THE SSH LIBRARIES: A NEW MOLECULAR TOOL TO IDENTIFY GENES ASSOCIATED WITH PRODUCTION AND REPRODUCTION TRAITS IN PIGS

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Introduction

In the last decade, considerable progress was made to unravel the pig genome. The pig genetic map now has more than 4000 loci of which 1588 are designated as genes (www.thearkdb.org/browser?species=pig). More recently, gene transcription profiling techniques were also developed to fully understand the physiological complexity of the pig transcriptome (i.e. complete set of mRNA molecules present in a cell, tissue or organ). These new techniques have the potential to illuminate the molecular processes that govern phenotypic characteristics of porcine production and reproduction traits (Whitworth *et al.,* 2005). Among these new techniques, the suppressive subtractive hybridization (SSH) allows the identification of differentially expressed genes between two types of cells or tissues (Diatchenko *et al*., 1996). Results obtained from 2 research projects in which the SSH technique was used are presented herein. The first example concerns the identification of genes involved in embryo survival (Project 1), whereas the second example shows how the SSH technique allowed us to identify genes associated with swine muscle steatosis (i.e. abnormal fat infiltration in ham muscles, Project2).

Materials and Methods

Project 1

***Animals*:** Three prolific multiparous Meishan X Landrace sows (ML; 16.8 piglets/litter) and 3 multiparous Landrace sows (LL; 11.2 piglets/litter) were inseminated twice with LL mix semen. Sows were slaughtered on Day 15 of gestation and the time of the first insemination was considered as Day 0. Both horns were flushed with PBS to recover the filamentous embryos. Strips of epithelial endometrium were collected at five different sites of implantation, which were chosen at random, for each horn.

***Suppressive subtractive hybridization*:** Total RNA was extracted from endometrial and embryonic tissues. Two SSH libraries were performed with the PCR-Select cDNA Subtraction Kit (Clontech, Palo Alto, CA); one for endometrial and one for embryonic tissues. PCR amplifications were performed and the PCR products generated were cloned into the pT-Adv vector (Clontech).

***Differential screening*:** The cDNA inserts obtained from the different clones were amplified by PCR and were dotted onto Hybond N+ membranes. Forward and reverse subtraction PCR products as well as nonsubtracted cDNA from testers and drivers were used as ^{32}P-labeled probes, using PCR-Select Subtraction Hybridization Screening Kit (Clontech). Hybridization signals were analyzed with a densitometer and subsequent DNA sequencing was performed on differentially expressed clones. The sequences obtained were compared against the GenBank database using online computer BLAST program (http://www.ncbi.nlm.gov/BLAST/). DNA sequences obtained from both SSH libraries were submitted to GenBank.

Project 2

***Animals*:** After slaughter, 48 commercial pigs (105 ± 3 kg) were allotted to treatments according to a 2 x 2 factorial design with muscle fat infiltration (normal or severely affected by muscle steatosis) and backfat thickness (fat or lean) as main factors. Samples of semitendinosus muscle of the right ham were removed and stored in liquid nitrogen.

***Suppressive subtractive hybridization*:** The SSH technique was performed on a pool of mRNA (3 normal and 3 affected fat pigs) that was extracted from muscle samples. Muscle fibres and intra-muscular fat were separated manually under a binocular. Two SSH libraries were then completed as described above, one for muscle fibres and one for intramuscular fat.

***Differential screening*:** The cDNA inserts obtained from the two libraries were amplified by PCR and dotted onto Hybond N+ membranes. Differential screening was performed as described in Project 1.

Results

Project 1

The SSH libraries resulted in a total of 137 clones for the endometrial library and 166 clones for the embryo library, which represented differentially expressed genes between ML and LL sows. After sequencing and analyzing each clone, we classified the cDNA sequences into different categories according to their sequence identities with GenBank database (Figure 1). Clones were considered as: 1) identified genes, when their similarity was > 95% with GenBank sequences; 2) High homology, when similarity was 90-95%; 3) Low homology, when their similarity was 80-89%; 4) Novel gene, when clones had no significant similarity to any GenBank sequence.

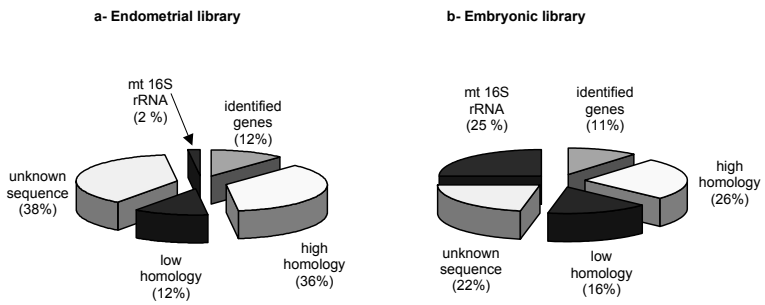

Figure 1. Pie diagrams of the cDNAs sequences found in the subtracted libraries for (a) endometrial and (b) embryonic tissues (Project 1)**.**

Project 2

Among the cDNA sequences obtained in the muscle fibres library, 10 clones were considered as identified genes, 20 clones showed high homology, whereas 23 others had low homology with GenBank sequences. Fifteen clones had no significant similarity to any GenBank sequence and were considered as novel genes. Sequence analysis of the cDNA clones obtained from the intramuscular fat library resulted in 7 clones which were considered as identified genes, 5 clones that had high homology and 7 others with low homology to GenBank sequences. Forty-four

clones were classified as novel genes. These included 12 clones with homologies to hypothetical and EST sequences, 15 clones with homologies to repetitive elements and 27 clones with no significant homology with any GenBank sequences.

For both research projects, the most promising candidate genes were then chosen according to the following specific selection criteria: 1) having hybridization signal difference higher then 10 on membranes; 2) having over 90% homology with known genes; 3) having involvement in different pathways known or suspected to be critical for embryonic survival or intramuscular fat infiltration; 4) novel genes were also selected for further characterization. Validation of promising genes was performed by using real-time PCR analyses to confirm that they were true differentially expressed genes. Table 1 only shows a few examples of genes that were validated by real-time PCR analyses.

Table 1. Relative mRNA Levels of SSH clones validated by real-time PCR analyses

Project 1					
Identification	**Tissue**	**ML**	**LL**	**SEM**	**ML vs LL[a]**
MHC class II SLA-DQ	Endometrial	2.03	4.81	0.09	0.0001
Stanniocalcin	Endometrial	0.86	1.78	0.04	0.0001
Sterol carrier protein 2	Embryonic	1.50	0.89	0.03	0.0001
Transferrin	Embryonic	1.20	0.54	0.04	0.0002
Project 2					
Identification	**Tissue**	**Steatosis**	**Normal**	**SEM**	**St vs N [a]**
New member of the aldo-keto reductase family	Muscle fibres	1.87	0.86	0.04	0.001
CDK4-binding protein	Muscle fibres	0.61	0.97	0.05	0.021
Mammalian suppressor of sgv1 (MSS1)	Intramuscular fat	0.64	1.08	0.04	0.009
SFRS2 splicing factor	Intramuscular fat	0.74	1.23	0.07	0.024

[a] *P* values, Statistical analyses were considered significant when $P < 0.05$.

Discussion and Conclusion

In this study, we have isolated novel and known genes that are differentially expressed in endometrial and embryonic tissues of ML and LL sows during the implantation period (Project 1). We believe that some of these genes could become promising candidate for selection of high litter size breeds since the Meishan breed is recognized for its higher embryonic survival rate. Known and novel genes were also identified in Project 2 and the differentially expressed genes that were validated are now considered as promising candidates that are affected by the development of swine muscle steatosis. The main advantages of using the SSH technique is that it allows the identification of novel genes and of genes that are weakly expressed (low copy number) in particular cells or tissues. This is not the case for commercial cDNA-microarray, another gene profiling technique (Dugan *et al*., 1999), because the genes that are spotted on microarray chips are only those that can be found in databases. Further characterization will be needed for the most promising genes in order to understand the molecular mechanisms involved in embryonic survival or in the development of swine muscle steatosis.

References

Diatchenko *et al*. (1996) Proc Natl Acad Sci U.S.A. 93:6025-6030.
Dugan *et al*., (1999) Nat Genet 21:10-14.
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