NRSP-8 Annual Report: Swine Species

University of Minnesota

Reporting Participants: Michael Murtaugh, Department of Veterinary and Biomedical Sciences
Scott Fahrenkrug, Department of Animal Science
Mark Rutherford, Department of Veterinary and Biomedical Sciences

Accomplishments.

1. Transcriptional profiling of stress-response in cultured porcine islets.

Xenotransplantation with adult porcine islets could meet the increasing demand for cell-based diabetes therapy. A better understanding of the perturbations of the cell biology encountered during islet processing will aid the rational design of cytoprotective strategies aimed at improving transplant outcomes. Isolated adult pig islets were exposed in vitro to inflammatory cytokines (IL-1b: 2ng/ml; TNFa: 1,000 U/ml; and IFN-g: 1,000 U/ml) and/or elevated glucose (5.6 vs 16.7 mM glucose). Islet gene expression profiles were assessed using a new porcine 70-mer oligonucleotide microarray and real-time PCR. Microarray data were analyzed by direct pairwise comparison between culture conditions and by loop design using GeneSpring and R/maanova, respectively. Our results revealed distinct gene expression patterns in response to exposure to cytokines and glucose for 48 h. Cytokine treatment resulted in increased expression of genes involved in stress, immune response (e.g. MHC-related genes), apoptosis, and cellular defense (e.g. MnSOD). Islets cultured under conditions of elevated glucose showed increased expression of genes involved in intracellular protein transport, glucose and lipid metabolism, and stress response. A decrease in intracellular ATP and insulin content was detected at 48 h in response to cytokines and at 96 h in response to high glucose. In conclusion, transcriptional profiling of the response of porcine islet beta cells to inflammatory and hyperglycemic conditions will help identify molecular targets that are likely to protect porcine islets during islet isolation and engraftment.

2. Technical issues encountered using a porcine 70mer-oligonucleotide array

Microarray-based analysis of gene expression provides the opportunity to simultaneously assess the expression of thousands of genes in porcine tissues. We have been using SSWG1 (sus scrofa whole-genome 1.0), a set of 70-mer oligonucleotides containing 13,297 distinct elements designed by Qiagen, for porcine gene expression studies. Several technical issues were encountered in using this set of array, including annotation accuracy, image analysis and data processing for experiment with multifactor and loop design.

SSWG1 probes were designed against version 5 of the TIGR Porcine Gene Index assembled by the USDA Meat Animal Research Center and the Institute for Genome Research (1). To extend the annotation for this oligo set, we have implement an annotation pipeline thought homologue identification with human genome, transcriptome and proteome information. As the first critical step for array data processing, image capture and data acquisition were performed with GenePix and QuantArray software. The performances of two software were compared by examining the signal distribution of negative controls and the consequence of bias caused by QuantArray were demonstrated by further data analysis. Because loop design effectively increases the number of replicates for a given condition, it delivered twice more data than reference design. Mixed ANOVA model were shown be to a powerful statistical approach

for microarray data analysis (2,3). We have implemented a loop design and analyzed the expression data using pairwise (GeneSpring) and multifactorial (mixed ANOVA model) approaches.

In conclusion, more than 29% redundancy were estimated for SSWG1. The redundancy issue could have been avoided ab initio by homologue identification for the oligos designed with the genome, transcriptome and proteome information of other species. Data generated with QuantArray image analysis software comprises a significant arbitrary component, which results in the loss of sensitivity in detecting significant genes with low or intermediate signal level. Mixed ANOVA model (implemented in R/Mannova) is suitable for the analysis of array data of experiments with loop and multifactorial design. Mixed ANOVA model is able to use all the data from loop design in data analysis Mixed ANOVA model is able to determined the main effect of each factor and the interaction between factors

3. Porcine somatic-cell transgenesis in vivo and in vitro using the Sleeping Beauty transposon system

Because the size of piglets approximates that of human neonates, they should provide excellent models for the development of gene therapy procedures. In addition, a potentially unlimited supply of pigs argues for the use of porcine tissue in therapeutic xenotransplantation. These diverse applications would benefit greatly from the development of technologies for efficient porcine somatic-cell transgenesis. We are using transposons and recombinases to engineer pig cells in vivo and in vitro, and will present current data regarding use of the Sleeping Beauty (SB) transposon system for improved efficiency and precision in transgene delivery and expression.

The effectiveness of in vivo delivery of transgenes to the liver (via the umbilical vein) and lungs (via tracheal nebulizer) of neonatal piglets using the SB transposon system are being investigated. Rates of transgene delivery and expression are being monitored using the Xenogen in vivo imaging system. The influence of functional SB transposase on transgenesis rates, short and long-term transgene gene expression, and the molecular architecture of transgenes are being analyzed and will be discussed.

We are also investigating the use of SB for transgenesis of isolated porcine islets to improve islet engraftment and tolerance. Transfection of islets with transposons encoding luciferase resulted in the uniform expression of 15,000 - 30,000 molecules per cell. Transfected islets transplanted under the kidney capsule rescued diabetic nude mice and maintained luciferase expression for at least 5 weeks. Transposons encoding immune regulatory molecules are being developed and will be tested for their ability to protect islets transplanted into immune-competent mice.

4. A transgenic porcine model of cystic fibrosis

Cystic fibrosis (CF) is the most common life-shortening disease in Caucasians, affecting between 1 in 2,000 and 1 in 4,500 individuals. The gene involved in CF, the transmembrane conductance regulator (CFTR), was identified in 1989. Although mouse models of CF have been generated which manifest some of the electrophysiologic characteristics of CF, their benign pulmonary phenotype renders these models somewhat irrelevant. We have undertaken the development of a porcine model of CF.

A two pronged approach has been adopted for ablation of porcine CFTR function; homologous recombination to introduce the *F508 mutation, and RNAi of porcine CFTR using

the Sleeping Beauty (SB) Transposon system. Our cellular resources include traditional porcine fetal fibroblasts (PFF) and pig multipotent adult progenitor cells (pMAPC). pMAPCs may be ideal for the extended culture required for double selection for homologous recombination, as they can proliferate extensively in culture (>130 PDs) without obvious senescence or loss of differentiation potential.

We have used BAC recombineering to construct a series of replacement vectors harboring the *F508 mutation in combination with both positive and negative selection cassettes for homologous recombination. For RNAi, shRNA-expressing transposons have been developed and tested in a porcine cell line that expresses CFTR. CFTR mRNA knockdown (90-94%) was demonstrated by quantitative PCR, in agreement with an equivalent loss of function based on apical membrane Cl- current. The status and characterization of PFFs and pMAPCs transfected with constructs for both approaches will be presented, along with plans for developing a pig genetic model by nuclear transfer.

5. Use of transposons and recombinases in pig cells. To 1) annotate genes important for regulation of nutrient partitioning, stress response and muscle development, and 2) the development of methods for enhancing animals performance and quality by somatic and germline transgenesis.

Gene-traps based on the Sleeping Beauty transposon system have been developed. We are assessing the frequency of transposon-based transgenesis in various adult and fetal porcine cell lines, including cells from the skeletal, reproductive, respiratory, endocrine and digestive systems. Parameters affecting the efficiency of in vitro transgenesis are being examined to characterize tissue specific amenability to transposon-based transgenesis. In addition, methods for chromosome manipulation using bacterial recombinases are under investigation and show promise in porcine cells. Special emphasis is being focused on annotation of secreted proteins due to their importance in intercellular communication. In vitro annotation of secreted proteins is being conducted in parallel with our efforts to identify sequences that encode them in the public data repositories using bioinformatics.

In vivo transgenesis via the respiratory system and the digestive tract are being investigated as routes for DNA-based gene supplementation. Delivery of DNA to the lungs could serve as a route for vaccination against respiratory viruses and bacteria. Delivery of DNA to the liver provides an experimental method for studying and manipulating piglet metabolism. Protocols for transposon delivery via these routes have been developed and tested. Further refinements are under way.

6. Gene discovery and expression profiling in porcine Peyer's patch.

Peyer's patches of the intestinal mucosa are essential for host defense and immune regulation in the enteric system. To better understand molecular mechanisms of Peyer's patch function, we have screened for differentially expressed genes specific to Peyer's patch. cDNA libraries were created from normal Peyer's patch, immune stimulated Peyer's patch, and pooled cDNA subtracted with fibroblast RNA. 3687 expressed sequence tags (ESTs), representing 2414 unique nucleotide sequences, were isolated from the subtracted library, identified by BLAST searches against public databases, and spotted onto a microarray for gene expression profiling. Approximately 30% of these ESTs BLAST to genes of unknown function and 20% have no known homology in the public databases (novel genes). Of the novel genes, 70% are expressed in normal immune tissues by microarray analysis, suggesting that at least 371 of the unidentified

EST sequences from the subtracted library are novel porcine genes and can now be further characterized to determine their function in the porcine Peyer's patch. We surmise that the products of these genes participate in biochemical and cellular functions related to the unique immunological and gastroenterological functions of the small intestine. The BLAST and gene ontology information for each of the subtracted library EST sequences, the normal and immune stimulated libraries, and the microarray are all valuable resources that will facilitate further examination of the biological function of porcine Peyer's patch tissue.

7. Radiation hybrid mapping of porcine Peyer's patch expressed sequences.

In an effort to identify genes responsible for anti-microbial responses in the gut, we isolated expressed sequence tags (EST) from an activated porcine Peyer's patch cDNA library. Approximately 30% of these ESTs did not display significant homology to known genes or ESTS, while an additional 15% matched ESTs of undefined function. To determine chromosomal location for genes expressed in porcine Peyer's patches, PCR-based mapping was performed across a swine radiation hybrid panel. A total of 125 ESTs were mapped with a lod score > 6.0. Northern blot or real-time PCR confirmed altered regulation of transcripts for several of these ESTs. These ESTs therefore provide insight into early immune mechanisms and processes activated in Peyer's patches. Placement of these ESTs on the porcine map will assist in development of high density and comparative genetic maps for positional cloning of genes responsible for immune function in the gut

Dvorak, C.M.T., K.A. Hyland, J.G. Machado, Y. Zhang, S.C. Fahrenkrug, and M.P. Murtaugh. 2004. Gene discovery and expression profiling in porcine Peyer's patch. Vet. Immunol. Immunopathol. In press.

Klee EW, Carlson DF, Fahrenkrug SC, Ekker SC, Ellis LB. 2004. Identifying secretomes in people, pufferfish and pigs. Nucleic Acids Res. 32:1414-21.

Liu G, Geurts AM, Yae K, Srinivasan AR, Fahrenkrug SC, Largaespada DA, Takeda J, Horie K, Olson WK, Hackett PB. 2005. Target-site Preferences of Sleeping Beauty Transposons. J Mol Biol. 346:161-73.

Nadershahi A, Fahrenkrug SC, Ellis LB. 2004. Comparison of computational methods for identifying translation initiation sites in EST data. BMC Bioinformatics. 5:14.

Wang, C., C. Reilly, and M.S. Rutherford, 2005. A method for the comparison of cluster analyses. Statistica Sinica, in press.

Zhang, X., N. Reilly, and M.S. Rutherford, 2005. An RNA interfering system for primary porcine alveolar macrophages. Anim. Biotechnol, in press.

ABSTRACTS

Flickinger, G.H., C.M.T. Dvorak, J.A. Hendrickson, M.P.Murtaugh, and M.S. Rutherford, 2004. Radiation hybrid mapping of porcine ESTs from porcine Peyer's Patch. Plant and Animal Genome XII, San Diego, CA, January 11-14.

Zhang, X., N. Schreiber, M.P. Murtaugh, and M.S. Rutherford, 2004. Suppression of porcine reproductive and respiratory syndrome virus (PRRSV) infection by RNA-mediated gene silencing. Amer. Soc. Virol. 23rd Annual Meeting, Montreal, Canada, July 10-14.