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G. R. Bertani
University of Nebraska-Lincoln

C. D. Gladney
University of Nebraska-Lincoln

R. K. Johnson
University of Nebraska-Lincoln, rjohnson5@unl.edu

Daniel Pomp
University of Nebraska-Lincoln, dpomp1@unl.edu

Evaluation of gene expression in pigs selected for enhanced reproduction using differential display PCR: II. Anterior pituitary[1]

G. R. Bertani[2], C. D. Gladney, R. K. Johnson, and D. Pomp[3]

Department of Animal Science, University of Nebraska, Lincoln 68583-0908

ABSTRACT: The objective of this study was to identify differentially expressed genes in the anterior pituitary (AP) of sows selected for enhanced reproductive phenotypes. Selection in the Index (I) line was based on an index of ovulation rate and embryo survival, whereas random selection was used in the Control (C) line. Average numbers of fully formed piglets at birth were 12.5 ± 1.5 and 9.9 ± 2.0 for Line I and C sows used in this study, respectively. In order to induce luteolysis and synchronize follicle development, sows were injected (i.m.) with 2 mL of prostaglandin $F_{2\alpha}$ analog between d 12 and 14 of the estrous cycle. Tissue was harvested 2 d (d2) or 4 d (d4) after injection, resulting in four experimental groups: Cd2 (n = 6), Cd4 (n = 4), Id2 (n = 6), and Id4 (n = 7). Differential display PCR (ddPCR) was used to search for transcriptional changes between selection lines in the AP, using samples within line but pooled across days. Northern hybridization was used to confirm ddPCR results. For ddPCR, two pools were used from each line (C and I). Three genes were confirmed to be differentially expressed between Lines I and C: *G-beta like protein*, *ferritin heavy-chain*, and *follicle stimulating hormone beta subunit*, whereas many other expressed sequence tags were observed to be differentially expressed but still require confirmation. Our findings indicate that long-term selection to increase ovulation rate and decrease embryo mortality has altered transcriptional patterns in the anterior pituitary, most likely as correlated responses.

Key Words: Gene Expression, Pig, Pituitary, Reproduction, Selection

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Introduction

Differential display PCR (**ddPCR**) was first described (Liang and Pardee, 1992) as a way of conducting gene expression studies by comparing eukaryotic messenger RNA (mRNA) levels from different sources. This technique enables the large-scale screening of the transcriptome without prior knowledge of existing information regarding sequence and identification of transcripts.

Gene expression analysis is a functional genomics approach that is useful for discovering novel expressed genes and for comparing mRNA levels of transcripts regulating fertility. The ddPCR method has been used for transcriptional analysis to understand reproductive processes in mouse (Lee et al., 2001; Minami et al., 2001), cow (Robert et al., 2001), human (Xu et al., 1999), Rhesus monkey (Ace and Okulicz, 1999), and pig (Li et al., 1996). Gene expression analysis is also useful for understanding the biological basis that underlies polygenic traits and the response to long-term genetic selection. Such traits as heat loss in mice (Allan et al., 2000) and reproduction in pigs (Gladney et al., 2004) have been investigated using ddPCR analysis in selection lines. These studies enabled the identification of a variety of differentially expressed genes, demonstrating that selection for polygenic traits can cause detectable changes in the transcriptome at various temporal and spatial coordinates related to the physiology of the trait.

The anterior pituitary is an important reproductive gland that intermediates communication between hypothalamus and ovaries. We investigated the effect of selection for enhanced reproduction on gene expression in the anterior pituitary of pigs. Differential display PCR was used to search for differences in transcription levels between lines, under the hypothesis that gene expression in the anterior pituitary gland of females has changed during the period of ovarian

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[3]Correspondence: A218 Animal Science (phone: 402-472-6416; fax: 402-472-6362; e-mail: dpomp@unl.edu).

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follicular development, as either a direct or correlated response to selection. Northern hybridization was used to confirm ddPCR results.

Materials and Methods

Resource Population, Animals, and Treatments

A general description of selection lines and sows used in this experiment is described in the companion article (Gladney et al., 2004) for a concurrent study evaluating the ovarian transcriptome. Briefly, 23 second-parity sows from the 16th generation of selection in the Index (**Line I**; n = 13) and Control (**Line C**; n = 10) lines were used. Sows were checked daily after weaning for estrus, and the average weaning-to-estrus interval was 6.4 ± 0.7 d for Line C sows and 8.9 ± 4.4 d for Line I sows. All sows from both lines were injected (i.m.) with 2 mL of the prostaglandin $F_{2\alpha}$ analog Lutalyse (Upjohn, Kalamazoo, MI) between d 12 and 14 after detection of estrus in order to induce luteolysis and synchronize follicle development. Anterior pituitaries from sows were harvested at d 2 or d 4 after Lutalyse injection, resulting in four treatment groups: Line C, **d2** (n = 6); Line C, **d4** (n = 4); Line I, **d2** (n = 6); and Line I, **d4** (n = 7).

Tissue Collection and RNA Extraction

Sows were weighed before harvest. Anterior pituitary lobes were collected, within 20 min after stunning of the animal, by opening the cranial cavity, and the lobes were snap-frozen in a cryovial using liquid nitrogen. Tissues were stored at –80°C. The anterior pituitary was weighed before extraction of total RNA using Trizol LS (Gibco Life Technologies, Grand Island, NY). Poly(A) RNA was purified using the Qiagen (Valenica, CA) Oligotex mRNA midi kit. Total RNA and poly(A) RNA were quantified using the TD-700 fluorometer (Turner Designs, Sunnyvale, CA).

Differential Display PCR (ddPCR)

Differential display PCR (ddPCR) was used to search for differences in AP gene expression between lines, and Northern hybridization was used to validate selected ddPCR results. Two pools of poly(A) RNA (2.2 ng/μL) were used from each line, each having samples from one d2 sow and from one d4 sow. Pools were designated as CA and CB (Line C) and IA and IB (Line I). Although evaluation of line × day interaction would have been appealing, insufficient sample was available to afford adequate power for such an analysis.

Methods for ddPCR are described in the companion article (Gladney et al., 2004). Briefly, first strand complementary DNA (cDNA) synthesis was performed using 2.2 ng of poly(A) and 4 pmol anchor primer to a final volume of 10 μL. Template cDNA representing the four experimental groups were used for fluorescent ddPCR employing 10 anchor primers combined with 20 arbitrary primers, resulting in a total of 200 different primer combinations and PCR reactions per sample pool.

Evaluation of ddPCR Gels

Products of ddPCR (4 μL) were mixed with 1.5 μL of loading buffer, denatured at 95°C for 5 min, loaded in a 5.8% polyacrylamide denaturing gel, and electrophoresed at 3,000 V (100 W) and at 50°C for 2.5 to 5 h. Gels were dried and washed before scanning for fluorescence. Images were evaluated using Adobe (San Jose, CA) Photoshop 4.0 software. Analysis of banding patterns was based on subjective visual inspection, taking into account background intensities, consistency within pools of each line, and intensity of the differences between lines. A scoring system of 1 to 6 was used to rank and prioritize bands for further investigations, with score 1 representing marginal differences between selection lines and increasing values representing increasing robustness and consistency of differences. Selected bands were excised and placed in 50 μL of TE buffer (10 m*M* Tris-Cl, pH 7.4), incubated at 37°C for 30 min and stored at –40°C.

Reamplification, Cloning, and Sequencing

Bands with scores of 5 and 6 (n = 168) and a few bands with similar expression between lines (n = 12) were sequenced (n = 179) as previously described (Gladney et al., 2004). Sequences were managed using BioEdit version 5.0.0 (jwbrown.mbio.ncsu.edu/BioEdit/bioedit.html). Expressed sequence tags (**EST**) were searched for similarity against the nr databases using the BLAST nr database (www.ncbi.nlm.nih.gov/BLAST/). Threshold of the expected value used for significant similarity was less than 0.001. Sequences were characterized regarding the presence of repetitive elements using RepeatMasker (repeatmasker.genome.washington.edu) and were submitted to GenBank (accession numbers BE231450 to BE231460, BE231489 to BE231492, BE241013 to BE241061, and BE344516 to BE344576). Sequences in GenBank were incorporated by TIGR (the Institute for Genomic Research) to help construct the Porcine Gene Index (www.tigr.org/tdb/tgi/ssgi).

Northern Hybridization Validation of Gene Expression Differences

Three EST with putative gene expression differences between selection lines were chosen for further validation of ddPCR results using Northern hybridization. Total RNA from the same animals used in the ddPCR was used to form pools for Northern blots. One pool of total RNA (15 μg) was used for each line, representing equal amounts of RNA from two d2 sows and one d4 sow. The small number of EST confirmed with Northern analysis, and the use of RNA pooled across

days in the validation process, were the necessary result of the very limited amounts of pituitary samples available.

Asymmetric PCR was utilized for probe preparation with 10 μL of a specific PCR product, 0.012 μ*M* arbitrary primer, 0.12 μ*M* anchor primer, 5 U of *Taq* polymerase (Promega, Madison, WI), 1 × enzyme buffer, 1.5 m*M* $MgCl_2$, 0.2 m*M* each d(C,G,T)TP, 0.006 m*M* dATP, and 1 μCi of [α-^{32}P]dATP in a volume of 50 μL. The PCR mixture was incubated in a PTC-200 thermal cycler (MJ Research, Waltham, MA) as follows: initial denaturation for 2 min (94°C), followed by 41 cycles of 30 s at 92°C, 30 s at 60°C, and 2 min at 72°C, with a final extension period of 7 min at 72°C. Probes were denatured and hybridized with membranes overnight at 42°C. Membranes were washed and exposed to a PhosphoImager cassette (Molecular Dynamics, Sunnyvale, CA) and scanned in a PhosphoImager SF. The level of expression of the gene under investigation was quantified using the software ImageQuant version 3.3 (Molecular Dynamics). Membranes were then stripped by two successive washes at 95°C (20 min each) in a solution of 0.1 × SSC with 0.5% SDS, and probed with *G3PD* (*glyceraldehyde-3-phosphate dehydrogenase*) to normalize expression of the gene under investigation.

Statistical Analysis

Anterior pituitary weight was analyzed using the GLM procedure of SAS (SAS Inst. Inc., Cary, NC) with the model $Y_{ijk} = \mu + L_i + T_j + (LT)_{ij} + BW_{ijk} + e_{ijk}$, where Y_{ijk} is the response of sow k in line i injected with the prostaglandin analog on d j; μ is the constant; Li is the effect of the ith line; T_j is the effect of the jth day of slaughter after prostaglandin analog injection; LT_{ij} is the interaction between line and day; B is the regression on body weight, Wijk; and e_{ijk} is the residual.

Results

Anterior Pituitary Mass

Weights of anterior pituitaries were significantly different ($P < 0.01$) between Control (351.0 mg) and Index (453.8 mg) lines. No difference was observed between anterior pituitary weights at d 2 (407.4 mg) and d 4 (410.9 mg), and the line × day interaction was not significant. Body weights of sows were not different between lines or day of tissue collection (Gladney et al., 2004).

Differential Display and Sequence Characterization

A total of 372 bands was extracted from differential display PCR and assigned quality scores based on the prioritization system previously described. Bands scored as 5 (n = 100) and 6 (n = 68) amounted to 45.2% of the extracted bands. Examples of bands scored as 6 are in Figure 1. The majority of extracted bands originated from Index line samples (n = 246; 66.1%) based on stronger intensity. Some bands with similar expression between lines (n = 12) were also cloned and sequenced.

From the 179 bands processed, sequences were obtained for 162 clones. Sequence analysis indicated that 125 of the 162 (77.2%) anterior pituitary expressed genes characterized had distinct sequences, being either singletons or part of an EST cluster. Long interspersed elements, short interspersed elements, long terminal repeats, or DNA elements were found in 10.4% (13/125) of these, and simple repeats or sequences with low complexity were found in 4.8% (6/125). Table 1 displays information on 125 EST with their GenBank accession numbers and TIGR tentative cluster (**TC**) ID.

These EST represent clones with total or partial similarity to known genes (n = 73; 58.4%), to genes with an uncharacterized biological function (n = 43; 34.4%), or are novel (n = 9; 7.2%). Among the EST that matched sequences of known biological function, we observed EST related to mitochondrial function, signal transduction, splicing, endocrine activity, cell growth, and translation. Most (110 of 125) of the anterior pituitary EST are included in the TIGR Porcine Gene Index (March, 2003); 89 are clustered into 85 TCs, and 21 represent singletons.

Northern Hybridization Validation of Gene Expression Differences

Evaluation of gene expression using Northern hybridization was performed for the *G-beta like protein, ferritin heavy-chain*, and *follicle stimulating hormone beta subunit* genes. Differential expression of each gene was validated with line direction of expression changes in agreement with those observed in differential display. Results for two genes are presented (Figure 1). Signal observed for *G3PD* control probe showed that similar amounts of RNA were loaded for each line (data not shown). Selection for the reproduction Index decreased expression relative to Line C (*G-beta like protein* and *ferritin heavy-chain*) by 56 and 42%, respectively, and increased expression of *follicle stimulation hormone beta subunit* by 10%.

Discussion

We employed a thorough differential display evaluation to identify gene expression changes in the female anterior pituitary as a result of long-term selection for increased ovulation rate and embryo survival. Many differentially expressed transcripts were identified and characterized, and a sample of these was confirmed using Northern hybridization (*G-beta like protein*, *ferritin heavy-chain*, and *follicle stimulation hormone beta subunit*). In general, the range of mRNA expression ratios observed was small, as might be ex-

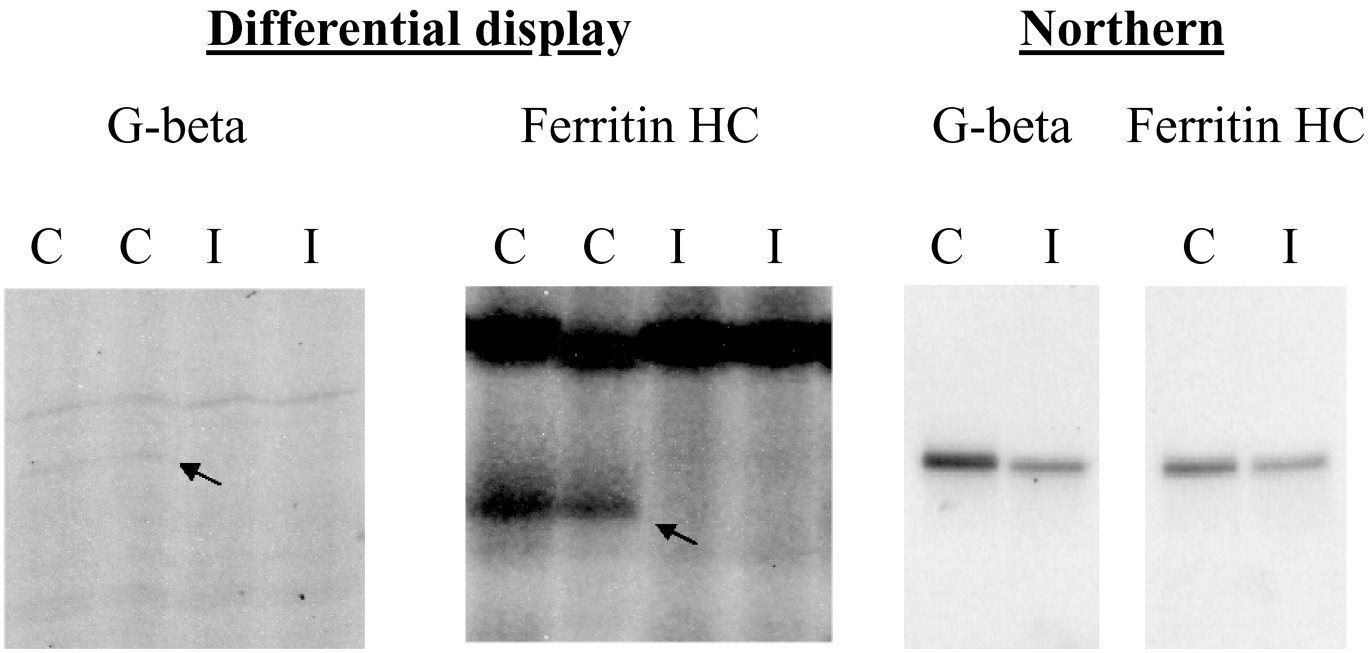

Figure 1. Differential display PCR and Northern hybridization results showing reduced expression (mRNA levels) of *G-beta like protein* and *Ferritin heavy chain* in anterior pituitaries of the Index selection line (I) relative to the Control line (C). Arrows point to the ddPCR bands that were excised from gels for sequence characterization. Two pools of samples were used to represent each line.

pected when evaluating selection response at the transcriptional level, and in agreement with a concurrent study using ovarian follicles (Gladney et al., 2004).

The Index line of pigs exhibits significant phenotypic improvements in ovulation rate and embryo survival (Johnson et al., 1999), which were the direct targets of selection. Increases in the number of pigs born alive have been partially limited to some extent by an increase in stillbirths (Johnson et al., 1999). Stillbirth in pigs can be caused by a variety of factors and has a genetic basis with several QTL localized in the Index and Control selection lines (Cassady et al., 2001). It has been suggested that iron deficiency is associated with an increased incidence of stillbirths in both pigs (Moore et al., 1965) and humans (Batu et al., 1972). Ferritin is a protein expressed in many tissues that plays an important role in iron storage and metabolism. Ferritin subunits (light and heavy chains) can be regulated at the transcriptional or translational levels (White and Munro, 1988). *Ferritin Heavy Chain* (*FTH*) transcription can be increased by transcription factors (Bevilacqua et al., 1994), thyrotropin (Chazenbalk et al., 1990), and progesterone (Zhu et al., 1995).

A negative correlation exists in boars between ferritin and iron concentrations and testis size, associated with changes in FSH levels (Ford et al., 2001; Wise et al., 2003). Iron overload is a genetic condition in humans caused by a mutation in the hemochromatosis gene (*HFE*; Feder, 1999), which encodes a membrane protein that can form a complex with endogenous transferrin receptor (TfR; Ikuta et al., 2000). Mice that have had *HFE* knocked-out have the iron overload phenotype (Zhou et al., 1998). Iron overload can result in lower levels of circulating LH and FSH leading to hypogonadotropic hypogonadism (Cundy et al., 1993), probably due to iron deposition in pituitary cells impairing their function (Bergeron and Kovacs, 1978; Charbonnel et al., 1981). In contrast, boars with elevated testicular ferritin have elevated FSH secretion (Ford et al., 2001).

We demonstrate that selection for increased ovulation rate and embryo survival has decreased mRNA levels of *FTH* in the anterior pituitary of sows. Although no evidence exists for a link between reduced FTH and increased incidence of stillbirths, it is possible that alterations in iron homeostasis are involved. Alternatively, it has recently been demonstrated that *FTH* is involved in the folate pathway by enhancing expression of serine hydroxymethyltransferase and de novo thymidine biosynthesis (Oppenheim et al., 2001). Supplementation of folic acid during gestation leads to increases in litter size, primarily due to improved embryo or fetal survival (Lindemann, 1993).

Expression of the *follicle-stimulating hormone beta subunit* (*FSHβ*) gene is up-regulated by selection for enhanced reproduction. This result is consistent with the role of FSH in regulation of ovarian follicle development (Hadley, 2000). Higher plasma levels of FSH have been found in both females and males (Cassady et al., 2000) from the Index line. Although large differences in allele frequencies were found for a genetic marker within the *FSHβ* locus between the Index and Control lines (Linville et al., 2001), influences of potential genetic drift were confounded with those results

Table 1. GenBank accession numbers, the Institute for Genomic Research (TIGR) cluster information, gene identification, and direction of expression change between selection lines for anterior pituitary EST characterized in this differential display study

Accession No.			
GenBank	TIGR[a]	Identification or clone information[b]	DD[c]
BE231450	TC56967	Replication factor C (activator 1) 1 (145Kd) (RFC1)[d]	C > I
BE231451	TC47390	Cytochrome c oxidase subunit I (COI)[d]	C < I
BE231452	TC47530	NADH dehydrogenase subunit 4 (ND4)[d]	C < I
BE231453	TC47699	Calmodulin 2 (CALM2)[d]	C < I
BE231454	Singleton	*Homo sapiens* chromosome 15, clone RP11-358L4[e]	C < I
BE231455	TC60706	Not available[f]	C < I
BE231456	Singleton	*Homo sapiens* chromosome 15, clone RP11-361D15 map 15q22[e]	C < I
BE231457	Singleton	U4/U6-associated RNA splicing factor (PRP3)[d]	C < I
BE231458	Singleton	Human chromosome 14 DNA sequence BAC R-124D2 of library RPCI-11[e]	C > I
BE231459	Singleton	*Sus scrofa* clone RP44-397B10[e]	C < I
BE231460	TC47530	NADH dehydrogenase subunit 4 (ND4)[d]	C < I
BE231489	TC63344	Not available[f]	C > I
BE231490	Singleton	Human DNA sequence from clone RP11-260H5 onchromosome 6[e]	C < I
BE231491	TC59847	Not available[f]	C > I
BE231492	TC47841	Neuronatin (NNAT)[d]	C < I
BE241013	TC47412	Cytochrome oxidase subunit II (COII)[d]	C > I/C < I
BE241014	TC47504	Cytochrome c oxidase subunit III (COX3, COIII)[d]	C > I
BE241015	TC47384	Cytochrome b (CYTB)[d]	C < I C > I/C < I
BE241016	TC47530	NADH dehydrogenase subunit 4 (ND4)[d]	
BE241017	TC47530	NADH dehydrogenase subunit 5 (ND5)[d]	C < I C > I/C < I
BE241018	TC47530	NADH dehydrogenase subunit 6 (ND6)[d]	
BE241019	TC47374	ATP synthase subunit 6 (MTATP6)[d]	C < I
BE241020	TC57468	16S ribosomal RNA[d]	C > I
BE241021	TC57217	*Homo sapiens*, similar to KIAA0663 gene product(LOC116284)[e]	C < I
BE241022	TC48993	Hypothetical protein FLJ12610 (FLJ12610)[e]	C > I
BE241023	Singleton	*Macaca fascicularis* brain cDNA, clone:QflA-11466[e]	C > I
BE241024	Singleton	Homo sapiens cDNA FLJ34215 fis[e]	C > I
BE241025	Singleton	*Homo sapiens*, similar to zinc finger protein 302[d]	C > I
BE241026	TC47371	*Homo sapiens*, similar to chromosome 14 open readingframe 3[e]	C > I
BE241027	TC56467	Human DNA sequence from clone CITF22-49G11 onchromosome 22[e]	C > I
BE241028	TC56935	*Homo sapiens*, similar to melanoma antigen, family D,1[d]	C < I
BE241029	TC56574	Splicing factor 3b, subunit 1 (SF3B1)[d]	C > I
BE241030	TC55967	Sus scrofa clone RP44-331G21[e]	C > I
BE241031	TC51044	*Homo sapiens*, similar to tuberin-like protein 1[d]	C > I
BE241032	TC52948	Immunoglobulin-like variable motif-containing protein(BIVM)[d]	C > I
BE241033	TC59793	*Homo sapiens* 3 BAC RP11-436A20[e]	C > I
BE241034	TC56394	PGP9.5 mRNA for ubiquitin C-terminal hydrolase[d]	C > I
BE241035	—	Not available[f]	C < I
BE241036	Singleton	Glycoprotein GPIIIa (CD61)[d]	C < I
BE241037	—	Regulatory factor X, 5 (influences HLA class IIexpression) (RFX5), mRNA[d]	C < I
BE241038	—	Tudor repeat associator with PCTAIRE 2(PCTAIRE2BP) [e]	C < I
BE241039	—	Mus musculus strain 129/SvJ BAC clone citb10i1from the MHC region[e]	C > I
BE241040	—	RIKEN cDNA 0610040D20 gene[e]	C > I
BE241041	TC59231	Suppression of tumorigenicity 13 (colon carcinoma)(Hsp70-interacting protein)[d]	C < I
BE241042	TC53537	Human DNA sequence from clone RP3-453D15on chromosome 6q16[e]	C < I
BE241043	TC47711	Heterogeneous nuclear RNA (hn-RNA)[d]	C > I
BE241044	TC56074	Synaptic vesicle glycoprotein 2 b (Sv2b)[e]	C < I
BE241045	TC51558	Gene dJ198K11.1[e]	C < I
BE241046	Singleton	*Homo sapiens* clone RP11-444D15[e]	C > I
BE241047	TC47599	RIKEN cDNA 2610003J06 gene[e]	C < I
BE241048	TC48755	*Homo sapiens* cDNA: FLJ22518 fis, clone HRC12216[e]	C < I
BE241049	TC62883	Six-transmembrane epithelial antigen of prostate 2(STEAP2)[d]	C > I
BE241050	TC51491	Uveal autoantigen with coiled-coil domains andankyrin repeats (UACA)[d]	C < I
BE241051	TC52924	Proteasome regulatory particle subunit p44S10[d]	C < I
BE241052	Singleton	*Homo sapiens* 12p BAC RP11-114G22[e]	C > I
BE241053	TC52950	Putative acid phosphatase F26C11.1 (LOC118924)[d]	C > I
BE241054	Singleton	Hypothetical protein FLJ13213 (FLJ13213)[e]	C < I
BE241055	TC47786	Pituitary glycoprotein hormone alpha subunit[d]	C > I
BE241056	TC53496	*Homo sapiens* cDNA FLJ13930 fis, cloneY79AA1000748[e]	C > I
BE241057	TC63728	*Homo sapiens* X BAC RP11-242C19[e]	C > I
BE241058	Singleton	*Homo sapiens* BAC clone RP11-533I8[e]	C < I

Continued

Table 1 (*Continued*). GenBank accession numbers, the Institute for Genomic Research (TIGR) cluster information, gene identification, and direction of expression change between selection lines for anterior pituitary EST characterized in this differential display study

Accession No.			
GenBank	TIGR[a]	Identification or clone information[b]	DD[c]
BE241059	Singleton	*Sus scrofa* clone RP44-123F10[e]	C < I
BE241060	Singleton	Mouse DNA sequence from clone RP23-419J18 onchromosome X[e]	C < I
BE241061	TC47523	Polyubiquitin (UBC)[d]	C > I
BE344516	TC58697	Not available[f]	C < I
BE344517	—	Not available[f]	C < I
BE344518	—	Not available[f]	C > I
BE344519	TC47674	NADH dehydrogenase subunit 3 (ND3)[d]	C < I
BE344520	Singleton	*Homo sapiens* chromosome 8, clone RP11-324E17[e]	C < I
BE344521	TC57464	*Homo sapiens* hypothetical protein FLJ10330(FLJ10330)[e]	C < I
BE344522	Singleton	Human DNA sequence from clone 879J18 onchromosome 11p13[e]	C > I
BE344523	TC56725	Sorcin (SRI)[d]	C < I
BE344524	—	Mus musculus chromosome 5 clone RP24-486D23[e]	C < I
BE344525	TC50478	Not available[f]	C < I
BE344526	—	Not available[f]	C < I
BE344527	—	*Homo sapiens* chromosome 10 clone RP11-564D11[e]	C < I
BE344528	TC55980	Steroidogenic acute regulatory protein gene[d]	C < I
BE344529	—	*Homo sapiens* clone RP11-296O14 on chromosome 1[e]	C > I
BE344530	—	Human DNA sequence from clone RP13-46M24 onchromosome Xp11.4-21.2[e]	C < I
BE344531	TC54556	Uroplakin II gene[d]	C < I
BE344532	TC47034	*Homo sapiens* cDNA from clone DKFZp434C0118[e]	C < I
BE344533	Singleton	Sus scrofa DNA for SINE sequence SSPRE[e]	C = I
BE344534	TC48023	Small acidic protein (SMAP)[d]	C = I
BE344535	TC49801	*Homo sapiens* hypothetical gene supported byAL833556 (LOC283778)[e]	C = I
BE344536	TC59446	*Homo sapiens*, clone MGC:9836 IMAGE:3863661[e]	C = I
BE344537	TC50173	Component of oligomeric golgi complex 3 (COG3)[d]	C = I
BE344538	TC50186	Protein phosphatase 3 (formerly 2B), catalytic subunit,alpha isoform (calcineurin A alpha) (PPP3CA)[d]	C = I
BE344539	TC53759	*Homo sapiens* chromosome 8, clone CTD-2544N14[e]	C = I
BE344540	TC47874	Ribosomal protein S23 (RPS23)[d]	C > I
BE344541	TC56277	Ribosomal protein S8 (RPS8)[d]	C < I
BE344542	TC56559	Ribosomal protein L31 (RPL31)[d]	C < I
BE344543	TC47353	Eukaryotic translation elongation factor 1 alpha 1[d]	C < I
BE344544	TC47550	Ribosomal protein S3a[d]	C < I
BE344545	—	Ribosomal protein S12 (RPS12)[d]	C < I
BE344546	TC56336	Ribosomal protein S24 (RPS24)[d]	C < I
BE344547	TC47583	Ribosomal protein L11 (RPL11)[d]	C < I
BE344548	TC56928	Ribosomal protein L12 pseudogene 4 (RPL12P4)[d]	C > I
BE344549	—	Ribosomal protein S25 (RPS25)[d]	C > I
BE344550	TC56335	Follicle stimulation hormone beta subunit[d]	C < I
BE344551	TC47383	Preprolactin[d]	C < I
BE344552	TC47694	Steroid membrane binding protein[d]	C < I
BE344553	TC49402	Schwannomin interacting protein 1 (SCHIP-1)[d]	C > I
BE344554	TC47179	Peptidyl-prolyl isomerase G (cyclophilin G)(PPIG)[d]	C > I
BE344555	TC56361	Superoxide dismutase[d]	C = I
BE344556	TC47497	Ferritin heavy-chain[d]	C > I
BE344557	Singleton	Scinderin (SCIN)[d]	C < I
BE344558	TC61710	G1-related zinc finger protein (G1rp)[d]	C < I
BE344559	TC52167	*Homo sapiens* cDNA FLJ39000 fis, cloneNT2RI2022468[e]	C < I
BE344560	TC48003	T-complex 1 (Tcp1)[d]	C < I
BE344561	TC59838	MADS/MEF2-family transcription factor (MEF2C)[d]	C > I
BE344562	TC56321	Activating transcription factor 4 (ATF4)[d]	C > I
BE344563	TC57604	Selenoprotein T (LOC51714)[d]	C > I
BE344564	TC56584	Protein inhibitor of neuronal nitric oxide synthase(PIN)[d]	C > I
BE344565	—	Amyloid beta (A4) precursor protein (APP)[d]	C > I
BE344566	Singleton	Ubiquitin-conjugating enzyme E2, J1 (UBC6 homolog,yeast) (UBE2J1)[d]	C < I
BE344567	TC57099	Conserved ATPase domain protein 44 (CADp44),Spermophilus tridecemlineatus 26S Proteasome S.U.[d]	C > I
BE344568	TC58868	Secretogranin III (SCG3)[d]	C = I
BE344569	TC56220	Beta 2-microglobulin[d]	C = I
BE344570	TC56366	Heterogeneous nuclear ribonucleoprotein F (HnRPF)[d]	C = I
BE344571	TC47243	Calpain I light subunit[d]	C = I

Continued

Table 1 (*Continued*). GenBank accession numbers, the Institute for Genomic Research (TIGR) cluster information, gene identification, and direction of expression change between selection lines for anterior pituitary EST characterized in this differential display study

Accession No.			
GenBank	TIGR[a]	Identification or clone information[b]	DD[c]
BE344572	TC49039	F1Fo-ATP synthase complex Fo membrane domain f subunit[d]	C > I
BE344573	TC47591	G-beta like protein[d]	C > I
BE344574	TC57182	*Homo sapiens* BAC clone RP11-746I5 from 2[e]	C < I
BE344575	TC52833	Rapamycin associated protein (FRAP2)[d]	C > I
BE344576	TC63812	SPARC-like 1 (mast9, hevin) (SPARCL1)[d]	C < I

[a]TIGR, March 2003 (www.tigr.org/tdb/tgi/ssgi).
[b]GenBank BLAST Nucleotide, March 2003 (www.ncbi.nlm.nih.gov/Genbank/index.html).
[c]Direction of differential display difference in expression. For example, C > I indicates that greater banding intensity was observed in the Control line. Both C > I and C < I indicates that in one instance the EST was found to be up-regulated in the Control line (C > I; band was excised and sequenced), whereas in another instance, the EST was found to be up-regulated in the Index line (C < I; band was excised and sequenced). Only after analysis of sequence data was it determined that these EST had similarity to the same gene.
[d]Total or partial similarity to known genes.
[e]Total or partial similarity to uncharacterized genes.
[f]Novel.

because the additive and dominance effects of the alleles did not differ from zero for any trait. Rohrer et al. (2001) identified several *trans*-acting QTL regulating plasma FSH levels in boars, but no evidence for a *cis*-acting QTL within or near the *FSH*β locus was found. In follicles of sows from the Index line, *follistatin* (*FST*) was found to be down-regulated (Gladney et al., 2004).

Taking into account these findings along with those of others studies conducted with these selection lines (Cassady et al., 2000), it is possible that low levels of follicular *FST* mRNA could be related to the elevated levels of *FSH*β pituitary mRNA in the Index line. However, Schneyer et al. (2003) recently found that follistatin has lower affinity for activin B, the pituitary form, than for activin A, the ovarian form. Also, Li et al. (1998) determined that *FST* mRNA concentration in the pituitaries of boars is not associated with FSH secretion.

Levels of mRNA for the *G-beta like protein* transcript were lower as a response to Index selection. G-Proteins are coupled with different receptors, and the beta subunit can be coupled with alpha subunits, which could stimulate or inhibit a given signaling pathway (Garrett and Grisham, 1999). Given the potential involvement of *G-beta like protein* in many important physiological processes, it will be difficult to assign relevance to the down-regulation of this gene in the Index line until a global gene expression analysis is conducted and cluster analysis is performed.

A novel finding of this study was that selection for the reproductive Index led to significantly increased weight of anterior pituitary in sows. There may be a correlation between this finding and the fact that twice as many differentially expressed bands were isolated from Line I vs. Line C, with the increased pituitary weight representing a physiological response to an increased metabolic demand in the Index line pituitary.

Many EST with known functions in reproductive physiology were isolated, including *pituitary glycoprotein hormone alpha subunit*, *steroidogenic acute regulatory protein*, *follicle stimulating hormone beta subunit*, *preprolactin*, and *steroid membrane binding protein*. In addition, many EST with undefined functions or that are novel were isolated. Future characterization of this latter subset of genes may contribute to understanding the molecular architecture of litter size in swine.

In summary, selection for increased ovulation rate and embryo survival has acted in part by altering gene expression of many transcripts in the anterior pituitary during a period of follicular development. Concurrent alterations in the ovarian follicle transcriptome have also been identified in the same animals (Gladney et al., 2004). The use of gene expression analysis represents a new vista to dissect polygenic traits and the nature of long-term selection response in livestock species. Although many potentially significant gene expression differences were uncovered using ddPCR, only a small sample of these was confirmed using Northern hybridization due to limited availability of anterior pituitary samples. This limitation also led to pooling of results across the two time points of follicular development used in this study, potentially confounding temporal differences that may have existed between the selection lines. Further confirmation of differential expression on a larger scale, as well as a deeper analysis of the anterior pituitary transcriptome, will be made possible by future large-scale expression profiling using microarrays.

Although application of transcriptional analysis to understanding selection response is a powerful tool, it is not possible to determine whether genes differentially expressed between selection lines represent QTL or the downstream consequences of QTL actions. The integrated approach of expression profiling in QTL mapping populations (e.g., Schadt et al., 2003a,b) would help determine the nature of transcriptional changes and their relationship to genetic variation

that contributes to complex traits and long-term response to selection. Furthermore, such integration would help partition gene expression results into those that are true responses to selection and those that result from genetic drift.

Implications

Differential display PCR was used to analyze gene expression differences in anterior pituitaries between pigs selected for increased ovulation rate and embryo survival and pigs of a randomly selected control line. Combined with concurrent analysis of the ovarian follicle transcriptome, our results indicate that a large number of gene expression changes may be involved in long-term selection response for a complex trait such as reproduction. Several individual gene expression changes were confirmed and present novel insights into pathways regulating reproductive phenotypes and the nature of the genetic architecture of complex traits with low heritability such as ovulation rate.

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