridizations were performed on each sample using Affymetrix Bovine GeneChips as described by the manufacturer (Affymetrix, Santa Clara, CA, USA). Briefly, complementary DNA (cDNA) synthesis was performed from 700 ng total sperm RNA using the Two-Cycle cDNA Synthesis Kit (Affymetrix). The MEGAscript T7 Kit (Ambion) was used for the first in-vitro transcription. GeneChip IVT Labeling Kit was used for the second in-vitro transcription and labelling of RNA. Complementary RNA (cRNA) was fragmented and 10 μg of fragmented cRNA were hybridized to the Genechips in a hybridization oven set to a temperature of 45°C with rotations (at medium rotation setting in Isotemp oven from Fisher Scientific, Pittsburg, PA, USA) for 16 h. The chips were then washed and stained with streptavidin/phycoerythrin antibody solution using an Affymetrix FS-450 fluidics station. GeneChips were scanned using the Affymetrix GeneChip scanner 3300. Images were processed with Affymetrix GeneChip operating software, which is based on a statistical expression algorithm that gives information on a signal (measure of the abundance of a transcript) detect present, absent or marginal results. *P*-value ≤ 0.05 indicates the significance of the detection for a probe set.

Metrics like noise, background, scale factor and the fold change of signals from β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) 3′ probes compared with 5′ probes were analysed. Information about the intensities of the spiked controls (*Bacillus subtilis* genes *lys*, *phe*, *thr* and *dap*), which were added to the total RNA at the beginning of the experiment at known concentrations, was used to monitor the linear amplification and labelling process independently from the target samples. Additionally, the performance of the hybridization control genes (*Escherichia coli* genes *bioB*, *bioC* and *bioD* and P1 bacteriophage *cre*) was used for determining the quality of each hybridization process.

Analyses of microarray data

For data visualization, the raw GeneChip signals were uploaded into GeneTraffic UNO (Iobion Informatics LLC, La Jolla, CA, USA), which generated scatterplots of pairwise hybridization comparisons and heat maps from all hybridizations using hierarchical clustering. Power Atlas, a web-based resource from the University of Alabama at Birmingham, was used to estimate the power of the hybridization given the sample size (Page et al., 2006). Probe sets corresponding to differentially expressed genes were uploaded into the Affymetrix Netaffx Analysis Centre (Bovine GeneChip annotation from Nov 2007) (Liu et al., 2003) to retrieve updated information regarding gene symbol, gene title and gene ontology annotation including biological process, molecular function and cellular component. To complement the annotation from Netaffx, GOAnna tools from AgBase, a Mississippi State University-curated, web-accessible resource for functional analysis of agricultural plant and animal gene products (http://agbase.msstate.edu/cgi-bin/tools/GOAnna.cgi) were used. For data visualization, all the GO terms associated to each gene were uploaded into GOSlimViewer, another AgBase tool (http://agbase.msstate.edu/cgi-bin/tools/goslimviewer_select.html) that provides a high-level summary of the gene ontology categories found in the dataset allowing a better visualization of the data.

Molecular modelling

The Ingenuity Pathway Analysis 5.0 software (IPA; Ingenuity Systems, Redwood City, CA, USA) designed for human, mouse and rat genes was used for data modelling and analysis of any networks related to the data sets generated from this study. Differentially represented transcripts in both bull samples were uploaded in the software. Some gene symbols were recognized by the software, while the corresponding human orthologues (gene symbol) of non-recognized bovine genes were manually introduced.

Confirmation of microarray data using quantitative real-time PCR

Quantitative real-time PCR (qPCR) was performed to assess the transcript abundance. Primer sets were designed using Primer Premier 5 software (Premier Biosoft International, Palo Alto, CA, USA) spanning two exons to avoid genomic DNA amplification (**Table 1**).

Here, RNA samples were obtained in triplicates from five low and five high-fertility bulls. All samples (15 low fertility and 15 high fertility) were normalized to 4 ng and reverse transcribed (10 min at 25°C, 50 min at 42°C and at 85°C for 5 min) using oligo(dT) primers. Then two units of *E. coli* RNase H were added to each tube and incubated at 37°C for 20 min (SuperScript III Platinum Two-Step qRT-PCR Kit; Invitrogen) according to the manufacturer's protocol. Five μl of cDNA were used for qPCRs (SYBR GreenER qPCR SuperMixes for iCycler; Invitrogen) with specific primers (10 μmol/l). The cycling parameters were 50°C for 2 min, 95°C for 8 min 30 s for denaturation and 40 cycles of 15 s at 95°C, 30 s at 60°C and 30 s at 72°C for amplification and extension. The melting curve was performed starting at 55°C with 0.5°C increase for 10 s in 80 cycles. Expression values were calculated using the relative standard curve method. Standard curves were generated using 10-fold serial dilutions by measuring the cycle number at which exponential amplification occurred.

Statistical analysis

Microarray data were analysed using the HDBStat (free software from the University of Alabama). Data were

Table 1 Genes used for validation of microarray data through qPCR.

Gene	*Primer sequence 5′—3′*	*Product length*	*Accession number*
CD36	F-CAGACCTCTTGGCAACCACT R-CTGGCATTAGAATCCCTCCA	170	NM_174010.2
CENPA	F-ACACTTTGCCAACAACCACA R-AAACGTCTTCAGGTGGCACT	161	XR_027462.1
CSN2	F-ACAGAGGATGAACTCCAGGATAAA R-AGCAGAGGCAGAGGAAGGTG	299	NM_181008.2
PRM1	F-CGAAGAAGATGTCGCAGACG R-TTCAAGATGTGGCAAGAGGGT	175	NM_174156.2

F = forward; R = reverse.

quantile—quantile normalized and examined for outliers using Pearson's correlation. Quality-control statistics included a deleted residuals approach (Chen, 2004; Persson et al., 2005; Trivedi et al., 2005). False discovery rates for the genes were calculated using the *t*-test (Benjamini et al., 2001). Fold changes were calculated based on the unadjusted data means. Transcripts in any single comparison with a $P < 0.01$, a false discovery rate of <20% and a fold change in excess of 2.0 were considered to be significant and examined further. qPCR data were analysed by one-way ANOVA using the SAS 9.1.3 (SAS Institute, Cary, NC, USA). A relative expression software tool (REST) was used to compare all samples of each group. The mathematical model used by REST is based on the PCR efficiencies and the crossing point deviation between samples (Pfaffl, 2001; Pfaffl et al., 2002). Average data of microarray and qPCR are given as mean signal ± SD.

Results

Selection of male donors of semen and sperm RNA isolation

Determination of bull fertility

Fertility data have been established for the progeny-testing programme AltaAdvantage, which is the industry's most reliable source of fertility information, consisting of insemination records collected from 180 well-managed dairy farms located in different geographical regions across the USA. AltaAdvantage provides paternity verification and pregnancies are diagnosed by veterinary palpation, instead of relying on non-return rates following 60—90 days after breeding. A subset of data was generated consisting of 962,135 insemination records from 934 bulls to predict the bull fertility. Average numbers of breedings were 1113 and 1507 while the fertility rates were −10.8% and +5.1% for the low- and high-fertility bulls used in this study, respectively.

Transcriptome of bull spermatozoa

The employed-protocol allowed isolation of high-quality ($A_{260/280}$ and $A_{260/230}$ ranging from 1.7 to 1.8 each) and high-quantity (about 1.4 ± 0.4 μg per 10^7 spermatozoa; 140 ± 40 fg/spermatozoa) total sperm RNA. The isolation of RNA free of genomic DNA contamination was evaluated through PCR of the *PRM1* gene. The amplification of RNA samples did not generate any amplicons as shown in **Figure 1A** and **B**, for high- and low-fertility bulls, respectively. RNA devoid of any genomic DNA contamination was subsequently used for the synthesis of cDNA, whose amplification revealed an amplicon band of 120 bp, shorter by 100 bp than that obtained with genomic DNA used as a PCR template (220 bp; **Figure 1C**). This observation was indicative of RNA samples devoid of any genomic DNA contamination. A further analysis of RNA samples on the bioanalyser instrument revealed the absence or presence at low quantities of ribosomal RNA (28S and 18S). This latter observation confirmed the absence of somatic cells contamination in the sperm samples.

Detection of global mRNA using DNA microarray

The Affymetrix GeneChip Bovine Genome Array contains 24,129 probe sets representing over 23,998 bovine transcripts, including assemblies from approximately 19,000 UniGene Clusters. Averages of 800 and 1400 (equal to 4% and 6% of the total) probe sets were detected on each individual replicate, but only 340 (1%) and 921 (4%) were present in all three replicates of each high- and low-fertility bulls, respectively. No clusters were detected among the samples (**Figure 2**). Transcripts of casein beta 2 (*CSN2*), keratin 8 (*KRT8*), *PRM1* and amelogenin X or Y (*AMELX* or *AMELY*) genes were, in comparison to other fully annotated probe sets, the most abundant transcripts in the sperm population irrespective of the bull fertility status. These transcripts belonged to a wide range of cellular biological processes such as transportation, apoptosis and signal transduction, as well as other unknown categories (**Table 2**). Interestingly, these highly detected mRNA were not among the strongest significantly differentially detected (fold change ≥2.0, $P > 0.01$). Data analysis revealed a total of 415 differentially detected transcripts between both bulls, of which 211 were detected ≥2.0 fold higher in the high-fertility bull spermatozoa and 204 transcripts were detected as ≥2.0 fold higher in the low-fertility bull spermatozoa

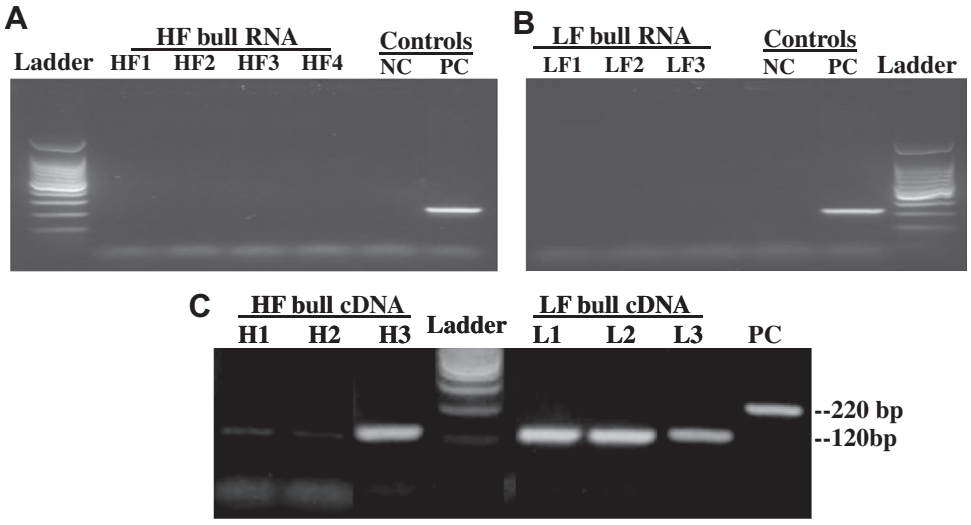

Figure 1 Representative gel electrophoreses following polymerase chain reaction (PCR) using total sperm RNA, complementary DNA and genomic DNA. PCR amplicons of protamine-1 gene in high-fertility (HF) and low-fertility (LF) bull spermatozoa. The use of total RNA as a template resulted in absence of amplifications in (**A**) HF and (**B**) LF bulls, while in (**C**) the cDNA and sperm DNA (for positive control) templates generated amplicon sizes of 120 bp and 220 bp, respectively. NC = negative control (water); PC = positive control (total rat embryo RNA).

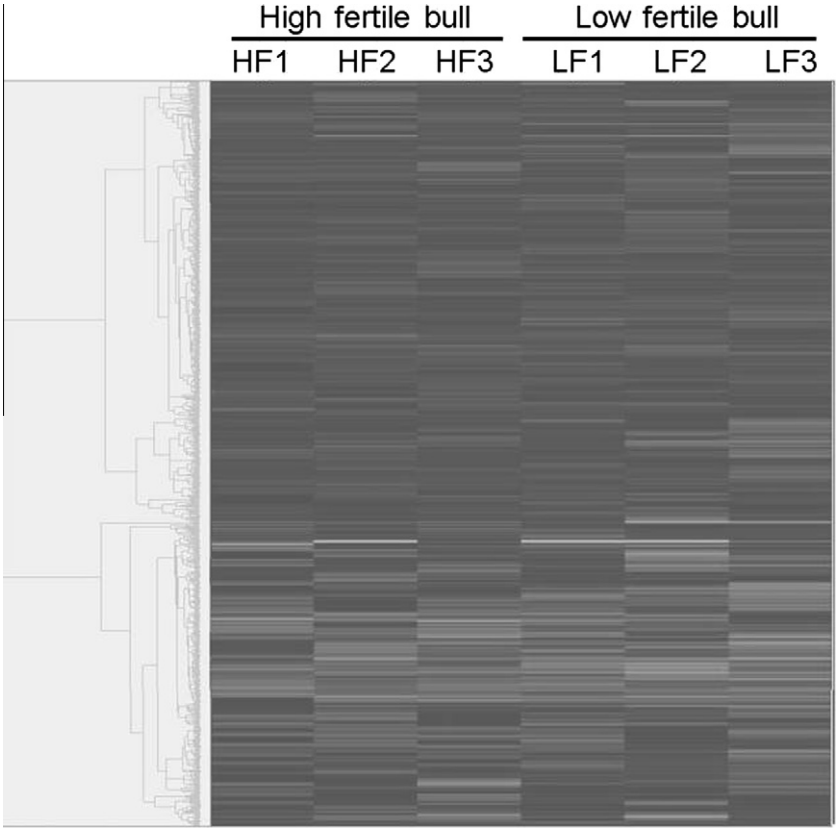

Figure 2 Hierarchical clustering of genes. Cluster analysis of hybridizations and genes using GeneTraffic UNO. Three replicates of microarray analysis performed with sperm transcripts from one high-fertility bull (HF1, HF2 and HF3) and one low-fertility bull (LF1, LF2 and LF3) are presented as columns. Each individual horizontal line represents a probe set with a high (green) or a low (red) signal above than average. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2 Genes with the highest mRNA concentration in both bulls.

Biological process and gene	*Accession number*
Transport	
Casein beta (*CSN2*)	NM_181008
Neutrophil cytosolic factor 4, 40 kDa	NM_001045983
Organic anion transporting polypeptide 1a2	NM_174654
Der1-like domain family, member 1	NM_205789
FXYD domain containing ion transport regulator 7	NM_001037627
Chromatin condensation during spermatogenesis	
Protamine 1 (*PRM1*)	NM_174156
Apoptosis	
Fas-activated serine/threonine kinase	NM_001035077
FAST kinase domains 3	NM_001024528
Apoptosis inhibitor 5	XM_583981
Signal transduction	
ADP-ribosylation factor-like 2	NM_001038079
Amelogenin X/Y (*AMELX* and *AMELY*)	NM_001014984 and NM_174240
rRNA modification	
CGI-41 protein	NM_001015641
Regulation of cell shape	
Myosin, heavy polypeptide 10, non-muscle	NM_174834
Keratine 8 (*KRT8*)	NM_001033610
Protein glycosylation	
Sialyltransferase ST3Gal-III	NM_001002882
Nucleotide catabolism	
5′ nucleotidase, ecto	NM_174129
mRNA processing	
Small nuclear ribonucleoprotein polypeptide G	NM_001040543
Cell cycle	
Cell division cycle 10	NM_001001168
Unknown	
Coronin, actin binding protein, 1B	NM_001024510
Replication protein A2, 32 kDa	NM_001045984
HMT1 hnRNP methyltransferase-like 1	NM_001024495
Ribosomal protein S8	NM_001025317

(**Table 3**). Transcripts such as HSP70, calcium channel voltage-dependent beta 2 subunit, citrate synthase, claudin 1 and solute carrier family 2 were among the most abundant mRNA population in high-fertility bull spermatozoa, while those of zinc finger MYND domain containing 11, galactokinase 1, mitochondrial fission regulator 1 and platelet-activating factor acetylhydrolase alpha subunit (45 kDa) characterized the low-fertility bull spermatozoa (**Tables 4 and 5**).

Functional classification of genes

Full information on differentially expressed genes was obtained from NetAffx Analysis Centre (Bovine GeneChip annotation from July 2007) and GOAnna tool (GOSlimViewer; AgBase resource, Mississippi State University) and classified as biological process (BP), molecular function (MF) and cellular component (CC) (**Figure 3**). Spermatozoa from the low-fertility bull appeared deficient in transcripts associated with extracellular space (CC), transporter (MF), transcription factor (MF) and translation machinery (BP), while spermatozoa from the high-fertility bull lacked cell cycle transcripts (BP). This classification also revealed that high-fertility bull-derived spermatozoa were characterized by high concentrations of cellular transportation (BP), receptor activity (MF) and extracellular space transcripts (CC).

Confirmation of microarray data using quantitative real-time PCR

Of the 13 genes selected for their relevance in spermatogenesis, fertilization or their expression pattern in the microarray experiment (**Table 1**), only four, *PRM1*, *CSN2*, *CD36* molecule and centromere protein A (*CENPA*), were successfully analysed by qPCR and normalized to *PRM1* because of the inability to detect traditional housekeeping genes (e.g. GAPDH and β-actin); therefore, *PRM1* data was not normalized (**Figure 4**). The expression levels of *PRM1* and *CSN2* were similar between high- and low-fertility bull-derived spermatozoa, whatever the technique used. The low concentration of *CD36* molecule in low-fertility

Table 3 Classification of differentially expressed probe sets between both of the bulls according to NetAffx data (July 2007 annotation).

Probe set category	*Higher in high-fertility bull*	*Higher in low-fertility bull*
Known bovine genes	70	56
Unknown transcripts	22	16
Similar to known genes	80	81
Hypothetical	16	19
Transcribed locus strongly similar to...	0	0
Transcribed locus moderately similar to...	1	0
Transcribed locus weakly similar to...	0	0
Transcribed locus	22	32
Total	211	204

Table 4 Top 10 transcripts with highest mRNA concentration in the high-fertility bull compared with the low-fertility bull, sorted by fold change.

Gene title	*Accession number*	*Mean signal in high-fertility bull*	*Mean signal in low-fertility bull*	*Fold change*	*P-value*
Heat shock 70 kDa protein 3	NM_174344	1737.50	94.93	18.30	0.002991
Solute carrier family 2, (facilitated glucose transporter) member 8	NM_201528	1482.53	139.37	10.64	0.001884
Calcium channel, voltage-dependent, beta 2 subunit	NM_175789	1390.63	172.20	8.08	0.004476
F-box and leucine-rich repeat protein 6	XM_867842	1833.83	231.77	7.91	0.001577
Claudin 1	NM_001001854	1199.70	158.83	7.55	0.004063
Citrate synthase	NM_001044721	1316.73	235.23	5.60	0.008602
Erythrocyte membrane protein band 4.9 (dematin)	NM_001034431	1646.33	332.40	4.95	0.008628
Pleckstrin homology domain containing, family Q member 1	NM_001076283	1258.07	272.97	4.61	0.009153
Biphenyl hydrolase-like (serine hydrolase; breast epithelial mucin-associated antigen)	NM_001045918	1362.10	301.87	4.51	0.008115
UDP-*N*-acetyl-alpha-d-galactosamine	NM_001017943	1195.20	296.17	4.04	0.009915

bulls was confirmed ($P < 0.05$), but the *CENPA* microarray results could not be reproduced.

Discussion

Spermatozoa are highly specialized cells produced through a long and complex process, ending with dramatic morphological remodelling (i.e. extensive chromatin condensation, reduction in nuclear and cytoplasmic volume, formation of an acrosome system and tail production). These modifications shape a highly motile spermatozoon able to migrate through the female reproductive tract to fertilize and deliver the paternal contributions to the egg (Parks et al., 2003; Sassone-Corsi, 2002). Recent reports have revealed that the spermatozoon also delivers a subset or the totality of its mRNA to the oocyte (Eddy, 2002; Gur and Breitbart, 2006; Miller and Ostermeier, 2006a,b; Ostermeier et al., 2002, 2004). The quality and quantity of these mRNA, as well as their potential roles in male fertility, are still largely unknown. At present, few studies have been conducted to determine the global profile of bull sperm mRNA population using techniques such as suppression subtractive hybridization, microarrays or human microarrays (Bissonnette et al., 2009; Gilbert et al., 2007; Lalancette et al., 2008). As far as is known, the present study is the first to use the high throughput bovine microarray (Affymetrix) which is widely exploited for analyses of other bovine embryonic tissues. The present study was limited to the evaluation of sperm-specific gene transcripts and the highly differentially expressed ones in extreme fertility bulls, as a means of finding potential mRNA that may help to design molecular bio-

markers of bull fertility and transcripts contributing to early embryonic development in bovine and in other mammals.

Determination of bull fertility

The outcome of this research approach resides on an accurate selection of extreme fertility bulls determined with the powerful AltaAdvantage progeny test. This programme includes parameters such as DNA verification of paternity and pregnancy diagnoses by palpation which is used in place of the non-return rates at 60—90 days after breeding, as a unique parameter generally used to determine bull fertility (Lalancette et al., 2008; Oliva, 2006). The AltaAdvantage progeny allows the retrieval of well-characterized low-

Table 5 Top 10 most abundant transcripts in spermatozoa of the high-fertility bull compared with the low-fertility bull, sorted by fold change.

Gene title	*Accession number*	*Mean signal in high-fertility bull*	*Mean signal in low-fertility bull*	*Fold change*	*P-value*
Zinc finger. MYND domain containing 11	NM_001045940	157.70	1630.43	10.34	0.002301
Small ubiquitin-like modifier 1	NM_001035458	209.53	1580.97	7.55	0.005753
15 kDa selenoprotein	NM_001034759	275.60	1676.93	6.08	0.002191
Mitochondrial fission regulator 1	NM_001035412	197.57	1169.50	5.92	0.000498
Galactokinase 1	XM_869646	303.57	1422.30	4.69	0.004812
Inhibitor of DNA binding 2, dominant negative helix-loop-helix protein	NM_001034231	293.33	1223.40	4.17	0.000578
Zinc finger protein 54	BC108220	301.13	1141.07	3.79	0.007186
Mucosal address in cellular adhesion molecule-1	NM_001037821	648.57	2155.57	3.32	0.006596
Metal response element binding transcription factor 2	NM_001046556	336.03	1071.43	3.19	0.005715
Platelet-activating factor acetylhydrolase, isoform Ib, alpha subunit (45 kDa)	NM_174663	436.77	1336.70	3.06	0.000855

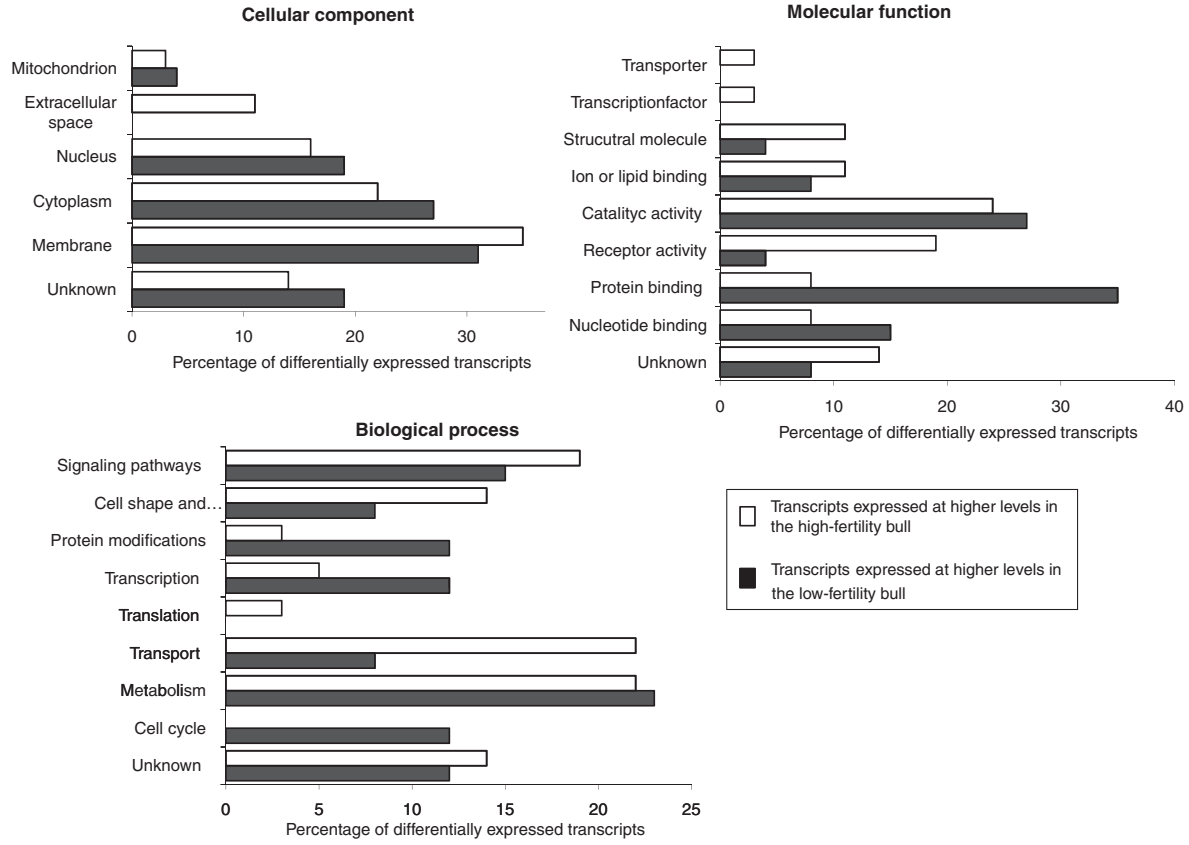

Figure 3 Distribution of the transcripts found in the high-fertility bull (open bars) and low-fertility bull (solid bars) according to the gene ontology terms cellular component, molecular function and biological process.

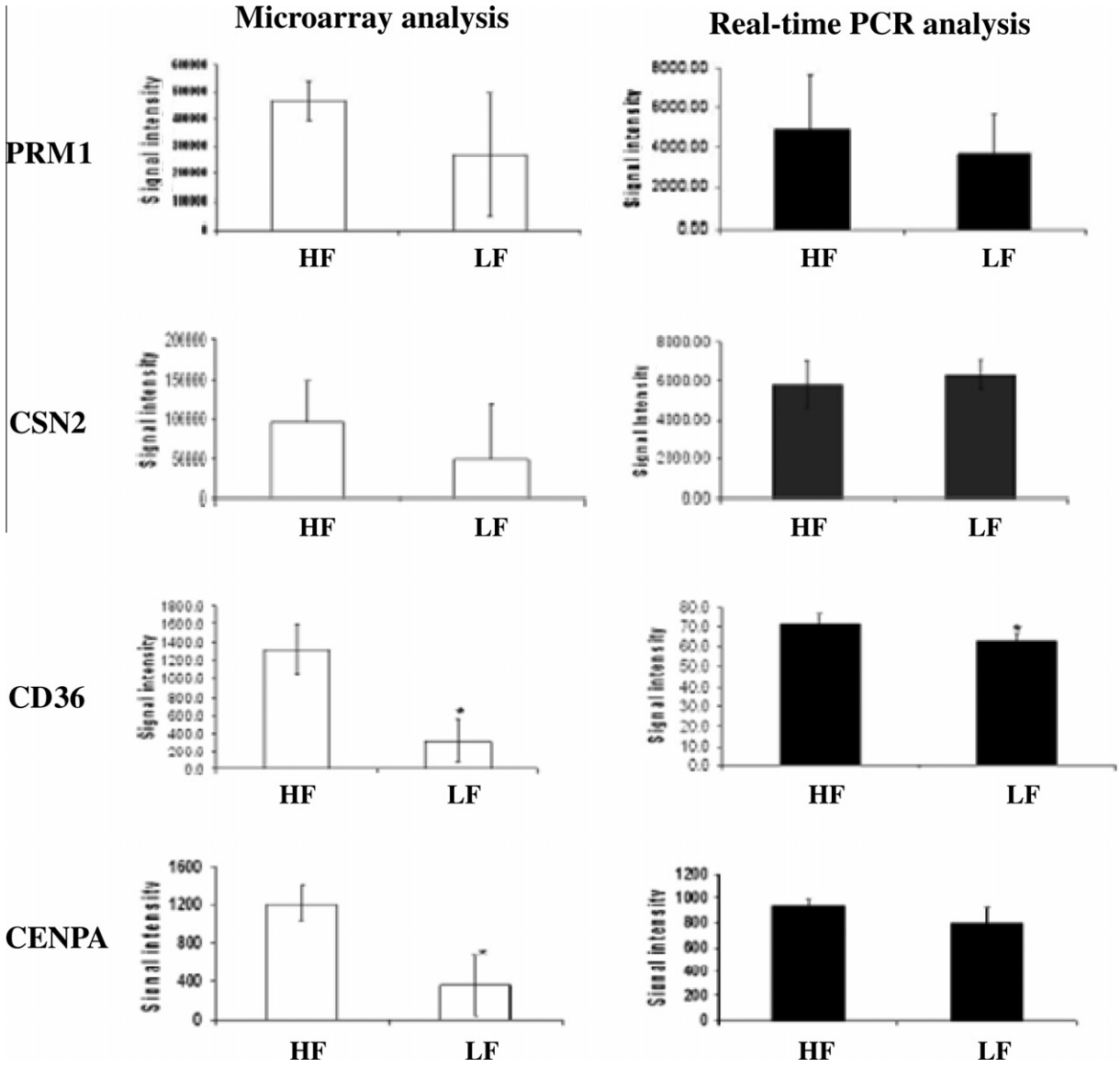

Figure 4 Quantitative results from microarray and real-time quantitative PCR analyses of four genes, showing gene expression analysis data for protamine 1 (*PRM1*), casein beta 2 (*CSN2*), thrombospondin receptor gene (*CD36*) and centromere protein A (*CENPA*). *Indicates significant difference between high-fertility (HF) and low-fertility (LF) bulls for the same gene ($P < 0.05$). *Indicates statistically significant differences with a *P*-value <0.05.

and high-fertility sires from bulls with appreciable average breeding number.

A suitable protocol for bull sperm RNA isolation

The great demand and cost of semen derived from high-fertility bulls strictly limit their availability for research needs. Therefore, studies on such materials required a redefinition of protocols for rare or costly materials. The determination of transcript abundance in mammalian spermatozoa is the most challenging aspect of gene expression analysis. Several techniques including TRIzol, guanidine isothiocyanate and commercial kits (i.e. RNeasy) have been used to improve both the purity and the recovery rate of total sperm RNA extraction (Gilbert et al., 2007; Lalancette et al., 2008; Ostermeier et al., 2002, 2004). In the present study, the recovery of high concentrations of pure total RNA (60—140 fg) per spermatozoon was achieved by introducing a grinding step and RNA carrier (glycogen) during the TRIzol incubation. The glycogen molecule binds to RNA as soon as they are available in solution and, therefore, enhances the yield of RNA recovery. This total amount of RNA is lower than that reported in human spermatozoon (more than 2830 fg RNA) (Roudebush et al., 2004; Wild and Roudebush, 2000), but greater than that (0.18 fg) reported by Gilbert et al. (2007) in bovine spermatozoon. Sperm RNA profiles in both low- and high-fertility bulls revealed the absence or low amounts of ribosomal RNA (28S and 18S) which is consistent with previous reports in human and bovine (Gilbert et al., 2007; Ostermeier et al., 2002).

These concentrations of ribosomal RNA (i) correlate with the idea of low translational activity in mature spermatozoa (Boerke et al., 2007) and (ii) remain the principal signature of sperm RNA profile compared with somatic cells (which contain both types of rRNA). All together, these results indicate an optimal protocol for quantitative and qualitative isolation of total RNA (average of 1.4 ± 0.4 μg) from a pool of spermatozoa equivalent to the content of a single commercialized bovine semen straw.

Microarray study

Microarray analyses were performed on two bulls with extreme fertility records (19.7% difference) (Feugang et al., 2009; Peddinti et al., 2008). The proportions of probe sets that were successfully detected or called 'present' were low in both bulls' total RNA samples (less than 6%). This poor detection could be a reflection of low amounts of mRNA in spermatozoa (121 ± 1.1 fg of mRNA/spermatozoon from the low-fertility bull and 146 ± 22 fg mRNA/spermatozoon from the high-fertility bull). This could also be explained by the absence of spermatogenesis genes on commercial microarrays, which are not enriched with specific testis/sperm transcripts. Therefore the analysis of sperm or testis tissue using commercial microarrays may provide a biased picture of the transcriptome.

The spiked controls, added to the mRNA sample before amplification and in-vitro transcription, produced hybridization signals several times greater than has been observed when somatic cell mRNA has been used for hybridizations. This could indicate a competition between the spiked mRNA controls and the scarce sperm mRNA for enzymes and nucleotides during the amplification and in-vitro transcription. Affymetrix Bovine GeneChips are reliable for detection of highly and marginally represented transcripts and have been successfully used to analyse gene expression of a variety of cells and tissues, including embryos and oocytes (Hamatani et al., 2006; Hashizume et al., 2007; Mamo et al., 2006; Misirlioglu et al., 2006; Patel et al., 2007; Ushizawa et al., 2007).

Nonetheless, the detection of several transcripts at the greatest quantities in both samples could be indicative of cell-specificity. However, five of them are involved in protein or ion transport: *CSN2*, neutrophil cytosolic factor 4, organic anion transporting polypeptide 1a2, Der1-like domain member 1 and FXYD domain containing ion transport regulator 7. Interestingly, *CSN2* was the highest expressed gene in both bulls. The high concentrations of such mRNA indicate the importance of protein and ion transport for a successful fertilization.

The presence of PRM1 mRNA amongst the highest expressed genes was not a surprise given its critical roles in packaging and stabilizing the sperm DNA (D'Occhio et al., 2007; Galeraud-Denis et al., 2007; O'Brien and Zini, 2005). These high concentrations could be explained by either remnant protamine messenger RNA from spermatogenesis. Lambard et al. (2004) found a significantly higher amount of *PRM1* mRNA in the low-motility fraction of the spermatozoa compared with the high-motility fraction. The authors attribute this finding to the presence of immature spermatozoa with residual cytoplasm in the low-motility fraction. An additional explanation for the presence of the *PRM1* transcript is a short transitory translational activity in spermatozoa to allow replacement of protamine presenting defects during the spermatozoa transit into the female genital tract. The abundance of *PRM1* could be influenced by the fertility status. The concentrations of *PRM1* transcript and protein were lower in testicular biopsies of infertile men (Steger et al., 2003).

Moreover, the detection of transcripts of other genes such as *KRT8* and *AMELX* and *AMELY* are somewhat surprising. However, some members of the superfamily of keratins are involved in the shape of the sperm structure (Hinsch et al., 2003; Kierszenbaum, 2002; Kierszenbaum et al., 2007), while amelogenin seems to play a role in male fertility and genetic divergence in X—Y pairing (Colley et al., 2008; Matsuda et al., 1991).

On the other hand, more than 200 transcripts among the higher represented transcripts in both bulls were differentially detected in low-fertility (204) and high-fertility (211) bulls. Interestingly, only a few of these transcripts (7.8% and 10.0%) were classified as unknown, leaving room for investigation on the relevance of remnant gene transcripts stored in mature spermatozoa (Table 3).

Indeed, this study found that a great proportion of gene products of high fertile bull-derived sperm transcripts have extracellular and membrane localizations that have crucial roles at the moment of fertilization. These transcripts mostly belong to the cellular (ion/lipid) transportation, structural molecules, cell shape and development and receptor activity. The sperm membrane is rich in receptors that ensure its motility during the female tract transit and spermatozoon—egg interaction (Baldi et al., 2000; Ensslin et al., 2007; Goncalves et al., 2008; Shur et al., 2006; Wassarman, 2002). Despite the importance of ion/lipid movements across the gamete membrane at the moment of fertilization, no gene products involved in the transportation process were detected in the low-fertility bull (Baldi et al., 2002; Florman et al., 1998). Moreover, the gene products corresponding to transcription factors or associated with the translational activity were found only in the high-fertility bull. Several transcription factors such as zinc finger protein have already been reported in spermatozoa and many of them may play a role during embryonic and fetal development through new transcriptional/translational activities mediated before, during or after fertilization (Boerke et al., 2007; Gur and Breitbart, 2008; Miller, 2007; Thomas et al., 2007). Although the present study lacks replicates for microarray analysis, it is believed that the results obtained are still strong given the powerful selection of the fertility of the candidate bulls for the study. The current results are somewhat consistent with the literature and suggest that a high transportation activity across the membrane (via its receptors and transporters) as well as a high transcriptional activity in spermatozoa may be indicators of high fertile status of the male.

Validation of microarray differential gene expression

Because of the low RNA material required for qPCR, the sample sizes were increased (from one to five bulls) in each high-

or low-fertility average-breeding groups of more than 553 ± 199 in both groups and the fertility difference between both groups was 13.2% on average. For microarray validation, four genes from microarray data were selected based on criteria such as their abundance, differential detection and biological relevance in sperm function (Table 1).

The expression of four genes was confirmed. The expression patterns of *PRM1* and *CSN2* after qPCR were similar to the microarray data. The results with *PRM1* were interesting with a tendency of decreased expression in low-fertility bulls, which is in line with recent studies in humans suggesting different amounts of *PRM1* between subfertility and fertility males (Aleem et al., 2008; Aoki et al., 2006a; Bissonnette et al., 2009; Lambard et al. (2004); Steger et al., 2008). This difference may reflect the amount of *PRM1* protein packaging the sperm DNA and, consequently, the level of sperm DNA protection. Indeed, a low protection of the paternal genome is suggested to cause delayed effects on embryonic and fetal development (Aoki et al., 2006a,b). Therefore, and in agreement with the current study's result above, spermatozoa derived from low-fertility or subfertility males with defects in the *PRM1* protein will be less apt for replacement given its lack of translational machinery.

Moreover, the decrease of both *CD36* molecules and *CENPA* in the low-fertility bull was replicable for *CD36* only. None of the commonly used housekeeping genes (i.e. β-actin, GAPDH) were detected in the samples analysed. Similar observations have been reported by Lalancette et al. (2008) who suggested the need to identify new 'housekeeping-like' genes, if there could be any, in spermatozoa.

In conclusion, a commercial bovine microarray was used to profile the global transcripts of spermatozoa originated from well-characterized low- and high-fertility bulls, followed by validation using qPCR. The transcripts represented were identified at (i) highest concentrations in spermatozoa, irrespective of their origin, and (ii) highly differential concentrations between high- and low-fertility bulls. These transcripts are associated with different cellular functions and biological processes. This study found that either transcriptional and translational machineries or the activity of membrane receptors and transporters might characterize high-fertility spermatozoa. In addition to contributing to the discovery of new molecular biomarkers of bull fertility, these results pave the road for making further insights into the roles of paternal transcripts during embryo development and beyond.

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