RNA EXPRESSION PROFILING OF OVARIAN FOLLICLE DEVELOPMENT IN SWINE LINES SELECTED FOR INCREASED OVULATION RATE

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INTRODUCTION

Improvement of reproductive traits is of major importance to the pork industry. Relatively low responses have been obtained when selection experiments were based on direct selection for litter size, with realized heritabilities ranging from 10 to 15 % (Bolet et al., 1989 ; Lamberson et al., 1991). However, the University of Nebraska (UNL) has developed unique swine lines with superior reproductive qualities, by selecting animals derived from a Landrace x Large White base population, with basis on a selection index for components of litter size (i.e. ovulation rate, embryonic survival, etc ; Johnson et al., 1999). The dissection of the genetic/biological basis controlling such traits can potentially lead to new approaches for improving the reproductive capability of pigs. Even though the UNL selection lines show a large phenotypic difference in ovulation rate (the Index line ovulates 7.4 more ova then the control line), only one QTL with significant effect on ovulation rate was detected in a study performed with an F2 population derived from these animals (Cassady et al., 2001). A study to evaluate the follicular developmental dynamics of the UNL selection lines indicated that the ovulatory advantage of the Index line is the result of changes in the dynamics of follicle maturation during the follicular phase of the estrous cycle (Yen, 1999). We designed a strategy to utilize cDNA microarray technology to identify genes that are differentially expressed between the UNL lines, in ovarian/follicular tissue collected during the follicular phase of the estrous cycle.

MATERIALS AND METHODS

Collection of tissues and RNA extraction. Gilts from the UNL swine lines selected on an index of high ovulation rate and embryonic survival to day 50 of gestation (I), and the respective randomly selected control (C) line (Johnson et al., 1999), were injected with PGF2α on day 13 of the estrus cycle (d0 = 1st day of estrus) to induce luteal regression. Ovaries were harvested by ovariotomy two to six days after PGF2α injection (d2, d3, d4, d5 and d6), weighted and numbers of corpora albicantia (CA) were counted to determine ovulation rate in the previous cycle. One ovary was randomly selected from each animal, snap-frozen in liquid Nitrogen and stored whole at -80°C. From the remaining ovary, all follicles ≥ 2 mm were dissected, measured, snap-frozen in individual tubes and stored at -80°C. Total RNA was isolated from ovaries/follicles with Trizol (GibcoBRL). Follicles were pooled within animals during the extraction process based on diameter : small (SF, 2-2.9 mm), medium (MF1, 3-4.9 mm; MF2, 5-6.9 mm) and large (LF, ≥ 7 mm). RNA samples were quantified by fluorometry.
Source of cDNA clones and preparation of probes for printing microarrays. A total of 3,636 unique cDNA clones derived from a normalized ovarian follicle cDNA library (see Pomp et al., 2001) were used. In addition, 816 clones were used in duplicate. Considering positive and negative controls, and a few follicle cDNA clones from other sources, a total of 4,608 probes were prepared for printing. cDNA inserts were PCR-amplified with primers located on the cloning vector in 100 µl reactions. A small aliquot of each PCR reaction was run in agarose gels for quality control. Inserts which failed to amplify (< 10 %) were not replaced. PCR products were precipitated with a solution of ethanol/NaAcetate at -20°C, centrifuged, rinsed with 70 % ethanol, dried at room temperature and resuspended in 30 µl of printing buffer (3xSSC and 0.1 % N-lauroylsarcosine).

Preparation of microarrays. Microarrays were printed with a GMS417™ Arrayer on Poly-L-lysine coated slides prepared with standard protocols. A total of 4,608 probes were printed in duplicate on each slide. Printed slides were exposed to UV light for cross-linking, blocked in a Succinic Anhydride/Sodium Borate solution and excess DNA probe material was washed off in a water bath at 95°C. Blocked slides were dehydrated in a bath of 100 % ethanol, centrifuged and stored in conventional slide boxes in a dark, cool, and dry location until use.

RNA pooling and generation of cDNAs. Pools of ovary RNA were made by combining samples from different animals within selection line and treatment day. Similarly, pools of RNA from follicles of the same size class were made. Polyadenylated RNA complementary to probes on the microarray derived from yeast untranslated genomic sequences (Incyte Yeast Controls) were spiked into each pool of RNA as positive controls. The Submicro™ expression array detection kit (Genisphere) was used for generating cDNAs from each RNA pool.

Experimental design, microarray hybridizations and scanning. Equimolar amounts of cDNA samples from the I and C Nebraska Selection Lines, each labeled with Cy3 or Cy5, from ovaries collected on the same treatment day (e.g. d2), were co-hybridized on the same microarray slide. This process was repeated with samples from each treatment group. Each hybridization was replicated a total of three times, and three additional hybridizations were performed with a reversal of the Cy dyes. Thus, a total of six hybridizations were performed with each pair of samples. Hybridizations were conducted overnight at 45°C in humidified chambers, according to the instructions provided by the kit manufacturer. Following the appropriate post-hybridization washes in SSC buffer, microarray slides were centrifuged for drying and scanned in a ScanArray5000 (GSI Lumonics) Scanner with the following parameters: Cy5 (Laser Power- 85 %, PMT- 85 %), Cy3 (Laser Power- 95 %, PMT- 85 %).

Microarray image analysis, data extraction and statistical analysis. Microarray images were analyzed with the software Imagene 4.2™ (Biodiscovery). Statistical analysis of the data was performed with a mixed model approach proposed by Wolfinger et al. (2001) that is based on two interconnected analysis of variance (ANOVA) models. The first model allows for the normalization of systematic effects that impact all the probes in a particular microarray. The residuals from the first model are used as input in a second model to allow the estimation of sample effect and significance levels for each individual probe on the array. Briefly, let Yijkl be the base-2 logarithm of the background-subtracted measurement (mean pixel value) from array
i (i=1,…,6), swine line j (j=1,2), dye k (k=1,2) and probe l (l=1,…,4608) for each treatment group. Then,

\[ Y_{ijkl} = \mu + A_j + L_k + (AL)_{ij} + \varepsilon_{ijkl} \]

where: \( \mu \) represents an overall mean value, \( A \) is the main effect for dyes, \( L \) is the main effect for swine line, \( AL \) is the interaction effect of line and arrays, and \( \varepsilon \) is stochastic error.

The residual of the first model \( (r_{ijkl}) \) is then fitted in a second model:

\[ r_{ijkl} = \mu_i + D_{ij} + L_{ij} + S_{ijkl} + \gamma_{ijkl} \]

Here, \( \mu, D, \) and \( L \) serve the same role as in the previous model, \( S \) is a main effect for spots, and \( \gamma \) is the stochastic error. To minimize the rate of false positives the Bonferroni method was used to calculate a cutoff value of significance to achieve an experiment-wide false positive rate of 0.05. The same approach was used to analyze data obtained from follicle samples.

**Tertiary analysis of the significant expression differences.** Expression profiling experiments generate large amounts of data with high levels of complex biological/statistical complexity. Several approaches to identify patterns of gene expression from microarray data have been proposed. We utilized the program Cluster (Eisen et al., 1998) to analyze our data with the Self-Organizing Maps (SOM) method proposed by Tamayo (1999).

**RESULTS AND DISCUSSION**

After an initial analysis of the gene expression data obtained from the hybridizations with whole ovary RNA samples, a total of 115 probes were found to be significantly differentially expressed between the UNL swine lines on one or more treatment days. Several quality checks were performed to test data quality and validate the statistical methods utilized for data analysis. None of the yeast probes used as positive controls showed significant differences between samples, as expected. Several clones that were duplicated within the set of 4,608 probes used for printing the microarray showed the same pattern of expression, corroborating the results and providing an internal check for our clone/probe tracking protocols. After removal of duplicated probes from the dataset, a total of 92 clones showed significant expression differences between swine lines in one or more treatment days. No expression differences higher than 4-fold were found. After analyzing the data with the SOM approach, several groups of probes showing similar patterns of expression were identified. Figures 1a and 1b are examples of these observations. More complex analysis, including putative biological function of the genes found to be differentially expressed based on comparative sequence analysis with mouse/human functional data, will be performed to provide the foundation for future hypothesis-based studies of mechanisms which may be controlling ovulation rate in pigs. Interpretations of our results should consider that differences in RNA expression are not necessarily correlated with differences in protein expression and function. Further studies to corroborate our results at the RNA, protein and phenotypic levels will be necessary. Data from the hybridizations with follicle RNA are currently being processed and analyzed.
Figures 1a, 1b. Examples of groups of genes that show similar patterns of differential RNA expression between the UNL swine lines during a period of 5 days of the follicular phase of the estrous cycle

CONCLUSION

Our approach was successful in identifying genes that are differentially expressed in the ovarian tissue of swine lines selected for high ovulation rate. The strategy we applied produced results that will be useful for uncovering biological mechanisms that orchestrate ovulatory follicle maturation in pigs and therefore, may lead to innovative methodologies to improve the reproductive performance of commercial pigs. This was a novel approach to study the biological control of a complex quantitative trait and will be an important tool for understanding the nature of selection response.

REFERENCES