SNP AND PROTEIN MARKERS FOR EMBRYO DEVELOPMENT AT EARLY STAGE IDENTIFIED FROM FUNCTIONAL GENOMICS IN LANDRACE

En-Chung Lin[1], Ruei-Shyan Wu[1], Qi-Zhen Xu[1], Shih-Torng Ding[1], Rong-Huav Juang[1], Borrung Ou[2], Winston Teng-Kuei Cheng[2]
[1] Dept. of Animal Science and Technology, National Taiwan University, Taipei, Taiwan
[2] Dept. of Animal Science and Biotechnology, Tunghai University, Taichung, Taiwan
e-mail: eclin01@ntu.edu.tw

ABSTRACT

Reproductive success is an important indicator for pig production. To insure the viability of porcine embryo is the most important task in improving the efficiency of female reproduction in swine industry. Several approaches of functional genomics were conducted in Landrace sows to investigate the transcriptome profiles of different developmental stages before implantation of the embryo. There were many differential expressed genes found in such functional genomic approaches with different physiological functions annotated. In order to improve the female reproductive efficiency in selection programs, discovery of single nucleotide polymorphism was studied in those selected 20 genes from previous functional genomic and metabolism studies as well as published literature. There were more than twenty polymorphic sites located at 13 genes with possibility as the candidate markers for female reproductive success. Several protein antibodies were produced to test the management for sow reproductive process of pregnancy, farrowing, weaning and consecutive conception. These studies would be of important findings as well as techniques for swine industry in Taiwan.

Keywords: Embryonic Development, Sow Reproductive Efficiency, Functional Genomics, Single Nucleotide Polymorphism (SNP), Protein Marker, Landrace

INTRODUCTION

The first two weeks of embryonic development are critical for a successful pregnancy in domestic pigs. Majority of embryonic loss frequently takes place in this period and results in decreasing litter size significantly (Perry and Rowlands 1962). There are three major causes of developmental events occurring in mammalian preimplanted embryos, including maternal-to-zygotic transition of the transcriptomic and proteomic sources, compaction of embryonic cells caused by cell-cell interaction and genetic determination, and the first tissue differentiation of the morula to the blastocyst (Schultz *et al.* 1999; Hamatani *et al.* 2006). The last event including differentiation of blastomeres and formation of the inner cell mass and trophectoderm enables the embryo to acquire the ability to implant. Therefore, identification of key genes expressed using functional genomic analysis is important to understand normal early embryonic development. In addition, to insure the viability of porcine embryo is the most important task in improving the efficiency of female reproduction in swine industry.

Reproductive success is an important indicator for pig production. Litter size, a major component of reproductive performance, is a polygenic, lowly heritable and sex-limited trait which causes low selection efficiency with traditional selection program. Many studies have attempted to improve a variety of reproductive traits, including ovulation rate (Johnson *et al*., 1999), litter size (Bidanel *et al*., 1994), uterine capacity (Bennett and Leymaster, 1990), and number of piglets weaned (Peterson, 1989). Unfortunately, the rate of improvement in most of these

traits was very low; in fact, it is still difficult to make rapid genetic improvement in most female reproductive traits. The genetic architecture of female reproductive traits is very complex. As with all physiological traits, reproductive performance is a combination of genetic and environmental factors. Improving the low heritability traits in sows by quantitative traits locus (QTL) or candidate gene approach, such as marker-assisted selection (MAS), in conjunction with traditional selection methods, might be one of more effective approaches for such traits. The candidate gene approach allows the identification of polymorphisms in genes likely to cause variation in a trait based on physiological, immunological or endocrine evidences. These polymorphisms can contribute to a better understanding of the genetic basis of phenotypic differences among individuals.

In consequence, we conducted a series of studies from functional genomics of embryo in early stages using sequencing of expressed sequence tag (EST) libraries and oligonucleotide array to find out the transcriptome profile during the important events of embryonic development. Thereafter, discovery and validation of genetic and protein markers for further breeding program as well as management techniques based on the previous results.

FUNCTIONAL GENOMICS FOR EMBRYONIC DEVELOPMENT AT EARLY STAGES

The EST technique is an effective approach to study functional expression of genes in various cells and tissues (Adams *et al.*, 1992). Therefore, our research team conducted several experiments to clone fulllength-enriched cDNA sequences of genes expressed in porcine embryo at the 4-cell, 8-cell, morula and blastocyst stages to test the hypothesis that in addition to housekeeping genes, those genes involved in embryonic development before implantation are abundantly expressed in such embryonic cells. There were totally 56,739 cDNA clones sequenced to have around 25,000 valid ESTs after eliminating those housekeeping genes, low quality and repeated sequences. As shown in Table 1, there were 1,115, 893, 888 and 840 unique sequences (total 3,105) found by sequence cluster analysis in 4-cell, 8-cell, morula and blastocyst, respectively. Those 3,105 unique sequences were annotated by Blast against several sequence databases on NCBI (National Center for Biotechnology Information) such as the non-redundant nucleotide (NR/NT), Unigene, Gene, etc. (Benson *et al.*, 2009). After predicting their gene function using Gene Ontology (GO) database, there are around 12.7~33.0%, 11.9~30.0%, 2.3~4.9% and 5.5~9.7% of genes among those unique sequences in the four stages involved in metabolism, cellular activity, immune function and regulatory mechanism, respectively.

Table 1. Quality description of multiple stages libraries of porcine preimplantation embryo.

	Embryonic stage				
Parameter	4-cell	8-cell	Morula	Blastocyst	Total
No. of trace files	11,134	14,592	18,064	12,949	56,739
No. of valid ESTs*	3,745	3,936	8,863	8,061	24,605
	Cluster analysis				
No. of contigs	348	382	505	372	1,369
No. of singletons	767	511	383	468	1,736
No. of unique sequences	1,115	893	888	840	3,105

* The item indicate the number of high quality sequences undergo the quality check processes.

Many stage-specific expressed genes were found in this experiment, such as splicing factor similar to arginine/serine-rich 7, H2B histone and DNA methyltransferase 1 (DNMT1) in the 4-cell stage; ubiquitin/ribosomal fusion protein (UBA52), transmembrane protein 34, ubiquitin-conjugating enzyme E2Q, heterogeneous nuclear

ribonucleoprotein A3, activated RNA polymerase II transcriptional coactivator and Sorting nexin-10 in the 8-cell stage; RNA pseudouridylate synthase domain containing 2, signal sequence receptor, translocon-associated protein alpha, pro-opiomelanocortin messenger RNA (POMC), transaldolase 1 (TALDO1) and tumor-associated calcium signal transducer 2 (TACSTD2) in the morula stage; cysteine-rich protein 1 (intestinal) (CRIP1) and beta-actin (ACTB) in the blastocyst stage.

Four oligonucleotide arrays were constructed in-house and each contained 6,000 genes including internal controls; each gene was represented by two different probes of 35~40 nucleotides. The sequences of designed probes were extracted from four porcine EST cDNA libraries (4-cell, 8-cell, morula and blastocyst from the NTU swine functional genomic project) and the genes were identified using the Unigene database clustered from embryonic EST libraries and mRNA sequences from RefSeq. The RNA samples were extracted from the embryos flushed at morula stages of three Landrace sows and at blastocyst stages of the other three Landrace sows at similar age (16.7 ± 0.9 months). Two repeated hybridizations for the 24,000 genes indicated that 2.36% of the genes were expressed to a greater extent in the morula (M) than in the blastocyst (B) and 71.83% of the genes were expressed to a greater extend in the blastocyst than in the morula (Fig. 1a). There were 24.04% of genes removed without further analyses because of low-quality signals. Under more stringent selection conditions, that is equal to or greater than 5-fold differential expression after log2 transformation, 2,288 genes were differentially expressed, including 162 genes displaying higher expression levels in the morula (Fig. 1b) and 2,126 genes displaying higher expression levels in the blastocyst (Fig. 1c). These 2,288 genes represented approximately 9% of the total tested genes; whereas most of the genes (49.35%) had lesser differential expression$(-3 \leq x \leq 3,\ x = \log_2(M/B))$. It is possible that some of these lesser differentially expressed genes may be important in support of the developmental process. The genes mapped to a biological process were mostly involved in macromolecule metabolism, but also in cell signaling, cellular transportation and cell differentiation. Some differentially expressed genes with large increase or decrease in blastocyst or morula were selected for further study including MELK, SNRNP200, ATM, ZFP462, LASP1, SLC37A1, DYNLRB2, FBXO32, ATP2B2, OSCP1, and PLA2G7 (Table 2).

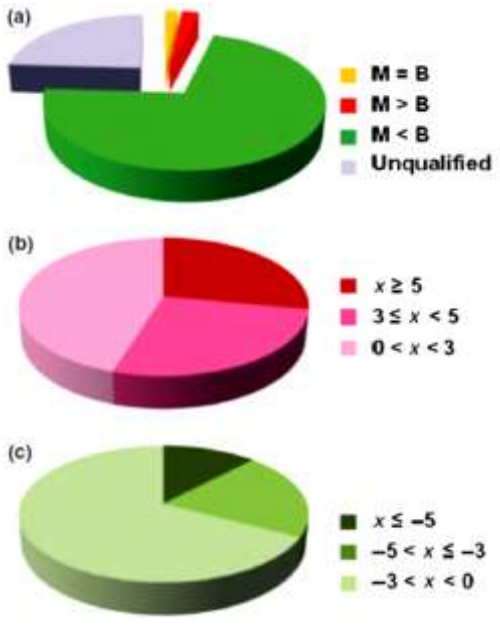

Fig. 1. Analysis of the change in embryonic transcription profiles of the morula (M) and blastocyst (B) collected from Landrace sows in vivo using microarray. (a) A chart was made to illustrate the total population of greater or equally expressed transcripts in the morula or in the blastocyst. Higher expression was shown for 2.36% of the genes in the morula, whereas 71.38% of the genes had higher expression in the blastocyst. Additionally, 1.77% of the genes had similar expression in both stages. The low quality signals, 24.04% denoted as unqualified, were removed from further analysis. (b) Genes with higher expression in the morula were further classified into three levels ($x \geq 5, 3 \leq x < 5, 0 < x < 3$) according to the signal ratio after log 2 transformation $x = \log_2(M/B)$. (c) Genes with higher expression in the blastocyst were further classified into three levels ($x \leq -5, -5 < x \leq -3, -3 < x < 0$)according to signal ratio after the log 2 transformation $x = \log_2(M/B)$. (Hsu *et al.*, 2012)

Table 2. The differentially expressed genes selected with annotated identity.

Origin of sequence	Related RefSeq Accession number	Possible identity	Gene symbol	Embryonic stage with Higher expression level
Ssc.22206	NM_010790.2	Maternal embryonic leucine zipper protein	MELK	Blastocyst
Ssc.13633	NM_177214.4	Small nuclear ribonucleoprotein 200 (U5)	SNRNP200	Blastocyst
Ssc.21876	NM_001123080.1	Ataxia-telangiectasia mutated protein	ATM	Blastocyst
Ssc.11839	NM_172867.3	Zinc finger protein 462 isoform 2	ZFP462	Blastocyst
PLAbI1012I16	NM_010688.4	LIM and SH3 protein 1	LASP1	Blastocyst
Ssc.24911	NM_153062.2	Solute carrier family 37 (glycerol-3-phosphate transporter), member 1	SLC37A1	Morula
Ssc.43557	NM_029297.1	Dynein light chain roadblock-type 2	DYNLRB2	Morula
Ssc.4368	NM_001044588.1	F-box protein 32	FBXO32	Morula
Ssc.25241	NM_001036684.1	ATPase, Ca++ transporting, plasma membrane 2	ATP2B2	Morula
Ssc.28385	NM_172701.2	Organic solute carrier protein 1	OSCP1	Morula
Ssc.19691	NM_001113013.1	Phospholipase A2, group VIIA (platelet-activating factor acetylhydrolase, plasma)	PLA2G7	Morula
Ssc.40797	N/A	Novel sequence	N/A	Morula

Four Agilant 44K Pig Gene Expression microarray sets were used to identify the differential mRNA expression in the white blood cells sampled in 1 day after weaning (W1) and 7 days after conception (C7) between four higher reproductive Landrace sows with records of 2 litters (H) of average litter size (9.3 ± 0.6) and four lower reproductive Landrace sows with records of 2 litters (L) of average litter size (5.1 ± 1.3). In the results, we found that 69 genes expressed significantly higher both in W1 $y_{W1} \geq 4, y_{W1} = H_{W1}/L_{W1}$ and in C7 $y_{C7} \geq 4, y_{C7} = H_{C7}/L_{C7}$ of H sows with most of them involved in metabolism and genetic regulation of white blood cell. There were 188 genes expressed significantly higher both in W1 $y_{W1} \leq -4, y_{W1} = H_{W1}/L_{W1}$ and $y_{C7} \leq -4, y_{C7} = H_{C7}/L_{C7}$ in C7 of L sows with most of them involved in different immune responses. These functional genomic studies provided abundant information and indication of porcine embryonic development before implantation for further studies of genetic and functional markers in female reproductive performance.

DISCOVERY OF CANDIDATE SNP MARKERS

To apply the results of those functional genomic studies into swine breeding program, our research team investigated the candidate genetic markers on the promoter region of each gene, such as single nucleotide polymorphism (SNP), associated to female reproductive efficiency of total number born (TNB), number born alive (NBA) and average birth weight (ABW). Using microarray data of differentially expressed mRNA between the morula and blastocyst stages in sows, we chose seven candidate genes more highly expressed in the morula than in the blastocyst stages and four candidate genes more highly expressed in the blastocyst than in the morula stages. In addition, some obesity metabolism related gene such as adiponectin, visfatin, perilipin, prolactin and serum amyloid A (SAA) which were interested for another candidate genes. Furthermore, we collected data for several published PCR-restriction fragment length polymorphism (PCR-RFLP) molecular markers associated with reproductive traits in pigs, including estrogen receptor (ESR), prolactin receptor (PRLR), retinol-binding protein 4 (RBP4) and leptin (LEP) (Korwin-Kossakowska, 2002; Rothschild *et al*., 1996; Rothschild, 1998; Rothschild *et al*., 2000). For the discovery of SNPs among those candidate genes, a total of 30 Landrace sows from four different purebred herds in Taiwan which were categorized by TNB per litter between the highest and lowest reproductive efficiency groups (15 sows per group with14.5 and 7.4 of average TNB, respectively). There were 88 SNPs found in those genes listed

above except from the ESR and PRLR genes: 39 SNPs from morula to blastocyst candidate genes, 45 SNPs from the obesity related genes, and 4 SNPs for the RBP4 and LEP genes. The GoldenGate assay (Illumina, San Diego, CA, USA) was applied to genotype these 88 SNP sites for the DNA samples collected from 456 Landrace sows of two commercial swine purebred herds in Taiwan.

The association analyses with genotype frequency and farrowing records were conducted using those 456 Landrace sows with 2,443 farrowing records from two commercial farms in Taiwan. The farrowing records for each sows included TNB, NBA and ABW from the first to ninth parity. We calculated their estimated breeding values (EBVs) of the three farrowing traits of each sow by a mixed linear model recorded:

$$y = Xb + Z_1u + Z_2c + e$$

where the vector of y represents the measures of the three traits, b is the vector of the fixed effects on the traits, including herd-season and parity for all traits, and TNB as the covariate for the trait of ABW, u is the vector of the additive genetic effect for all traits, c is the vector of litter common random permanent environmental effect for all traits, e is the vector of random residual effect. The incidence matrices X, Z_1, Z_2 associate elements of b, u, c with the measures in y. The estimated breeding values (EBVs) were obtained from the solutions of vector u by solving the mixed linear model equations for each trait using Multiple Trait Derivative-Free Restricted Maximum Likelihood package (Boldman *et al.*, 1995). The EBV calculated by Best Linear Unbiased Prediction (BLUP) here represents the sum of independent gene effects by eliminating most of the environmental effects from the phenotypic value of each trait.

The two extreme groups of 90 individuals with highest 20% EBV and another 90 individuals with lowest 20% EBV were selected to conduct the case-control association test (Sasieni, 1997) for modeling the trend of an additive allele in a single SNP site. In this study, the case represents the high EBV group, and the control for the low EBV group. For example, the genotypic frequencies of r_0, r_1, and r_2 for the genotypes with 0 (negative), 1 (heterozygous), and 2 (homozygous) major alleles in the high EBV (case) group are found in the SNP site, respectively. The genotypic frequencies of s_0, s_1, and s_2 for the genotypes with 0 (negative), 1 (heterozygous), and 2 (homozygous) major alleles in the low EBV (control) group are found in that SNP site, respectively. Then, the total genotypic frequencies of 0, 1 and 2 major alleles are n_0 (= $r_0 + s_0$), n_1 (= $r_1 + s_1$), and n_2 (= $r_2 + s_2$), respectively. The numbers of individuals in the high and low EBV groups as well as all animals in this statistical comparison are R (= $r_0 + r_1 + r_2$), S (= $s_0 + s_1 + s_2$), and N (= $n_0 + n_1 + n_2$), respectively. The case-control chi-square value (χ^2) with 1 degree of freedom, so called trend test, to test the effect of an additive allele for a particular trait is shown below:

$$\chi^2 = \frac{N[N(r_1 + 2r_2) - R(n_1 + 2n_2)]^2}{R(N - R)[N(n_1 + 4n_2) - (n_1 + 2n_2)^2]}$$

There were 17 SNPs located at 8 genes (ATM, B2M, PAFAH, F-BXO, LASP1, PMCA2, SAA and NOR1) with significantly different genotype frequency ($p < 0.05$) in the top 20% EBV group of TNB (1.29 ± 0.33) from that in the bottom 20% EBV group (-0.88 ± 0.34). Twenty SNPs located at 8 genes (ATM, B2M, F-BXO, LASP1, PMCA2, SAA, NOR1 and Visfatin) were found significantly different genotypic frequency ($p < 0.05$) in the top 20% EBV group of NBA (0.95 ± 0.23) from that in the bottom 20% EBV group (-0.72 ± 0.25). For ABW trait, there were 14 SNPs located at 8 genes (ATM, RBP4, PAFAH, ASCC3, ADN, SAA, DLC2B and NOR1) with significantly different genotypic frequency ($p < 0.05$) in the top 20% EBV group (0.09 ± 0.05) from that in the bottom 20% EBV group (-0.13 ± 0.04). These SNP markers might be useful for the selection program of female reproductive efficiency in Landrace population.

DEVELOPMENT OF PROTEIN MARKER

According to the results of functional annotation, our research team selected about 10 candidate protein markers, which were annotated for immune or metabolism regulatory processes, for monoclonal antibody production. The candidate markers included beta 2 microglobulin (β2M), insulin growth factor binding protein 3 (IGFBP3), etc. We

selected these candidate markers because these proteins are expressed in the serum and which makes the potential utilization in predicting sow reproductive ability. The monoclonal antibody production of these candidate markers was based on the protocol which was described in the previous report (Milstein, 1980), by using mouse immunization and spleen fusion to create hybridoma cell lines for developing, producing and purifying monoclonal antibody. For example, we successfully purified the monoclonal antibody of β2M for monitoring the health conditions of sows during pre-mating stage (Fig. 2). The results indicated that the expression profile of β2M in the prolific sow were significantly different from those low fertility sow which is consistent with the previous study in human (Giorgetti *et al.*, 2007). We conducted a blind test for confirming the monitoring efficiency of β2M for the health condition of sows during pre-mating stage in cold (February-March) vs. hot (July-August) seasons. We selected high (H), intermediate (M) and low (L) fertility sows according to their potential producing ability (PA) which equals to sum of estimated breeding value (EBV) and permanent environmental effect (PE), and applied the β2M monoclonal antibody to predict their reproductive performance in comparison with the actual NBA in the next parity. The results revealed that the prediction accuracy of this monoclonal antibody was better when the sow gestation period happened in the cold season, but such prediction accuracy in hot season was decreased during the hot season which may be significantly influenced by the environmental effects (Fig. 3). There have been different antibodies under investigation for the environmental effects due to temperature during pre-mating stage and gestation period.

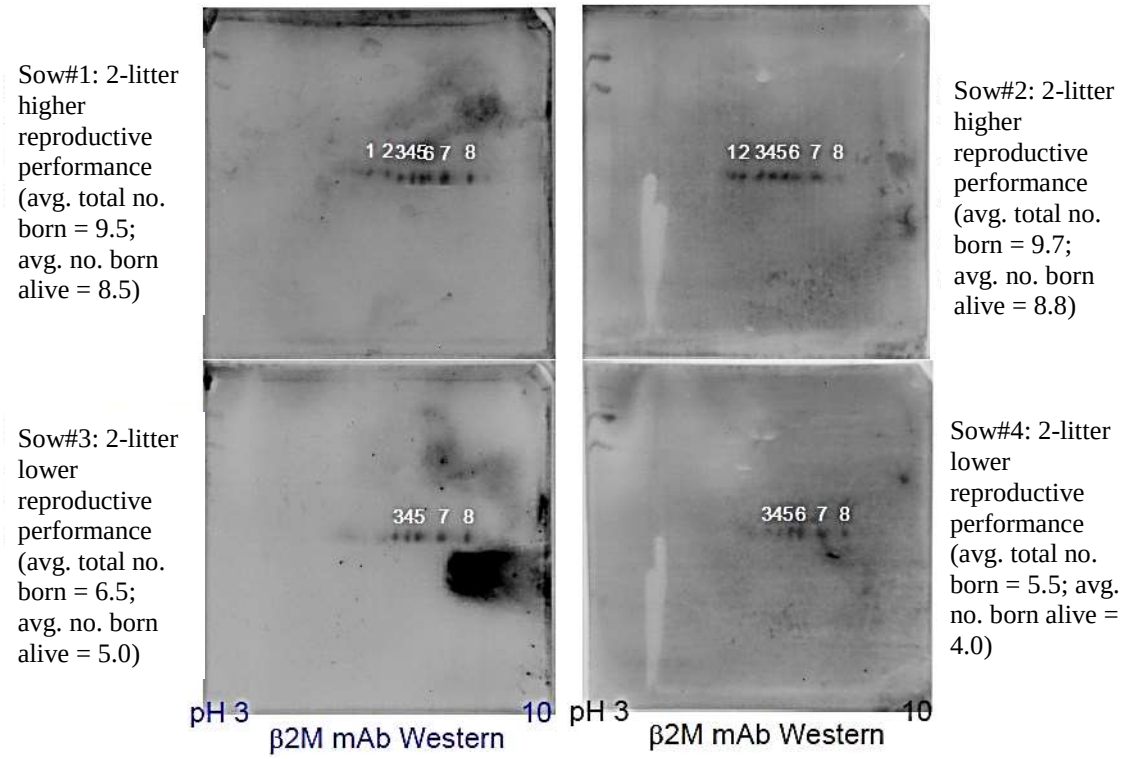

Fig. 2. The expression profile of 2M monoclonal antibody in prolific (sows#1 #2) and infertility sow (sows#3 & #4).

Blind Test-I (Cold Season)

ID	1	2	5	6	4	7	10	3	8	9
Ear notch	L1535-01	L1722-01	L1933-01	L1831-01	L1682-02	L1878-03	L1837-01	L1805-06	L1931-04	L1757-04
Parity	5	3	2	3	4	2	3	3	2	4
Average NBA	10.6	12.0	12.5	12.3	9.5	11.0	8.7	8.0	5.0	8.5
Group (PA=EBV+PE)	H (0.81)	H (0.80)	H (1.36)	H (1.95)	M (0.02)	M (0.27)	M (0.31)	L (-0.30)	L (-0.80)	L (-0.38)
Prediction of β2M	L	H	H	L	L ?	L	H	L	L	L
NBA in next parity	Fail	10	15	9	Fail	8	10	8	Fail	2

Blind Test-II (Hot Season)

ID	4	6	7	9	2	8	10	1	3	5
Ear notch	L0015-02	L1688-01	L1831-04	L1925-02	L1781-01	L1874-02	L1794-01	L1991-01	L1575-02	L1951-04
Parity	2	5	3	3	4	3	3	2	5	2
Average NBA	9.5	10.6	13.0	11.3	9.3	9.0	9.7	8.0	8.2	8.5
Group (PA=EBV+PE)	H (0.44)	H (0.73)	H (1.86)	H (1.02)	M (0.12)	M (0.14)	M (0.13)	L (-0.03)	L (-0.48)	L (-0.32)
Prediction of β2M	H	L	H	H	H	H	H	H	M ?	L
NBA in next parity	11	9	12	13	7	11	12	8	13	9

Fig. 3. The results of 2M prediction in different sow gestation seasons (cold and hot).

CONCLUSION

Our team showed a generally increased transcriptional activity in early blastocysts compared with morulae of pigs, and found a concurrent increased variety of genes expressed in the preimplantation stages of embryonic development in Landrace sows. The well-orchestrated gene expression pattern determines the final outcome of implantation, and the survival of the embryos. In addition, there have been many differentially expressed genes found during the porcine embryonic development. Focusing on promoter region related to regulation of those genes, some novel SNP markers were found by using the GoldenGate assay and association analyses of mixed linear model and case-control test. These novel SNP sites might provide new insights for genetic control of female reproductive efficiency. The final approach was an attempt to investigate the possibility of several protein markers, such as β2M, in prediction of management for female reproductive process. It might need several markers to find out the multiple causes of sow management problem in advance.

REFERENCES

Adams, M. D., M. Dubnick, A. R. Kerlavage, R. Moreno, J. M. Kelley, T. R. Utterback, J. W. Nagle, C. Fields, and J. C. Venter. 1992. Sequence identification of 2,375 human brain genes. Nature 355:632–634.

Bennett, G. L., and K. A. Leymaster. 1990. Genetic implications of a simulation model of litter size in swine based on ovulation rate, potential embryonic viability and uterine capacity: II. Simulated selection. J. Anim. Sci. 68: 980-986.

Benson, D. A., I. Karsch-Mizrachi, D. J. Lipman, J. Ostell, and E. W. Sayers. 2009. GenBank. Nucleic Acids Res. 37: D26-D31.

Bidanel, J. P., J. Gruand, and C. Legault. 1994. An overview of twenty years of selection for litter size in pigs using "hyperprolific" schemes. Proceedings of the Fifth World Congress on Genetics Applied to Livestock Production 17: 512-515.

Boldman K. G., L. A. Kriese, L. D. Van Vleck, C. D. Van Tassell, S. D. Kachman. 1995. Manual for Use of MTDFREML. A Set of Programs To Obtain Estimates of Variances and Covariances [DRAFT]. U.S. Dept. of Agriculture, Agricultural Research Service.

Giorgetti, S., Stoppini, M., Tennent, G. A., Relini, A., Marchese, L., Raimondi, S., Monti, M., Marini, S., Ostergaard, O., Heegaard, N. H., Pucci, P., Esposito, G., Merlini, G. and Bellotti, V. 2007. Lysine 58-cleaved b2-microglobulin is not detectable by 2D electrophoresis in ex vivo amyloid fibrils of two patients affected by dialysis-related amyloidosis. Protein Sci. 16: 343-349.

Hamatani, T., M. S. H. Ko, M. Yamada, N. Kuji, Y. Mizusawa, M. Shoji, H. Asada, T. Maruyama, and Y. Yoshimura. 2006. Global gene expression profiling of preimplantation embryos. Hum. Cell. 19: 98-117.

Hsu, C.C., E. C. Lin, S. C. Chen, S. C. Huang, B. H. Liu, Y. H. Yu, C. C. Chen, C. C. Yang, C. Y. Lien., Y. H. Wang, C. W. Liu, H. J. Mersmann, W. T. K. Cheng, and S. T. Ding. 2012. Differential gene expression between the porcine morula and blastocyst. Reprod. Domest. Anim. 47: 69-81.

Johnson, R. K., M. K. Nielsen and D. S. Casey. 1999. Responses in Ovulation Rate, Embryonal Survival, and Litter Traits in Swine to 14 Generations of Selection to Increase Litter Size. Faculty Papers and Publications in Animal Science.Paper 96.

Korwin-Kossakowska, A., M. Kamyczek, D. Cieslak, M. Pierzchala, and J. Kuryl. 2002. The effect of the polymorphism of leptin (lep), leptin receptor (lepr) and osteopontin (opn) genes on selected reproduction traits of synthetic line 990 sows. Anim. Sci. Pap. .Rep. 20: 159-168.

Milstein C. 1980. Monocloanl Antibodies. Scientific American. 243: 56-64.

Perry, J. S. and I. W. Rowlands. 1962. Early pregnancy in the pig. J. Reprod. Fertil. 4: 175-188.

Peterson, G. A. 1989. Evaluation of sow productivity index selection in Landrace and Duroc swine. Anim. Breed. Abstracts 59: 52.

Rothschild, M. F. 1996. Genetics and reproduction in the pig. Anim. Reprod. Sci. 42: 143-151.

Rothschild, M. F. and A. Ruvinsky. 1998. The genetics of the pig. CBA international, Wallingford, UK: pp. 199-294.

Rothschild, M. F., L. A. Messer, A. Day, R. Wahs, T. Short, O. Southwood, and G. Plastow. 2000. Investigation of the retinol binding protein (RBP4) gene as a candidate gene for litter size in the pig. Mamm. Genome 11: 75-77.

Sasieni P. D. 1997. From genotypes to genes: Doubling the sample size. Biometrics. 53: 1253-1261.

Schultz, R. M., W. Davis Jr., P. Stein, P. Svoboda. 1999. Reprogramming of gene expression during preimplantation development. J. Exp. Zoo. 285: 276-282.