The Role of Transcriptomics: Physiological Equivalence Based on Gene Expression Profiles

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(トランスクリプトミクスの役割：遺伝子発現プロファイルに基づく生理学的同等性)

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CHAPTER I

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1. RNA and transcriptomics

The “Central dogma of molecular biology”, as defined by Francis Crick, explains the flow of genetic information within a biological system (Crick, 1970). This concept states that genetic information encoded in DNA is transcribed to RNA, and RNA is translated to protein. The biological activities of cells, tissues, and organisms are based on the central dogma of molecular biology. RNA is an important factor required to mediate gene and protein expression. There are two types of RNA, non-coding RNA (ncRNA) and messenger RNA (mRNA or protein-coding RNA). ncRNAs play several key roles in gene regulation including transcriptional and post-transcriptional regulation, regulation of alternative splicing, control of transcription factor binding, chromatin modification, and protein-coding RNA stabilization (Louro *et al*., 2009; Pertea, 2012) (Table 1). ncRNAs include ribosomal RNA (rRNA) and transfer RNA (tRNA) and are classified into two broad groups by size. Long ncRNAs (lncRNA) are greater than 200 nucleotides and small ncRNAs (sncRNA) are 200 nucleotides or less. lncRNAs include large intergenic ncRNAs, long intronic ncRNAs, antisense RNAs, and pseudogene RNAs. lncRNAs play critical and specialized roles in numerous biological processes including the regulation of gene expression, and pre- and post-transcriptional modulation of epigenetic regulation (Rinn and Chang, 2012; Guo

et al., 2015). sncRNAs also have several functions: microRNAs (miRNAs) and small interfering RNAs (siRNAs) modulate post-transcriptional gene expression by binding to specific mRNAs; small nucleolar RNAs chemically modify rRNAs and other RNAs; and piwi-interacting RNAs inhibit transposon function through the PIWI protein, and maintain genomic constancy in germline cells. Other ncRNAs include small nuclear RNA, transcription initiation RNA, X-inactivation RNA, and promoter-associated RNA. Dysfunction of ncRNA is associated with complex diseases such as cancer, and neurological, developmental, and cardiovascular diseases (Taft *et al*., 2010; Esteller, 2011). More than 90% of the genome is transcribed into RNA, and it is estimated that mRNA constitutes approximately 62% of the transcripts (Pertea, 2012). mRNA plays a key role in transcription and reflects the information of almost all expressed genes. The complete set of mRNA, or primary transcripts, under a specific condition or in a specific cell is defined as the "transcriptome". Transcriptomics is the study of the transcriptome and enables researchers to elucidate gene expression dynamics under different circumstances. This leads to a deeper understanding of the function of each gene in the genome, and the regulation of gene expression, and aids in the elucidation of molecular mechanisms of abnormal states such as diseases.

In this study, we describe the general application of transcriptomics and recommend approaches for analyzing whole genome expression profiling data. Additionally, we performed two microarray experiments using pig model for evaluation change and constancy respectively.

2. Methods of transcriptomics

The transcriptome is complex. Over the past decade, several approaches have been developed to elucidate its intricacy and recent dramatic advances in analytical technologies have allowed researchers to further appreciate the transcriptome. In the 1990s, expressed sequence tag (EST) sequencing was employed to rapidly identify expressed genes and gene fragments (Schuler *et al*., 1996). Although EST sequencing is a high-throughput technique, it is expensive. Tag-based methods including serial analysis of gene expression, cap analysis of gene expression, and massively parallel signature sequencing were developed, but were unable to discriminate between genetic isoforms and were very expensive to apply on a large scale. Microarray was developed for genome-wide analysis, and has become the most widely used approach for transcriptomics. Recently, RNA sequencing (RNA-seq) using next generation sequencing technology has allowed the transcriptome to be characterized, and the

number of studies using RNA-seq have gradually increased (Ghosh and Qin, 2010; Yu and Lin, 2016). Microarray and RNA-seq have become the main tools used in transcriptome research. These tools allow us to simultaneously analyze the expression of a large number of genes and to focus on physiological equivalence. Several studies have compared the accuracy of microarray and RNA-seq measurements (Marioni *et al*., 2008; Fu *et al*., 2009; Su *et al*., 2011; Zhang *et al*., 2015). It was found that the biological interpretation of detected genes was mostly consistent between the data of the two technologies, and that RNA-seq provides better detection sensitivity than does microarray. To validate the accuracy of these techniques, it is necessary to quantitatively evaluate the expression levels of genes detected as having altered expression by DNA microarray or RNA-seq. Therefore, Real-Time Reverse-Transcriptase PCR is performed after global analysis in most studies.

2.1 DNA microarray

DNA microarray analysis was established in 1995 as a new technology to analyze gene expression (Schena *et al*., 1995). Since then, microarrays are being widely used across biological disciplines. The number of published papers using the technology continues to increase, as does the number of commercial suppliers of

microarrays, associated reagents, and analysis hardware and software (Kawasaki, 2006; Yauk and Berndt, 2007).

The microarray technique is based on the basic principle of "DNA Hybridization" and uses DNA chips consisting of many oligonucleotides (probes) spotted onto a glass slide. Probes are deposited onto glass slides via the direct (*in situ*) synthesis of oligonucleotide probes onto the chip surface using photolithographic methods (Gao *et al*., 2004) or by deposition methods, which include contact-spotting using pins and deposition by ink jet (Hughes *et al*., 2001; Sethi *et al*., 2008). Presently, these arrays comprise mostly 40- to 70-mer oligonucleotides spotted on a glass slide. This technique makes it possible to semi-quantitatively measure the expression levels of large numbers (1,000-40,000) of genes simultaneously. In conjunction with computational analysis tools, microarray analysis enables the identification of genes that vary in expression in different biological contexts (Schena *et al*., 1995; Quackenbush, 2001).

The microarray method consists of several processes. Total RNA is extracted from the sample and reverse-transcribed into complementary DNA (cDNA). cDNA is labeled with fluorescent dyes, hybridized to the DNA chip, and scanned to produce microarray image data. The intensity of fluorescence, which reflects the degree of

hybridization and transcript copy number, is digitized by appropriate software. However, microarray has several limitations including requiring information about genome sequence to produce the oligonucleotides, and inaccuracy of data owing to high background from nonspecific cross-hybridization.

2.2 RNA-seq

RNA-seq is a new approach involving next-generation sequencing and allows a quantitative analysis of all expressed genome regions (Wang *et al*., 2009). The RNA-seq method consists of several steps: extraction of total RNA, reverse transcription of RNA to cDNA, construction of a cDNA fragment library, sequencing using a high throughput sequencing platform, generation of single-end or paired-end reads 30–400 base pairs in length, and sequence alignment (Wang *et al*., 2009; Griffith *et al*., 2015). Recently, a method allowing direct single molecule RNA sequencing, without prior conversion of RNA to cDNA, was reported (Ozsolak *et al*., 2009).

RNA-seq captures RNA directly to build a sequence, allowing for the detection of new transcription products, fused sequences, and single nucleotide polymorphisms of unknown genes without gene-specific biases. Additional advantages of RNA-seq

include low background noise, large and dynamic signal range, and detection with no requirement for prior sequence information. More recently, RNA-seq has emerged as the preferred approach for genome-wide expression analysis (Wang *et al*., 2009; Rowley *et al*., 2011; Su *et al*., 2014) .

3. Transcriptomics for physiological difference

DNA microarray and RNA-seq technology provide a wide range of novel application opportunities relating to gene expression profiles, which can be applied to various studies. The availability of transcriptomic technology has provided new opportunities for researchers to characterize global gene expression profiles. Recent advances in transcriptomics have allowed us to identify specific genes, gene families, and pathways associated with biological responses. The mechanisms regulating biological reactions, as well as the identification of genes implicated in these responses, are of great interest to the research community. These techniques could serve to assign functions to previously unannotated genes and to allocate gene groups to functional pathways (DeRisi *et al*., 1996; Han *et al*., 2015). Additionally, these techniques contribute to our understanding of biological mechanisms and responses to environmental stimuli (Miller and Tang, 2009). The identification of differentially

expressed genes is helpful to show the biological distinction and physiological difference between two different sets of conditions.

3.1 Evaluation for genetic background

Organisms sustain biological activity based on information contained within the genome. The characteristics of the individual are distinctively determined by genetic information, the expression of which defines the phenotype (sex, aging, tissues, individuals, and species). Detection of genes differentially expressed between these phenotypes helps to characterize the sample and allows us to evaluate individual subjects.

Differences in gene expression resulting from changes in intrinsic conditions such as sex (Balakrishnan *et al*., 2012; Blekhman *et al*., 2010; Conforto and Waxman, 2012; Jansen *et al.*, 2014; Caetano-Anolles *et al*., 2015; Sakashita *et al.*, 2015) and aging (Brink *et al*., 2009; Wilson *et al*., 2010; Takahashi *et al.*, 2011; Naumova *et al.*, 2012; Steegenga *et al*., 2014; Roux *et al*., 2015; Wei *et al*., 2015) can be identified using microarray and RNA-seq techniques. Liu *et al*. (2013) reported that aging results in sexually dimorphic changes in the skeletal muscle transcriptome, and they detected differential expression of genes related to oxidative

phosphorylation, immune function, and muscle protein catabolism. Data show that gene expression dynamics related to aging vary according to sex, and suggest that older women tend to be more predisposed to loss of muscle function with aging. Many studies show that normal tissues have their own gene expression profiles and have identified organ specific gene sets that are highly expressed in a tissue selective manner in the mouse (Su *et al*., 2002), rat (Walker *et al*., 2004), dog (Briggs *et al*., 2011), pig (Hornshøj *et al*., 2007), and human (Hsiao *et al*., 2001; Shmueli *et al*., 2003; Son *et al*., 2005; Kilpinen *et al*., 2008). Transcriptomics can also be used to compare gene expression data across species (Chan *et al*., 2009; Merkin *et al*., 2012; Sudmant *et al*., 2015). These studies provide new insights into the molecular basis of tissue and organismal diversity. Transcriptomics can be used to identify genes that contribute to this diversity, and can be utilized to build a biological gene database.

3.2 Evaluation for environmental effects

Recent progress in transcriptomics enables us to identify genes and pathways associated with responses to exogenous abiotic stresses (Ma *et al*., 2012; Jogaiah *et al*., 2013; Deshmukh *et al*., 2014; Evans, 2015). Many studies have used DNA

microarrays to infer how organisms respond to different environments (Gracey and Cossins, 2003; Cossins *et al*., 2006; Gracey, 2007; Evans and Hofmann, 2012), such as temperature (Murata *et al*., 2006; Yang *et al*., 2010; Long *et al*., 2012; Aguado-Urda *et al*., 2013; Logan and Buckley, 2015), osmolality (Posas *et al*., 2000; Evans and Somero, 2008; Melamed *et al*., 2008; Halbeisen and Gerber, 2009), oxygen (Ton *et al*., 2003; Garnczarska, 2006; Swiderek *et al*., 2008; Otsuka *et al*., 2010; Gracey *et al*., 2011; Shinde *et al*., 2015) and pH (Leaphart *et al*., 2006; Serrano *et al*., 2006; Worden *et a*l., 2009; Evans *et al*., 2013). Detection of genes with expression changes in response to environmental change helps to predict the fragility, resistance, and adaptability of an organism, tissue, or cell in the environment. Additionally, genes with constant expression in the presence of environmental change can be identified and are potentially important for overall survival. Transcriptomics using RNA-seq in fishes have reported many new genes that participate in metabolic functions, protein folding and degradation, developmental processes, oxygen transport, and protein synthesis (Liu *et al*., 2013). These studies have also identified heat shock protein genes that are differentially expressed following alterations in temperature (Liu *et al*., 2013; Smith *et al*., 2013). Coble *et al*. (2014) identified differential expression of genes related to decrease

internal temperatures, reduced hyperthermia-induced apoptosis, and promotion of tissue repair occurring in the liver of heat-exposed broiler chickens. In addition, they also found that the expression of genes involved in the regulation of perturbed cellular calcium changes following heat exposure.

Moreover, an adaptive response to various exogenous environmental stresses, including osmotic pressure and starvation, was validated at a genome level using RNA-seq (Xia *et al*., 2013; Johnson *et al*., 2015). RNA-seq is being applied to study the stress response similar to how microarray is being used assess the physiological state.

Transcriptomics has been especially useful in the field of experimental embryology, where it has been used to evaluate the *in vitro* and *in vivo* environments. Transcriptomic data of embryonic cells produced *in vitro* and those developed *in vivo* have been compared in the mouse (Ren *et al*., 2015), pig (Østrup *et al*., 2013; Whitworth *et al*., 2015), bovine (Driver *et al*., 2012; Degrelle, 2015), and sheep (Wei *et al*., 2016). These studies have identified crucial discordances between the *in vitro* and *in vivo* expression of several genes and gene pathways. Individual genes, and pathways, function in complex biological processes. Minor changes in the expression of several genes may perturb a pathway and possibly have drastic

biological effects (Han *et al.*, 2015). Transcriptomics helps researchers to identify differences in embryo gene expression *in vivo* and *in vitro*. These analyses may assist in improving culture conditions so that *in vitro* analyses can more accurately represent *in vivo* physiological conditions.

3.3 Evaluation for toxicological damage

Evaluation of chemical stress or toxicogenomics is critically important for transcriptome analysis. Afshari *et al*. (1999) and Nuwaysir *et al*. (1999) demonstrated the efficacy of microarray as a tool for assessing chemical and environmental toxicity in a bioassay. Our group has applied microarrays to examine the molecular response of a yeast model using various toxic materials. We have detected differentially expressed genes and determined the mechanism against the toxic matter for each of terpinene (Parveen *et al*., 2004), dimethyl sulfoxide (Murata *et al*., 2003), mycotoxin citrinin (Iwahashi *et al*., 2007), thorium (Mizukami-Murata *et al*., 2006), cadmium (Momose and Iwahashi, 2001), and thiuram (Kitagawa *et al*., 2002) (Table 2).

The application of microarray to toxicogenomics is not limited to yeast cells. Various different models are being used to assess cadmium stress, including fungus

(Zhao *et al*., 2015), plants (Oono *et al*., 2014; He *et al*., 2015; Xu *et al*., 2015; Oono *et al*., 2016), fish (Bougas *et al*., 2013), mollusk (Meng *et al*., 2013), mouse (Hu *et al*., 2014), and human cells (Cartularo *et al*., 2016). Huang *et al*. (2012) reported that perfluorooctane sulfonate affects the expression of genes related to neurobehavioral defects, mitochondrial dysfunction, and the metabolism of proteins and fats. Identification of differentially expressed genes helps to reveal statistical significance (e.g. fold change and significance test) and/or biological significance (Tseng *et al*., 2012) and to clarify the mechanisms regulating adaptive responses. RNA-seq can also be applied to study toxicogenomics and has been used to evaluate changes in miRNA expression in response to multiple environmental factors including arsenic (Zhang *et al*., 2016), cigarette smoke (Beane *et al*., 2011; Hackett *et al*., 2012), the carcinogen benzo[a]pyrene (Van Delft *et al*., 2012) and gamma-irradiation (Moskalev *et al*., 2014).

We are exposed to many substances that can have a direct or indirect influence on us including drugs, additives, and toxic chemicals. Risk assessment for these materials using transcriptomics is a means to evaluate the degree of toxicity, or risk, that such substances pose to an organism.

3.4 Evaluation for diseases

Comparison of genome-wide expression patterns among patient samples presents us with measurable information and helps to identify genes that would be reasonable targets for therapeutic intervention (Afshari *et al*., 1999; Chin and Kong, 2002; Dudda-Subramanya *et al*., 2003; Saei and Omidi, 2011). The invention of technologies for transcriptomics, using genome-wide analysis and computational approaches, has made it possible to identify the prognostic significance of individual gene expression changes from thousands of markers. For example, in cancer studies, this technology is employed to obtain comprehensive gene expression profiles in both normal tissues (Saito-Hisaminato *et al*., 2002) and cancer tissues including those from hepatocellular carcinomas, pancreatic cancers, and esophageal squamous cell carcinomas (Okabe *et al*., 2001; Han *et al.*, 2002; Macgregor, 2003; Nakamura *et al*., 2004; Yamabuki *et al.*, 2006; D'Angelo *et al*., 2014; Zhu and Tsao, 2014; Nishimura *et al.*, 2015). Using microarrays, biomarkers for Parkinson's disease (Alonso-Navarro *et al*., 2014; Sun *et al*., 2014) and myocardial infarction (Devaux *et al*., 2010; Głogowska-Ligus and Dąbek, 2012) have been identified. Additionally, DNA microarrays are used to study complex diseases, in which hundreds of genes are often implicated, such as allergies,

diabetes, and obesity (Rome *et al*., 2009; S. Wang *et al*., 2009; Rodríguez-Acebes *et al*., 2010; Liu *et al*., 2013; Lu and Liao, 2015). Some reports indicate that these diseases rely on multiple gene interactions, rather than changes in a single causal gene, and that many different mechanisms and pathways are linked together (Benson and Breitling, 2006). While more illness biomarkers have been identified using microarray, RNA-seq is emerging as a very powerful tool to identify biomarkers of cancer (Wood *et al*., 2007; Berger *et al.*, 2010; Pflueger *et al*., 2011; Cancer Genome Atlas Research Network., 2013; Fumagalli *et al*., 2014; Kosti *et al.*, 2016), Alzheimer's disease (Satoh *et al*., 2014), tuberculosis (Zhang *et al.*, 2014), and cirrhosis (Tan *et al*., 2014). RNA-seq may be a more suitable platform for the search of precise biomarkers than traditional omics approaches, including microarray or proteomics because of its ability to detect novel genes/exons, RNA editing, fusion transcripts, and allele-specific expression. Still, RNA-seq does have limitations and issues resulting from several biases including experimental/technical procedures, downstream computational analyses, and informatics infrastructures (Costa *et al*., 2013).

Genome-wide analysis has boosted the biomarker diagnostics industry and contributes to disease subtype classification, disease diagnosis and prognosis,

selection of therapeutic treatments, and disease prevention (He *et al*., 2006; Sun *et al*., 2013; Su *et al*., 2014; Aibar *et al.*, 2015).

4. Transcriptomics for physiological equality

Most transcriptome analyses have been aimed at detecting genes with altered expression levels. Many researchers analyze expression patterns to identify a characteristic expression pattern following exposure to change, as detailed in the previous section. Although transcriptome analysis has been a powerful tool for biological and biomedical studies, it remains to be determined whether these technologies can be applied with high accuracy and precision. Proof of invariability or/and constancy of gene expression profiles provides internal evidence of biological stability. Transcriptome analysis enables us to check the technical/biological uniformity using genome-wide screening for gene expression.

4.1 Capacity

Gene expression profiles reflect biological capacity and vary according to the situation at the time. For example, expression profiles change during the process of differentiation and generation (Mansergh *et al*., 2009; Goggolidou *et al*., 2013;

Iruretagoyena *et al*., 2014; Shiraki *et al*., 2014; Alonso-Martin *et al*., 2016). DNA microarray is tool used to evaluate cell properties by comparing the expression profiles of all genes. In some stem cell research reports, the degree of differentiation and development is evaluated by examining the similarity of gene expression patterns. Global gene-expression patterns were compared between human induced pluripotent stem (iPS) cells and human embryonic stem (ES) cells using oligonucleotide DNA microarrays (Takahashi *et al*., 2007). A high correlation of global gene-expression patterns was found between iPS cells and ES cells, suggesting that established iPS cells are similar to ES cells. Hrvatin *et al*. (2014) reported that differentiated human stem cells are analogous to fetal β cells rather than adult β cells. Mishra *et al*. (2008) demonstrated that human bone marrow-derived mesenchymal stem cells exposed to tumor-conditioned medium over a prolonged period assumed a carcinoma associated fibroblast-like myofibroblastic phenotype. Handel *et al*. (2016) generated transcriptome data to compare iPS cell derived neurons to human fetal and adult brain and indicated that iPS cell-derived cortical neurons closely resembled primary fetal brain cells. Tanaka *et al*. (2013) confirmed that the human iPS cell derived myogenic differentiation cells were similar to those of perfectly differentiated human

myoblast cells and quite divergent from those of undifferentiated iPS cells. Therefore, transcriptomics provides evidence for the establishment of cell-specific identities.

Moreover, Datson *et al*. (2007) reported the comparison of gene expression profiles between tissues in the same individual. This study reported that a high correlation coefficient was obtained when comparing gene expression in marmoset neuronal tissues (hippocampus and cortex) indicating a high degree of similarity in expression profiles. Additionally, comparison of hippocampal gene expression with that of all peripheral tissues resulted in a severe drop in the correlation coefficient. Thus, transcriptome analysis is useful to demonstrate biological similarity between cells or tissues, and for the determination of genetic characteristics.

4.2 Reproducibility

Biological experiments need to be reproduced multiple times under the same experimental conditions. To demonstrate evidence of reproducibility is important for data to be comparable (Chen *et al*., 2007; Darbani and Stewart, 2014). The Micro Array Quality Control (MAQC) project was established to construct quality control and standardization tools using four titration samples which are measured

on seven microarray platforms and three alternative gene expression technologies. The MAQC project had already proven the reproducibility of microarray data by the quantitative signal values and the qualitative detection calls (MAQC Consortium *et al*., 2006). To validate and extend these observations, numerous researchers have independently validated microarray data (Yang *et al*., 2002; Burgoon *et al*., 2005; Guo *et al*., 2006; ’t Hoen *et al*., 2008).

In addition to the technical reproducibility of microarray results, biological and physiological reproducibility are also important. Iwahashi *et al*. (2009) and Takahashi *et al*. (2012) demonstrated the importance of reproducibility of expression profiles among individuals under the same experimental conditions. This reproducibility proved the stability of an experimental protocol that affected the biology and physiology. Therefore, the reproducibility of gene expression patterns observed under the same experimental conditions suggests that the experimental and analysis methods used are stable and robust. However, MAQC does not provide conclusions related to inter-platform compatibility. There are differences in the fluorescent intensities measured by different platforms, and even within each platform site-by-site variability exists (Chen *et al*., 2007).

Reproducibility of RNA-seq has been demonstrated by multi-group reports (Tang *et al*., 2015), across laboratories (’t Hoen *et al*., 2013), and among technical replicates (Marioni *et al*., 2008; Mortazavi *et al*., 2008; Anders and Huber, 2010; Bullard *et al*., 2010; Ozsolak *et al*., 2010; Roberts *et al.*, 2011). Danielsson *et al*. (2015) compared RNA-seq data sets of human brain, heart, and kidney samples from different laboratories and studies and concluded that RNA-seq expression measurements show global consistency after log transformation and elimination of batch effects. To establish the reproducibility and comparability of RNA-seq, the RNA Sequencing Quality Control (SEQC) project was constituted and coordinated by the Food and Drug Administration. The role of the SEQC is to assess the performance of RNA-seq across laboratories and to dissect different sequencing platforms and data analysis pipelines (SEQC/MAQC-III Consortium, 2014).

4.3 Stability

It is essential that there is a high correlation and reproducibility within and between replicated experiments (within established standards) for data to be considered reliable and robust (Yauk and Berndt, 2007). In 2001, the Functional Genomics Data Society (http://fged.org/) described the Minimum Information About a

Microarray Experiment (http://fged.org/projects/miame/) to establish a standard for recording and reporting microarray-based gene expression data (Brazma *et al*., 2001). They provide six factors for standardization: experimental design, array design, sample, hybridization, measurements, and normalization. However, this report does not indicate the most suitable standardized analytical methods, which may result in the variance of data.

To overcome the dispersion of microarray data, standardization is carried out in various experimental models such as yeasts (Mizukami *et al*., 2004; Taymaz-Nikerel *et al*., 2016), mice (Williams *et al*., 2004), rats (L. Guo *et al*., 2006), and non-human primates (Ebeling *et al*., 2011). Microarray is employed to prove the stability of both experimental conditions and experimental subjects. Iwahashi *et al*. (2009) used genomics to report on the physiology of *medaka,* which are used as a model animal for toxicity testing. *Medaka* mRNA expression was measured in individuals maintained within, as well as beyond, the Organization for Economic Cooperation and Development (OECD) guidelines for the fish acute toxicity test. They found that the toxic environment specified within the OECD guidelines did not affect the expression profiles of *medaka* and indicated that extraordinary conditions, beyond the guidelines, decreased reproducibility of data.

Takahashi *et al*. (2011) conducted microarray analysis to evaluate variations in whole blood gene expression patterns in different individual miniature pigs at different ages. The number of expressed genes and variation in gene expression intensity within miniature pigs of the same age were observed to converge with aging, and gene expression became uniform after 20 weeks of age. This report reveals the age at which genetic uniformity of the large animal model was reached. It is a basic concept of biological experiments that all conditions, except the variable being measured, must be the same. Demonstration of biological standardization and uniformity of genetic background using transcriptomics provides great value for laboratory animals in biological experiments. Such analyses allow for efficient and accurate experimental results and contribute to the standardization of breeding and rearing methods.

4.4 Margin of safety

Few studies have applied transcriptomics to the issue of food safety. We have introduced this new application of transcriptomics (Miura *et al*., 2016). We have used transcriptomics to demonstrate the safety of a diet consisting of a by-product of Japanese liquor production (shochu distilled water: SDW) for use as pig feed.

We evaluated the expression profiles of pigs fed with SDW, hyperlipid diet, and feed containing toxicant. We observed a high correlation between the gene expression profiles of the control and SDW feeding groups. Furthermore, the expression profiles of these two groups were different from those of the hyperlipidemia and toxicant model groups. These data indicated that feeding with SDW did not have a physiological effect on the pigs and assessed such feeding as safe. Therefore, microarray can be used to test foods and demonstrate proof of similarity with the normal state, making it a valuable approach for evaluating safety.

In the medical field, preclinical and clinical drug safety studies are a key prerequisite of the drug approval process. Non-human primates and pigs are important models for such studies. Ebeling *et al*. (2011) reported the genetic similarity of the non-human primate *Macaca fascicularis* to human. However, from the viewpoint of animal welfare, usage of these species in animal experiments has declined, and the opportunity of animal experimentation is limited. Active utilization of transcriptomics, which can obtain a large volume of information simultaneously, improves our understanding of the *in vivo* pharmacokinetics of model organisms and provides a significant contribution to the global “3R” animal

welfare initiative: reduce, refine, and replace animal experiments.

5. Conclusion

Transcriptomics has allowed us to simultaneously identify gene expression dynamics and differential gene expression. Transcriptomics is useful to identify illness biomarkers as well as biological responses to various stimulations and stresses, and plays a key role in advancing genomic and molecular biology research.

However, using this approach, we may overlook potentially important functions of genes that are not induced by the particular condition being examined. Not only should we concentrate on detecting specific differentially expressed genes, but we should also examine the entire expression profile. In addition, standardization of experimental conditions is essential and an absolute requirement for the legitimacy of the experiment. Uniformity of experimental conditions is brought about by adjustment of the technical, platform, and biological biases.

In conclusion, transcriptome analysis can be used to validate the standardization of an experiment by eliminating biological biases. As well as using transcriptomics to identify change or variability, we should take advantage of these approaches as

evidence of the invariance, constancy, and reproducibility of our system of interest (Figure 1).

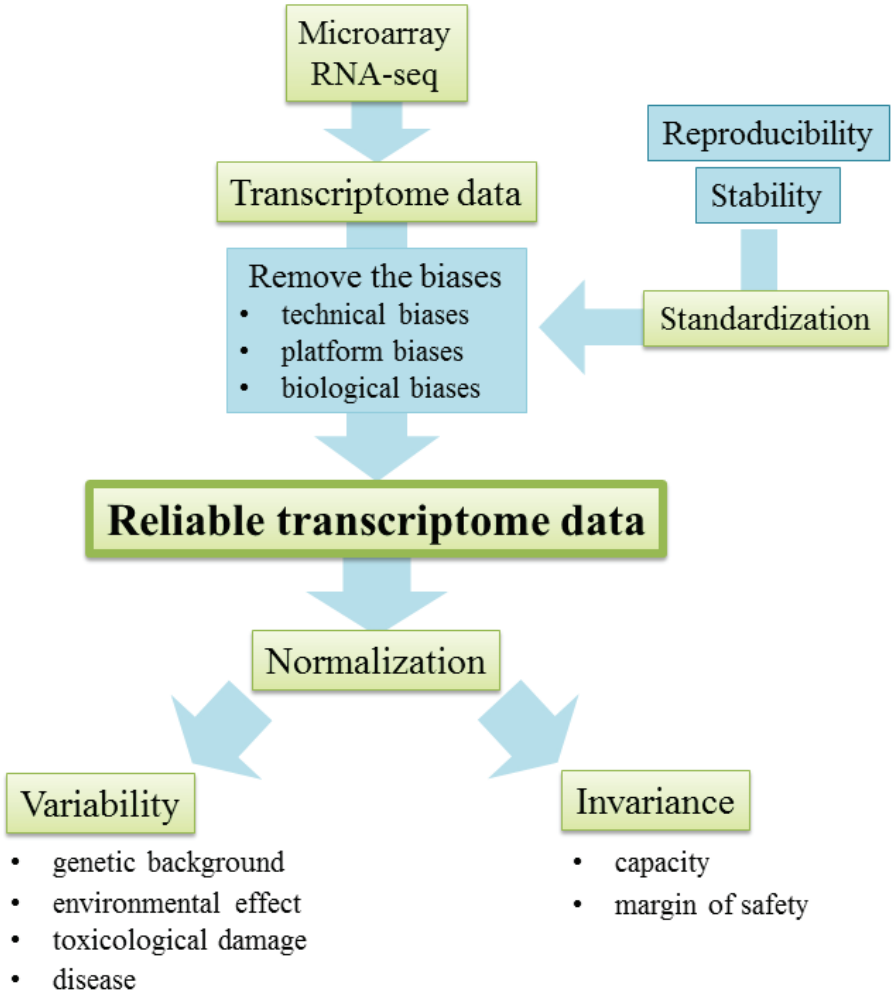
Table 1. List of major non-coding RNAs and their main functions

Function category	Type		Functions
protein synthesis	rRNA	ribosomal RNA	Constituent of ribosomes
	tRNA	transfer RNA	Transport of amino acids through ribosomes
transcriptional modification, replication of DNA	snoRNA	small nucleolar RNA	Chemically-modified of rRNA and other RNA; methylation and the genespseudouridine modification
	snRNA	small nuclear RNA	Functions of pre-mRNA splicing, rRNA processing and histone mRNA 3' end-formation
regulation of expression	piRNA	PIWI-interacting RNA	Inhibit the function of the transposon through PIWI protein, and maintains the genomic constancy of the germline cells
	miRNA	micro RNA	Modulate the post-transcriptional gene expression by binding to specific mRNA
	siRNA	small interfering RNA	Repress gene expression after transcription called the RNA interference (RNA interference: RNAi)
	xiRNA	X-inactivation RNA	Inactivate an X chromosome
	tiRNA	transcription initiation RNA	Regulation of transcription by targeting epigenetic silencing complexes
	lincRNA	large intergenic RNA	Repress the transcriptome response depend on p53
	pRNA	promoter-associated RNA	Mediate transcriptional gene silencing and transcriptional gene activation

Table 2. Evaluation of toxicity by yeast DNA microarray

toxic material		function of differentially gene up-regulate gene	down regulate gene	main mechanisms of action of toxicity	
terpinene	antifungal drug	lipid and fatty acid metabolism, cell wall structure and organization, detoxification, cellular transport	protein and carbohydrate metabolism, synthesis, transcription	cell wall structures	Parveen *et al*., 2004
dimethyl sulfoxide	cryoprotectant	phospholipid biosynthesis, the methionine synthesis	energy (mitochondrial respiration-related genes), ion homeostasis, protein synthesis	synthesis and maintenance of the cell wall	Murata *et al*., 2003
mycotoxin citrinin	fungal secondary metabolite	metabolism, cell rescue, defense and virulence, energy	glutathione synthesis, cell rescue, defense and virulence	oxidative stress	Iwahashi *et al*., 2007
thorium	radioactive material	oxidative stress, glycogen and trehalose metabolism, cell wall damage		cell wall damage, oxidative stress	Mizukami-Murata *et al*., 2006
thiuram	pesticide	cellular transport, carbohydrate metabolism, protein destination, transport facilitation, detoxification, stress response	transcription, cell growth, protein synthesis, carbohydrate metabolism, cellular communication, DNA, lipid etc. metabolism	membrane factors, transport activity, protein denaturing and degradation, oxidative stress, DNA damage	Kitagawa *et al*., 2002

Figure 1. Recommended workflow of transcriptomics



CHAPTER II

Oligonucleotide microarray analysis of dietary-induced hyperlipidemia gene expression profiles in miniature pigs

1 Introduction

Hyperlipidemia is well recognized as a risk factor for cardiovascular disease (CVD). As diet represents the most important determinant of hyperlipidemia, dietary animal models can be useful for the study of CVD progression (Lissner and Heitmann, 1995). High-fat, high- cholesterol, and high-sugar diets have been shown to induce hyperlipidemia, obesity, and insulin resistance in humans and rodents (Russell and Proctor, 2006; Oron-Herman *et al*., 2008; Radonjic *et al*., 2009). Dietary-induced hyperlipidemia pig models have also been established (Kobari *et al*., 1991; de Smet *et al*., 1997; Orbe *et al*., 2001; Bowles *et al*., 2004; Yin *et al*., 2004; C. Zhang *et al*., 2006; De Keyzer *et al*., 2009).

Compared to rodents, pigs are a useful animal model for elucidating the molecular mechanisms underlying the transition from a healthy state to the progression of diseases caused by hyperlipidemia because they are able to breed stably over a long period, and have a similar anatomy and digestive physiology to humans (Simon and Maibach, 2000; Lunney, 2007). In addition, miniature pigs are easier to breed and to handle than other non-primates are, making them a convenient species for preclinical tests (Vodicka *et al*., 2005). In September 2003, the Swine Genome Sequencing Consortium (SGSC) was formed to promote pig genome sequencing under international coordination (Schook *et*

al., 2005). The swine research environment has been enhanced since members of the SGSC announced a completed swine genome map in November 2009 (Archibald *et al*., 2010). To evaluate temporal changes in gene expression profiles with the progression of dietary-induced alterations, minimally invasive blood sampling, which allows for the direct measurement of immune-responsive blood cells, excels over other invasive biopsy techniques for disease diagnostics and assessment of drug responses, as well as health monitoring. If biomarker candidate genes can be identified from blood analyses, these may be useful for diagnosis in humans. Use of whole blood is preferable to other specimens on two accounts. Firstly, RNA expression and degradation are susceptible to artificial manipulations such as cell separation and extraction. Whole blood manipulation can reduce these risks via the use of RNA blood collection tubes. Secondly, whole blood is an attractive prime tissue due to its critical role in immune responses, metabolism, and communication with cells and the extracellular matrix in almost all body tissues and organs. Whole blood will depart from the normal state when a considerable alteration occurs in some blood cell subpopulations, tissues, or organs. Moreover, blood samples can be obtained repeatedly from miniature pigs, and blood RNA contains an enormous amount of information on the expression of messenger RNA and non-coding functional RNA molecules that are not translated into proteins.

Thus, analysis of blood RNA provides an opportunity to detect subtle changes in physiological state. We consider it particularly important to identify gene expression characteristics in whole blood. Microarray techniques allow the detection of genome-wide perturbations in response to different treatments and the measurement of various responses using a multitude of gene probes. Toxicogenomics, in which microarray techniques are specifically used in toxicology tests, has been widely recognized as one of the standard safety procedures for chemicals (Tong *et al*., 2003; Pennie *et al*., 2004; Williams-Devane *et al*., 2009). Gene expression microarrays have been used particularly for the screening of genes involved in specific biological processes of interest. Microarrays also allow the clustering of genes according to similar patterns of expression or functions. In this study, we conducted a series of whole blood microarray experiments to evaluate long-term alterations during 27-week feeding periods using specific pathogen-free (SPF) miniature pigs. There are two main types of dietary protocols for hyperlipidemia pig models, one with cholesterol and animal lipids (Kobari *et al*., 1991; de Smet *et al*., 1997; Orbe *et al*., 2001; Bowles *et al*., 2004; De Keyzer *et al*., 2009), and the other with cholesterol, animal lipids, and sucrose (Yin *et al*., 2004; Zhang *et al*., 2006). Some studies have focused primarily on a subset of genes, but this approach cannot elucidate whole blood RNA profiles during the process of

change. We selected two typical dietary protocols. One was a high-fat and high-cholesterol diet (HFCD) containing 15% lard and 2% cholesterol; the other was a high-fat, high-cholesterol, and high-sucrose diet (HFCSD) containing 15% lard, 2% cholesterol, and 37% sucrose. The present microarray analyses of whole blood were conducted according to the following aspects. The first analysis dealt with similarity among individuals based on the correlation coefficient. Variation among individuals of the same dietary group and between the different dietary periods was examined. The second analysis addressed the function of genes. Up- or down-regulated genes for each dietary protocol were examined by functional categorization. While whole blood RNA derives from white blood cell RNA, whole blood gene expression profiles may not entirely correspond to those of white blood cells (Takahashi *et al*., 2011). White blood cell microarray analyses conducted at the end of each dietary period are greatly influenced by diet, and the variations between the expression profiles of white blood cells and whole blood were assessed for each dietary group

2 Materials and Methods

Animals

Fifteen 12-week-old, male Clawn miniature pigs were housed individually in cages of

1.5 m^2 at the breeder's specific pathogen free (SPF) facility (Japan Farm Co., Ltd, Kagoshima, Japan) for 27 weeks. Body weights at the beginning of the experiment were 5.1 (2.6) kg (mean (standard deviation; SD)). During this period, 5 pigs were fed with 450 g/day standard dry feed (Kodakara73, Marubeni Nisshin Feed Co., Ltd., Tokyo Japan), and had unlimited access to water (control group). Five pigs were fed a high-fat, high-cholesterol diet containing 15% lard and 2% cholesterol (HFCD group). The 5 remaining pigs were fed a high-fat, high-cholesterol and high-sucrose diet containing 15% lard, 2% cholesterol, and 37% sucrose (HFCSD group). During dissections, the heart, liver, kidney, stomach, and spleen were excised and weighed immediately.

Hematology and clinical chemistries

Blood samples were collected from the superior vena cava after 5, 10, 14, 19, 23, and 27 weeks of the feeding period. Blood (EDTA), plasma (EDTA), and serum samples for hematology and biochemical tests were collected 24 hours after fasting. Hematology and biochemical tests were conducted by Clinical Pathology Laboratory, Inc. (http://www.patho.co.jp/index.html) (Kagoshima, Japan) using standard clinical methods.

MIAME compliance and data availability

The microarray experiments described in this manuscript were MIAME compliant and the raw data have been deposited in the Gene Expression Omnibus (GEO) database (Accession number GSE 32616, http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE32616.)

Preparation of samples and microarray assays

Whole blood samples for microarray analyses were collected from each subject in PAXgene™ tubes (Qiagen/BD GmbH, UK), incubated at room temperature for 4 hours for RNA stabilization, and then stored at -80°C. RNA was extracted from whole blood using the PAXgene™ Blood RNA System Kit (Qiagen GmbH, Germany) according to the manufacturer's guidelines. RNA from white blood cells was extracted from whole blood samples using a LeukoLOCK Total RNA Isolation kit (Ambion, Austin, TX). Isolations were performed according to the manufacturer's protocol. The quality of the purified RNA was verified using an Agilent® 2100 Bioanalyzer (Agilent Technolo- gies, Santa Clara, CA). RNA concentrations were determined using a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Fluorescent cyanine 3-CTP– labeled cRNA was used for hybridization onto porcine oligo microarray slides

(#G2519F#20109, Agilent Technologies) containing 43,603 oligonucleotide probes at 65℃ for 17 h. The hybridized microarray slides were washed according to the manufacturer's instructions and were scanned with an Agilent DNA Microarray Scanner (#G2565BA, Agilent Technologies) at 5-μm resolution. The scanned images were analyzed numerically using Agilent Feature Extraction Software version 9.5.3.1. (Agilent Technologies).

Microarray data analysis

Normalized data using quantile normalization were analyzed using GeneSpring GX software version 10.0.1 (Agilent Technologies). The Gene Ontology (GO) Database (http://www.geneontology.org/) was used to categorize gene expression profiles functionally. GO terms were obtained from the TIGR pig gene indices, Porcine version 14.0 3-11-10 (http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=pig). The TC Annotator List includes the gene number and the GO terms. Out of the 43,603 probes used in the Agilent porcine microarray (#G2519F#20109), GO annotations were available for 6,019 genes. Microarray cDNA probes were classified according to GO terms for different biological processes. For the microarray data analyses, we focused particularly on the variation of dietary-related gene expression profiles. Initially,

microarray spots of interest were divided into 2 groups: ‘‘absent’’ and ‘‘present,’’ using the flag values provided by the scanner. Background levels were determined from the spots outside of the gene probing area. ‘‘Absent’’ was assigned to spots with a signal intensity that was less than that of the background level, while the rest were marked “present”. Only data for ‘‘present’’ spots were used for the analyses. The intensity ratio of white blood cell gene expression to that of whole blood is a contribution indicator for white blood cell RNA to whole blood RNA. The relation of tissues or organs ESTs to the white blood cell contribution indicator was examined. To focus on obesity-related organs, i.e., the liver, adipose tissue, and muscle, the relative EST numbers of these organs to blood ESTs for each gene were calculated using EST profiles from the Unigene NCBI database of the transcriptome. An EST profile breakdown of 22,000 porcine genes by body site is available, comprising 40 organ types, such as the lung, ovary, liver, adipose tissue, muscle, and blood. The profiles show gene expression patterns inferred from EST counts and cDNA library sources (http://www.ncbi.nlm.nih.gov/UniGene/).

Statistical analysis

Continuous variables were analyzed using a one-way factorial ANOVA followed by a

Tukey-Kramer multiple comparisons test for multiple groups. After excluding the unexpressed genes from each set of array data, Pearson correlation coefficients were calculated to identify similarities in gene expression among individuals. Pearson correlation coefficients were analyzed by a one-way factorial ANOVA using Fisher's Z-transform to normalize the correlation distribution. Correlations were considered statistically significant for ANOVA tests among all groups and *t*-tests between 2 groups when $p < 0.05$. All values were expressed as non-transformed mean (standard deviation (SD)). Genes with a fold change greater than 2.0 ($p < 0.05$) and less than 0.5 ($p < 0.05$) after 10, 19, and 27 weeks were identified. These genes were mapped to the Gene Ontology and KEGG pathway in the Database for Annotation, Visualization and Integrated Discovery (DAVID Bioinformatics Resources 6.7, National Institute of Allergy and Infectious Diseases, http://david.abcc.ncifcrf.gov/) (Dennis *et al*., 2003; D. W. Huang *et al*., 2009). Chi-square tests were performed for feature extractions of GO terms. The expected values were the number of up- and down-regulated genes bearing all GO annotations, and the observed values were specific to each GO term. Simple linear regressions were performed for the scatter plots to obtain the slopes and intercepts, and the significance of each regression slope was verified.

Ethical considerations

All experimental protocols were approved by the Committee for the Care and Use of Experimental Animals at AIST (Permit Number: 2009-055A).

3 Results

Characteristics of study subjects

Temporal changes in mean body weights for the 3 dietary groups are shown in Figure 2. One-way ANOVA analysis for dietary-related variation revealed no significant difference at any feeding period except at week 12. In this study, the term ‘‘week’’ refers to the dietary period and not to the period since birth, unless otherwise stated. Table 3 lists the fasting plasma triglyceride concentrations for the group fed the high-fat, high-cholesterol diet (HFCD) and the group fed the high-fat, high-cholesterol, and high-sucrose diet (HFCSD). Almost no changes were observed in fasting plasma triglyceride levels. Fasting plasma total cholesterol concentrations had increased in the HFCD group and the HFCSD group by week 5 of the feeding period ($p < 0.001$) and were maintained between 350 and 1150 mg/dL from weeks 10–27 (Table 4). Fasting plasma high-density lipoprotein cholesterol (HDL-C) concentrations increased and showed significant differences ($p < 0.001$) from weeks 10–27 between two dietary

treatment groups and control (Table 5). Fasting plasma low-density lipoprotein cholesterol (LDL-C) concentrations also increased and showed significant differences from weeks 5–27 between two dietary treatment groups and control (Table 6). Fasting plasma glucose concentrations remained unchanged (Table 7). The number of white blood cells and the ratios of granulocytes (basophiles, eosinophils, neutrophils, lymphocytes, and monocytes) to white blood cells were not statistically significant among the three test groups (Table 8–13). The liver ($p < 0.001$) and spleen ($p < 0.01$) weights were increased significantly compared to the controls in both the HFCD and HFCSD groups. In contrast, the heart, kidney, and stomach weights remained unchanged (Table 14).

Microarray gene expression profiles – Correlation of gene expression

RNA analyses were conducted on blood samples obtained at weeks 10, 19, and 27 of the feeding periods to characterize the dietary effects on gene expression profiles in whole blood and white blood cells of miniature pigs. Each RNA sample was analyzed by porcine gene expression microarray consisting of 43,603 oligonucleotide probes. We evaluated variation in correlation coefficients among individuals on the same diet and between different diet groups.

Pearson correlation coefficients were used for the correlation analysis. Correlation coefficients for 45 microarrays in total were obtained for a normalized signals log-scale after excluding ‘‘absent’’ spots, definition of ‘‘absent’’ were described in Materials and Methods. A color-coded pairwise correlation matrix is displayed in Figure 3. Figure 4 illustrates the mean correlation coefficients for gene expression profiles among individuals within the same dietary group, showed the individual difference of the gene expression profiles within the dietary groups during dietary period. Figure 5 presents the mean correlation coefficients for gene expression profiles among different diet groups. The correlation coefficients of whole blood expression profiles within the same diet groups were 0.97 (0.01) (mean (standard deviation; SD)), 0.94 (0.05), and 0.97 (0.01) for the control, HFCD, and HFCSD whole blood at 10 weeks, 0.94 (0.03), 0.93 (0.06), and 0.95 (0.01) at 19 weeks, and 0.95 (0.02), 0.95 (0.03), and 0.98 (0.01) at 27 weeks, respectively. The correlation coefficients of white blood cell expression profiles within the same dietary groups were 0.94 (0.05), 0.95 (0.03), and 0.96 (0.02) for the control, HFCD, and HFCSD groups at 27 weeks, respectively. Using Fisher’s Z-transformation to normalize the correlation distributions, no significant differences in correlation coefficients among dietary groups were observed at any period during the treatments. This indicates uniformity of dietary- induced hyperlipidemia for our protocols. Next, we

analyzed expression profile correlations among the different diet groups. In Figure 5, ‘‘control vs. HFCD’’ represents the mean correlation coefficient between control and HFCD group individuals. The whole blood correlation coefficients among the different diet groups were 0.95 (0.04), 0.97 (0.01), and 0.96 (0.04) for control vs. HFCD, control vs. HFCSD, and HFCD vs. HFCSD at 10 weeks, 0.93 (0.03), 0.94 (0.02), and 0.95 (0.03) at 19 weeks, and 0.95 (0.03), 0.91 (0.03), and 0.95 (0.03) at 27 weeks, respectively. The white blood cell correlation coefficients among the different diet groups were 0.94 (0.04), 0.94 (0.03), and 0.96 (0.02) for control vs. HFCD, control vs. HFCSD, and HFCD vs. HFCSD at 27 weeks, respectively. Correlations of whole blood expression profiles were statistically significant according to an ANOVA test among all groups at 27 weeks, as a low correlation coefficient was obtained for the control vs. HFCSD groups. This indicates HFCSD differs much from control group and slightly from HFCD 27 weeks in whole blood gene expression profiles.

Figure 6 displays the average correlation coefficients between whole blood and white blood cell expression profiles within the same dietary group. The correlation coefficients were 0.83 (0.04), 0.79 (0.07), and 0.74 (0.05) for control, HFCD, and HFCSD at 27 weeks, respectively. Significant differences were observed between the control and HFCSD groups according to an ANOVA analysis using Fisher’s Z-transform

($p < 0.01$).

Assigning known functions to gene expression – Gene ontology annotation

We identified up- and down-regulated genes and classified these according to function using information from the Gene Ontology (GO) Database to understand the observed differences in whole blood gene expression profiles for the different dietary groups. Top-ranked genes with fold changes in expression greater than 2.0 ($p < 0.05$; HFCD, Table 15; HFSCD, Table 16) and less than 0.5 ($p < 0.05$; HFCD, Table 17; HFSCD, Table 18) were selected at 10, 19, and 27 weeks. Genes TC440907, TC448587 (ABCA1), and TC438339 were ranked highest in HFCD and HFCSD during the dietary period. These genes were analyzed using the Database for Annotation, Visualization and Integrated Discovery (DAVID; Table 19, HFCD; Table 20, HFCSD). As a result, the GO categories of many genes up-regulated at the end of the 19- week dietary period in both HFCD and HFCSD groups were related to nucleotide binding (GO: 0000166, GO: GO: 0005524, 0005525, GO: 0017076, GO: 0019001, GO: 00032553, GO: 00032555, GO: 0032561). The GO categories of gene up- regulated after 19 weeks in the HFCD group only were related to catabolic processes (GO: 0009057, GO: 0019941, GO: 0030163, GO: 0043632, GO: 0044257, GO: 0044265,). Many genes down-regulated after 27

weeks in both HFCD and HFCSD groups were in the GO categories related to biological adhesion (GO: 0007155, GO: 0022610). In addition, many genes down-regulated at the end of the 27-week dietary period in the HFCSD group only were related to steroid metabolism and lipid biosynthesis (GO: 0006694, GO: 0008202, GO: 0008203, GO: 0008610, GO: 0016125, GO: 0016126). To investigate potential reasons for the differences in gene expression among the diet groups during the dietary period, Chi- square tests were performed to identify whole blood GO categories for each treatment group vs. the control group. The expected values represented the number of up- and down-regulated genes bearing all GO annotations at each period of the diet, and the observed values represented the number of up- and down- regulated genes bearing each specific GO term. A difference of $p < 0.05$ between groups was considered significant. To identify up- and down-regulated genes, we compared levels of expression for each gene between the control vs. HFCD groups and between the control vs. HFCSD groups at each period using Student's *t*-tests. As the lowest number of genes for which the expectation frequency reached 1 or higher was 140 according to the conditions of observed value, the GO terms, which involve more than 140 genes, were used for the Chi-square tests. The results of the Chi- square tests for up- and down-regulated genes are listed in Tables 21–24. The correlation coefficients of

constituent gene between whole blood and white blood cells at 27 weeks were calculated for each GO term. Table 21 lists the GO terms for which significant differences were observed in the HFCD and HFCSD groups relative to the expected values. Inflammatory response elements (GO:0006954) were repressed in the HFCD group, and were both induced and repressed in the HFCSD group. The correlation coefficients between whole blood and white blood cells for expression levels of inflammatory response genes were 0.92 (0.03), 0.97 (0.02), and 0.95 (0.02) for the control, HFCD, and HFCSD groups, respectively. Genes involved in reproduction (GO:0000003) were induced in the HFCD group, and were both induced and repressed in the HFCSD group. The correlation coefficients for expression levels of genes involved in reproduction between whole blood and white blood cells were 0.91 (0.02), 0.93 (0.03), and 0.88 (0.03) for the control, HFCD, and HFCSD groups, respectively.

Table 22 lists the GO terms for which significant differences were observed in the HFCD group compared to the expected values. Muscle contraction (GO:0006936) and locomotor behavior (GO:0007626) elements were both induced and repressed. Muscle organ development (GO:0007517) and metabolic processes (GO:0008152) were repressed.

Table 23 lists the GO terms for which significant differences were observed in the

HFCSD group compared to the expected values. Translation (GO:0006412), embryonic development ending in birth or egg hatching (GO:0009792), electron transport (GO:0006118), and transcription from the RNA polymerase II promoter (GO:0006366) elements were both induced and repressed. Positive regulation of growth rates (GO:0040010), nematode larval development (GO:0002119), intracellular protein transport (GO:0006886) and growth (GO:0040007) elements were induced. A cell surface receptor-linked signaling pathway (GO:0007166) and responses to hypoxia (GO:0001666) were repressed.

Table 24 lists the GO terms for which ratios to the expected values were unchanged in the HFCD and HFCSD groups. In addition, the ratios of up- and down-regulated genes to the each observed values were unchanged at 27 weeks.

Figure 7 depicts a scatter plot of correlation coefficients between whole blood and white blood cells for each GO term, selected for the Chi-square tests, at 27 weeks of each dietary treatment group relative to the control group. The slope of the HFCD to the controls regression line was 1.007 ($p < 0.001$). The slope of the HFCSD to the controls regression line was 1.097 ($p < 0.001$), indicating that the correlation coefficients between whole blood and white blood cell expression levels for many GO terms were low. The predominant GO terms with low correlation coefficients in the HFCSD group

were nervous system development (GO:0007399), biological processes (GO:0008150), signal transduction (GO:0007165), regulation of transcription, DNA-dependent (GO:0006355), and cell proliferation (GO:0008283). In contrast, the predominant GO terms with high correlation coefficients in the HFCSD group were skeletal system development (GO:0001501), small GTPase mediated signal transduction (GO:0007264), synaptic transmission (GO:0007268), cell surface receptor linked signaling pathway (GO:0007166), and transcription from the RNA polymerase II promoter (GO:0006366). The intensity ratio of white blood cells to whole blood is a contribution indicator of the white blood cell RNA to whole blood gene expression. To focus on obesity-related organs, i.e., the liver, adipose tissue, and muscle, the relative numbers of ESTs for these organs to blood ESTs for each gene were calculated using EST profiles from the Unigene NCBI database of the transcriptome. The normalized EST values increase when the contribution indicator is small, as shown in Figure 8.

4 Discussion

This study aimed to evaluate the transition of gene expression profiles caused by dietary-induced hyperlipidemia through blood microarray analyses of miniature pigs during a 27-week dietary period.

Dietary-induced hyperlipidemia miniature pig models have previously been established. There are 2 main types of dietary protocol, one containing cholesterol and animal lipids (Kobari *et al*., 1991; de Smet *et al*., 1997; Orbe *et al*., 2001; Bowles *et al*., 2004; Keyzer *et al*., 2009), and the other containing cholesterol, animal lipids, and sucrose (Yin *et al*., 2004; Zhang *et al*., 2006). Some studies have focused their attention on certain kinds of candidate genes with specific functions, but this has not clarified a complete projection of whole blood RNA profiles of the transitions caused by diet-induced hyperlipidemia. Excessive exposure to dietary fats and/or sugars is an essential factor in the initiation of obesity and metabolic syndrome-associated pathologies, two typical conditions associated with diet-induced hyperlipidemia. The fasting plasma total cholesterol level in- creased within a month, and then, either remained high or decreased in the high-fat and high-cholesterol diet (HFCD) models (Kobari *et al*., 1991; Bowles *et al*., 2004; Keyzer *et al*., 2009). In contrast, fasting plasma total cholesterol levels increased throughout the dietary period in the high-fat, high-cholesterol, and high-sucrose diet (HFCSD) models (Zhang *et al*., 2006). Therefore, in the present study, hyperlipidemia was induced by the administration of a high- fat and high-cholesterol diet or a high-fat, high-cholesterol, and high sucrose diet to Clawn miniature swine.

Fasting plasma lipid values increased rapidly and were maintained at a high level during

the 27-week feeding period under both feeding treatments. However, fasting plasma glucose concentrations remained unchanged. The liver and spleen weights increased significantly after the 27 weeks, and fatty livers were reported based on autopsies of individuals from both treatment groups. There was no significant difference in body weight, hematology, or other biochemical aspects of blood between individuals from the 2 dietary treatments.

Gene expression profiles of dietary-induced hyperlipidemia for whole blood RNA

We used whole blood to evaluate the transition of gene expression profiles. Whole blood RNA is easy to handle compared to isolated white blood cell RNA. In addition, whole blood contains a heterogeneous mixture of subpopulations of blood cells. Associated changes will be reflected on whole blood RNA once a great change has occurred in the composition and expressing condition of subpopulations, tissues, or organs. We previously evaluated the ‘‘healthy state’’ gene expression profile by whole blood microarray analyses of miniature pigs of different age groups, and identified characteristics of age-related gene expression by taking into account the change in the number of expressed genes by age and the similarities of gene expression intensity between individuals (Takahashi *et al*., 2011). The report on the healthy state of

miniature pigs found that the correlation coefficients within the same age groups were 0.87 (0.04), 0.93 (0.03), 0.98 (0.01), and 0.96 (0.02), for the fetal stage, and for 12-, 20-, and 30-week-old male pigs, respectively. Variation in gene expression was greatest for younger subjects and diminished with age. These results indicate that uniformity of laboratory animals can be expected in miniature pigs after 20 weeks of age. In this study, feeding treatments commenced when the pigs were 12 weeks old, RNA analysis was conducted on whole blood sampled after 10, 19, and 27 weeks of the feeding period, and on white blood cell RNA after 27 weeks. Variation in whole blood gene expression intensity among individuals within either the HFCD or the HFCSD group was in the same range as that of the controls at any period, indicating uniformity of dietary-induced hyperlipidemia expression profiles in miniature pigs.

Effects of white blood cells on whole blood gene expression profiles in dietary-induced hyperlipidemia

Most of the nucleated cells in blood are white blood cells such as neutrophils, T cells, B cells, and monocytes. Min *et al*. (2010) reported highly correlated results (r^2=0.85) for 8,273 genes expressed in both whole blood RNA and peripheral blood mononuclear cell (PBMCs) RNA samples from healthy volunteers. Other researchers have conducted a

large-scale genome-wide expression analysis of white blood cell subpopulations (Cobb *et al*., 2005). That study indicated that correlation coefficients for T cells and monocytes among different healthy subjects were 0.98 (0.01) and 0.97 (0.01), respectively. However, the correlation coefficient between T cells and monocytes for the same subjects (n=5) was 0.88 (0.01), indicating varied correlations between white blood cell subpopulations (Cobb *et al*., 2005). We believe that no effects of composition ratio of white blood cell subpopulations were observed in our study, because the ratios of granulocytes (neutrophils, eosinophils, and basophils), lymphocytes, and monocytes to white blood cells were statistically insignificant among the three test groups.

In previous studies, tumor-derived RNA was detected in the circulation of cancer patients (Kopreski *et al*., 1999; Lo *et al*., 1999). It has also been demonstrated that fetal RNA can be detected in maternal plasma (Poon *et al*., 2000). These results indicate that whole blood RNA may contain RNA originating from the tissues and/or organs. Hyperlipidemia is one of the risk factors associated with atherosclerosis.

Atherosclerosis was induced by the administration of a high-fat and high-diet to Göttingen miniature swine for a 6-month period (Kobari *et al*., 1991). The liver and spleen weights were increased significantly compared to the controls in both the HFCD and HFCSD groups in our experiment at the end of each dietary period. Thus white

blood cell microarray analyses were conducted at the end of each dietary period, as the tissues and/or organs, such as the liver, spleen, and blood vessels, were presumed to be influenced by dietary treatment.

The average white blood cell correlation coefficients within the HFCD and HFCSD groups were in the same range as that of the controls after the 27-week feeding period. However, variation in whole blood gene expression intensity between the HFCSD group and the control group was statistically significant, whilst variation in white blood cell gene expression intensity between the HFCSD group and the control group was not significant after the 27-week feeding treatments. In addition, the HFCSD correlation coefficient between whole blood and white blood cells after 27 weeks was significantly lower than that of the control and HFCD groups.

The intensity ratio of white blood cell gene expression to that of whole blood shows the contribution of white blood cell RNA to whole blood RNA samples. The intensity ratio of white blood cells to whole blood is, therefore, considered as the contribution indicator. We assume that the low intensity ratio of white blood cell to whole blood gene expression indicates a greater contribution of tissues and/or organs RNA to whole blood RNA. We then compared the EST numbers of the tissue or organ with the contribution indicator, focusing on obesity-related organs such as the liver, adipose tissue, and

muscle. The number of gene ESTs for each tissue or organ normalized to blood ESTs becomes greater when the contribution indicator is small. As a result, we suggest that RNAs originating from tissues and/or organs are present in whole blood.

Characteristics of gene expression profiles in dietary- induced hyperlipidemia

It is generally acknowledged that excessive exposure to dietary lipids disrupts the homeostasis of cellular metabolism and triggers an inflammatory response in adipose tissue (Hotamisligil, 2006). An enhanced inflammatory response has been observed in the livers of mice fed on high-fat diets and in skeletal muscles of Otsuka Long-Evans Tokushima Fatty (OLETF) rats using microarrays (Hayashi *et al*., 2010). We examined dietary-induced transitions of gene expression profiles for genes bearing GO terms. Major changes included an induction of proteins involved in catabolic processes and protein metabolism after a 19-week dietary period, especially in the HFCD group, and a reduced expression of proteins involved in steroid metabolism and lipid biosynthesis after a 27-week dietary period, especially in the HFCSD group.

In whole blood samples, some genes involved in inflammatory responses (GO: 0006954) were down-regulated in the HFCD group, whilst some genes involved in inflammatory responses were up-regulated and others were down-regulated in the

HFCSD group. It has been established that skeletal muscle is an obesity-related organ, such as the liver and adipose tissue, in association with insulin resistance (Krotkiewski, 1994; Roberts *et al*., 2002; Hotamisligil, 2006). Indeed, 2 out of 4 GO terms (muscle contraction, GO: 0006936, muscle organ development, GO: 0007517) that were statistically significant in the HFCD group were related to muscle function. Genes involved in reproduction (GO: 000003) were induced in the HFCD group, and were either induced or repressed in the HFCSD group. Asexual reproduction is the process by which an organism creates a genetically similar or identical copy of itself without the contribution of genetic material from another individual, and some genes involved in asexual reproduction are linked to the repair of damaged organs. Genes involved in translation (GO: 0006412), positive regulation of growth rate (GO: 0040010), and growth (GO: 004007) were induced in the HFCSD group, and these processes are also linked to organ repair. Meanwhile, GO terms that were statistically significant in the HFCSD group were mainly associated with cellular volatility, such as cellular activity, cell growth, or cellular responses.

We examined correlations between whole blood and white blood cells for genes bearing GO terms. The correlation coefficients for each GO term were calculated for the control, HFCD, and HFCSD groups after the 27-week feeding treatments. As a result, GO terms

related to white blood cell function, including inflammatory responses (GO: 0006954), and cell surface receptor-linked signaling pathways (GO: 0007166) show high correlation coefficients in the control and dietary groups. In contrast, GO terms related to the repair of damaged organs, including translation (GO: 0006412), positive regulation of growth rate (GO: 0040010), and growth (GO: 004007), show low correlation coefficients in the HFCSD group.

The differences in the scatter plot regression slopes between the HFCD and control treatments and between the HFCSD and control treatments did not indicate a decrease in the extraction efficiency of RNA due to inhibitory substances in blood. In a previous study of microarray cDNA expression profiles using 23 healthy porcine tissue specimens, a large portion of the genes exhibited tissue-specific expression in agreement with mappings to gene descriptions (Hornshøj *et al*., 2007). In our study, the minimum correlation coefficient for each GO term was 0.737 (0.038), while the maximum was 0.989 (0.004), indicating different values related to functions. The reason for the lower correlation may be due to the differences in gene expression between blood cells and organs, and because a stronger tendency for a decrease in correlation strength was observed in the HFCSD group as compared to the HFCD group. Our EST profile analysis also supported this assumption.

Statistically significant differences in fasting plasma lipids and glucose levels between the HFCD and HFCSD groups were not observed. However, blood RNA analyses demonstrated differences in the characteristics of dietary components between these groups. By considering variation in the dietary-induced hyperlipidemia gene expression profiles of miniature pigs, we have established that whole blood RNA analyses can be used in practical applications. The blood RNA diagnostics under development may eventually be useful for monitoring human health.

5 Conclusion

In this study, no statistically significant differences in fasting plasma lipids and glucose levels between the HFCD and HFCSD groups were observed. However, blood RNA analyses revealed different characteristics corresponding to the dietary protocols. In conclusion, whole blood RNA analyses proved to be a useful tool to evaluate transitions in dietary-induced hyperlipidemia gene expression profiles in miniature pigs.

Figure 2. Subject body weights.

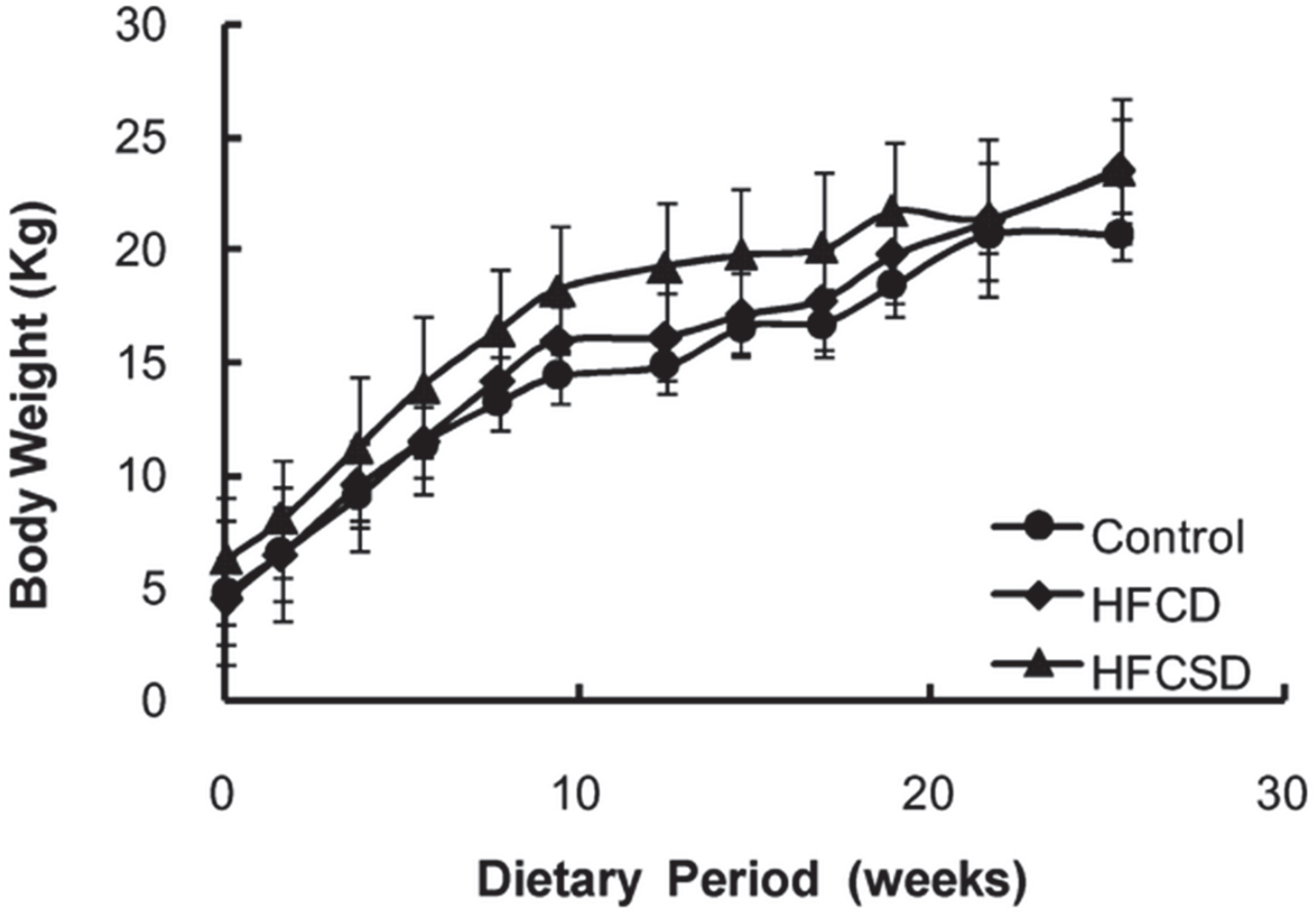


Figure 3. Correlation matrix of dietary-related gene expression profiles of whole blood and white blood cells.

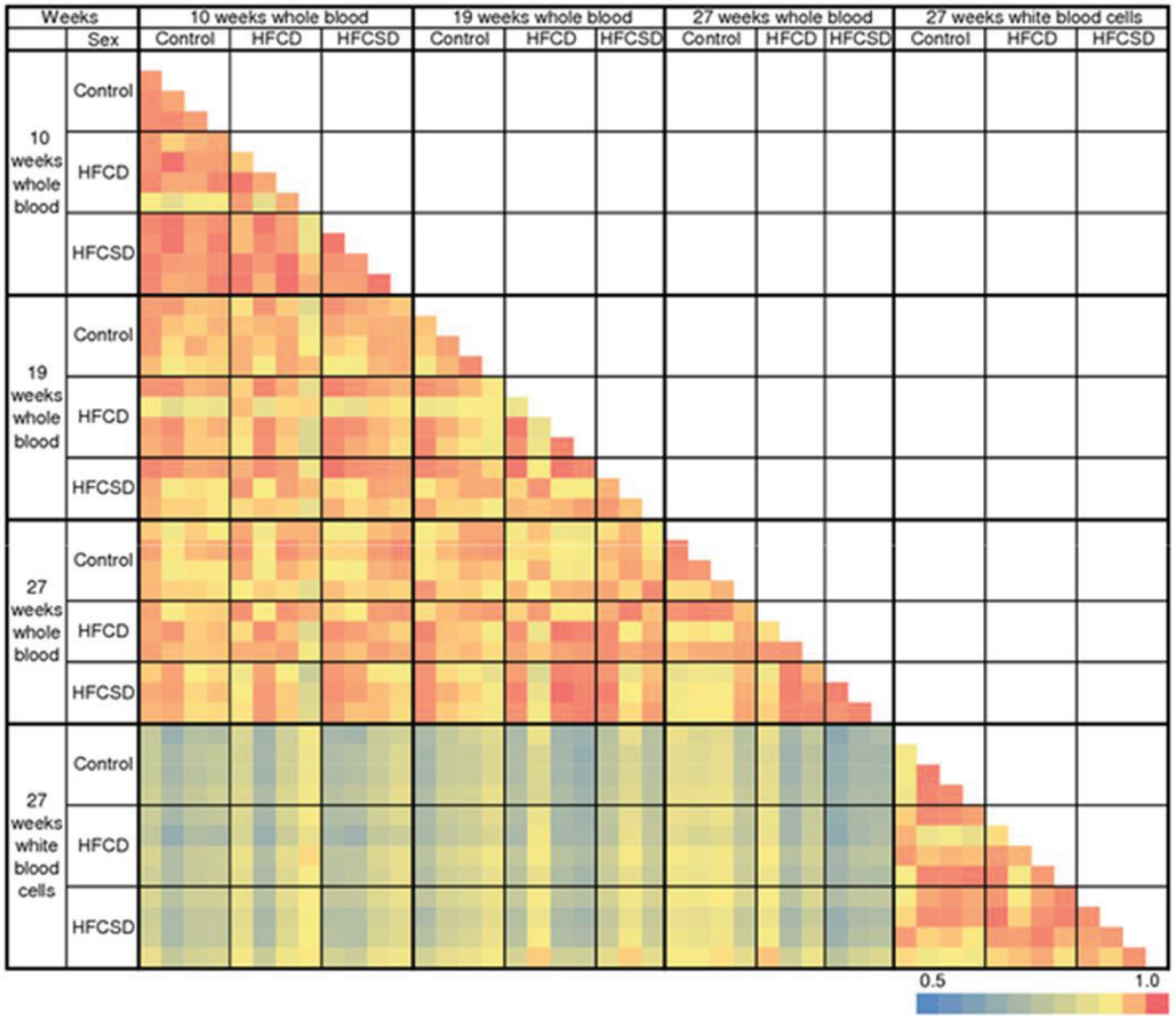


Figure 4. Summary of dietary-related correlation coefficients within the same diet groups.

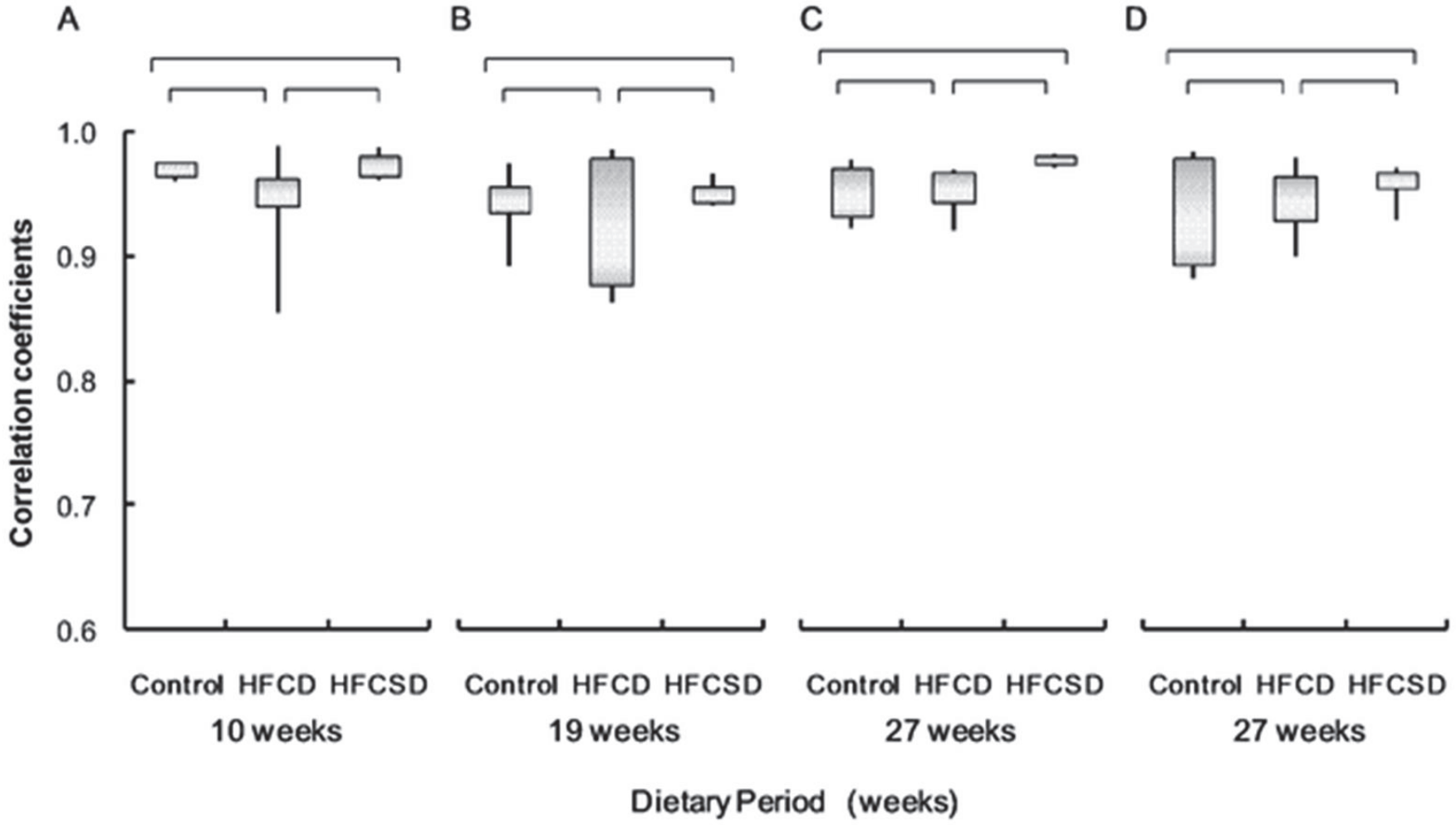


Figure 5. Summary of dietary-related correlation coefficients among different diet groups.

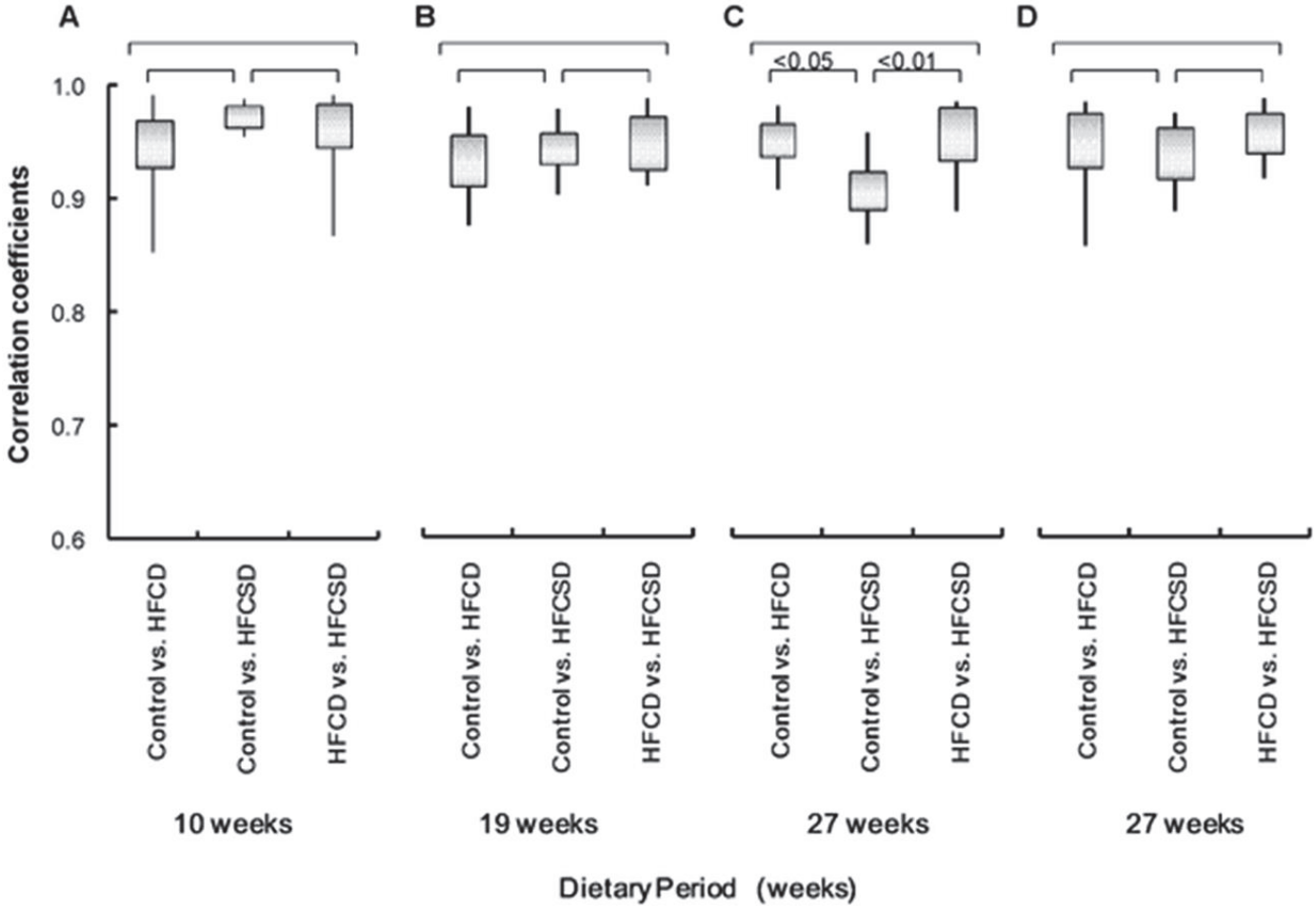


Figure 6. Correlation coefficients between whole blood and white blood cells within the same diet groups.

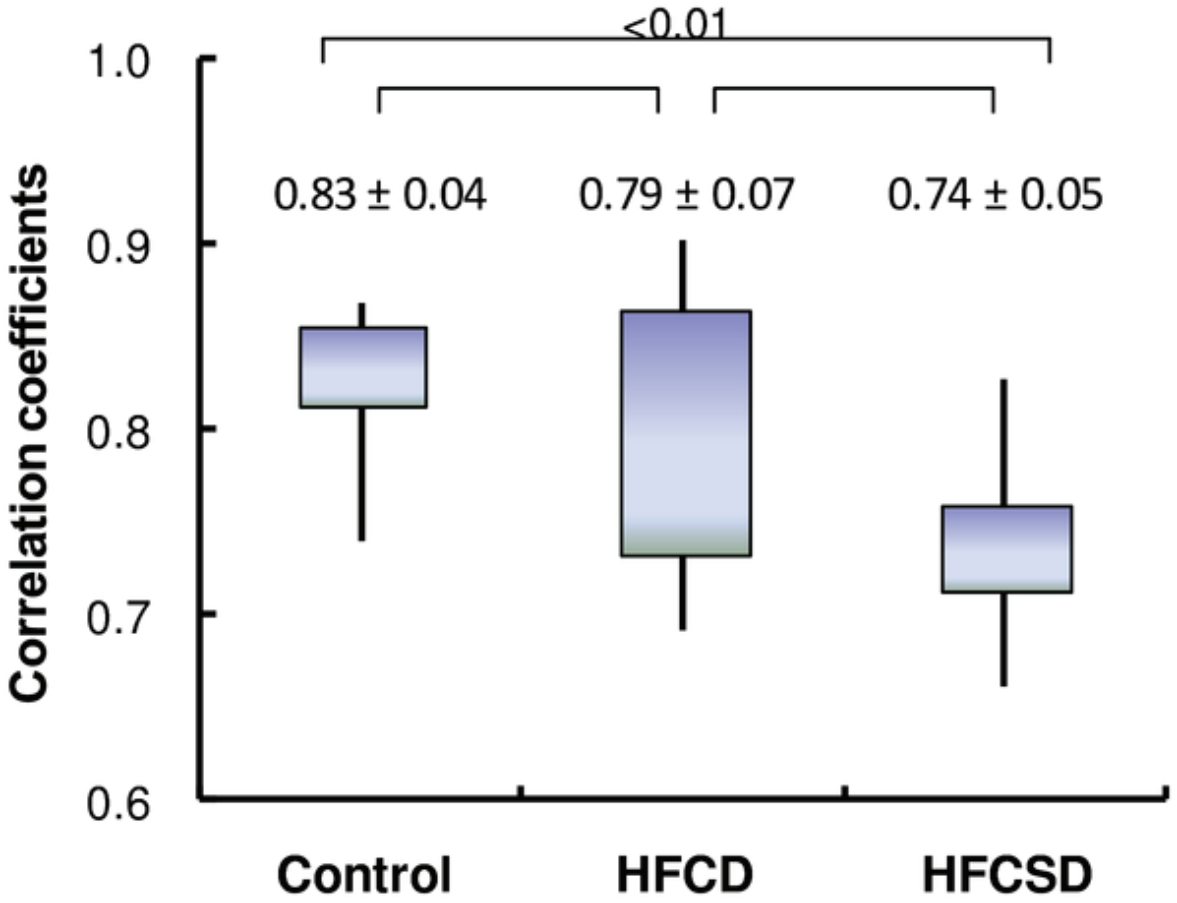


Figure 7. Scatter plot of dietary-related correlation coefficients.

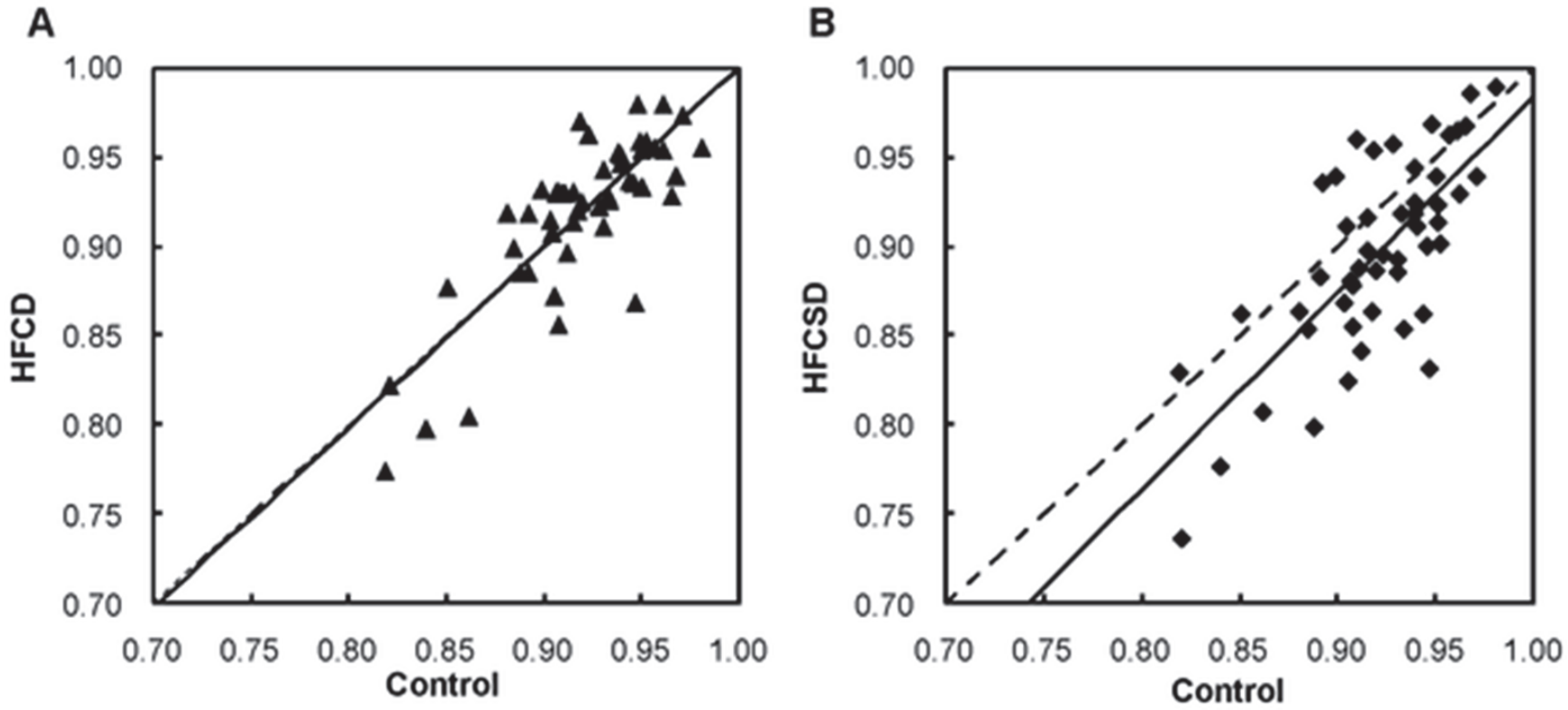


Figure 8. The relation of tissue or organ ESTs to the white blood cell contribution indicator.

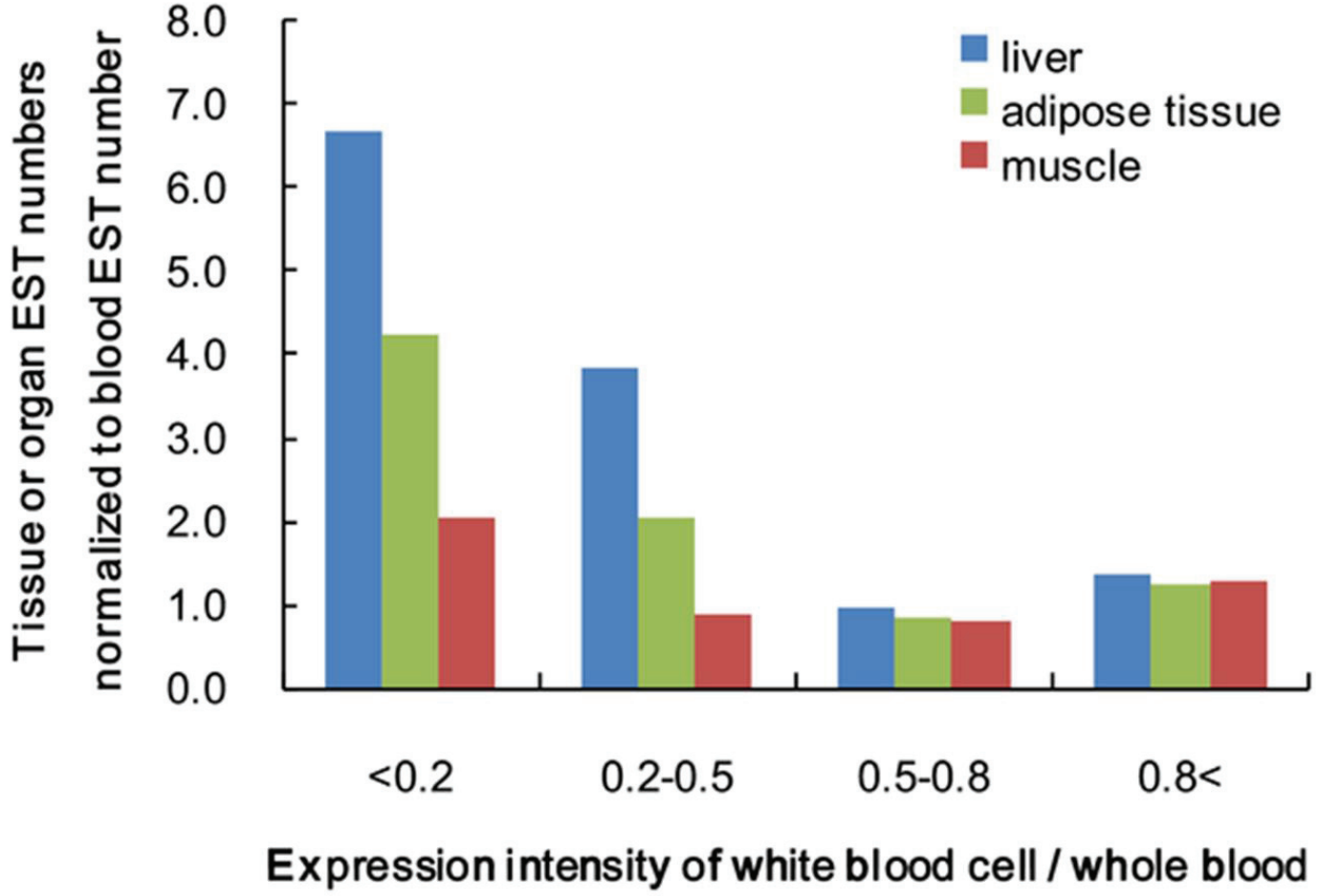


Table 3. Fasting plasma triglyceride levels (mg/dL).

Time point (weeks)	Control	HFCD	HFCSD	$P^{\dagger}$
0	44.5±16.9	42.4±14.4	37.8±21.4	NS
5	25.3±7.2	22.0±22.0	19.5±9.3	NS
10	21.0±2.3	30.4±18.9	30.2±14.7	NS
14	10.8±5.6	7.4±5.0	6.0±3.2	NS
19	10.2±3.6	14.2±14.0	15.6±7.2	NS
23	16.8±7.6	8.2±3.6	8.6±3.2	<.05
27	8.0±2.3	7.2±3.4	12.0±13.5	NS

Values are mean ± SD. NS; not significant.
$^{\dagger}P$ values were calculated using a one-way factorial ANOVA.

Table 4. Fasting plasma total cholesterol levels (mg/dL).

Time point (weeks)	Control	HFCD	HFCSD	$P^{\dagger}$
0	99.0±21.3	117.4±22.2	100.4±22.5	NS
5	103.8±4.8	620.0±292.6	605.0±131.2	<.001
10	81.2±11.1	780.0±239.3	619.0±205.3	<.001
14	54.4±12.4	646.7±141.4	480.3±43.3	<.001
19	54.6±11.9	745.4±172.7	874.6±208.4	<.001
23	54.2±16.9	562.6±144.9	654.4±219.2	<.001
27	53.0±8.8	541.4±148.5	689.4±267.3	<.001

Values are mean ± SD. NS; not significant.
$^{\dagger}P$ values were calculated using a one-way factorial ANOVA.

Table 5. Fasting plasma HDL cholesterol levels (mg/dL).

Time point (weeks)	Control	HFCD	HFCSD	$P^{\dagger}$
0	39.3±11.8	50.3±14.8	41.9±11.5	NS
5	50.3±3.1	81.1±6.3	70.7±19.8	NS
10	41.1±5.9	105.2±30.2	96.2±19.6	<.001
14	36.2±5.4	99.3±21.4	106.3±14.9	<.001
19	30.6±7.6	100.3±22.7	117.9±19.8	<.001
23	31.4±8.7	122.3±8.2	110.7±14.2	<.001
27	29.1±4.5	119.2±12.1	106.6±14.6	<.001

Values are mean ± SD. NS; not significant.
$^{\dagger}P$ values were calculated using a one-way factorial ANOVA.

Table 6. Fasting plasma LDL cholesterol levels (mg/dL).

Time point (weeks)	Control	HFCD	HFCSD	$P^{\dagger}$
0	56.8±11.1	68.4±20.6	56.4±12.9	NS
5	54.0±5.0	318.8±141.7	283.8±49.5	<.01
10	39.2±7.5	339.2±146.5	259.2±107.5	<.01
14	21.8±5.6	212.0±138.4	152.3±43.3	<.05
19	22.8±6.3	236.4±102.7	248.2±78.6	<.001
23	20.8±9.4	220.6±102.2	186.8±46.7	<.001
27	20.6±5.8	201.4±85.2	193.4±86.2	<.01

Values are mean ± SD. NS; not significant.
$^{\dagger}P$ values were calculated using a one-way factorial ANOVA.

Table 7. Fasting plasma glucose levels (mg/dL).

Time point (weeks)	Control	HFCD	HFCSD	$P^{\dagger}$
0	111.8±12.0	122.6±48.5	119.2±21.9	NS
5	98.6±17.2	100.5±12.0	100.0±23.2	NS
10	93.8±15.7	91.8±27.0	83.0±11.1	NS
14	116.4±32.2	104.6±15.6	108.6±30.5	NS
19	92.4±10.5	95.0±13.1	88.8±25.5	NS
23	87.6±19.8	77.2±7.2	92.0±19.2	NS
27	81.6±7.7	89.6±14.6	101.0±13.0	NS

Values are mean ± SD. NS; not significant.
$^{\dagger}P$ values were calculated using a one-way factorial ANOVA.

Table 8. White blood cell count ($10^2/\mu L$).

Time point (weeks)	Control	HFCD	HFCSD	$P^{\dagger}$
0	45.8±11.4	61.8±17.1	65.0±16.3	NS
5	68.6±15.5	85.7±14.6	90.0±19.9	NS
10	67.6±12.5	70.8±23.0	78.4±11.1	NS
14	69.4±5.2	87.2±25.9	85.2±13.3	NS
19	68.0±14.8	69.6±12.4	83.4±13.7	NS
23	86.0±21.6	90.2±32.0	100.6±26.1	NS
27	61.8±15.2	75.8±18.6	62.6±34.4	NS

Values are the mean ± SD. NS; not significant.
$^{\dagger}P$ values were calculated using a one-way factorial ANOVA.

Table 9. The ratio of basophils to white blood cells (%).

Time point (weeks)	Control	HFCD	HFCSD	$P^{\dagger}$
0	0.0±0.0	0.3±0.5	0.0±0.0	NS
5	0.2±0.4	0.0±0.0	0.2±0.4	NS
10	0.3±0.4	0.2±0.4	0.3±0.4	NS
14	0.2±0.4	0.0±0.0	0.4±0.5	NS
19	0.0±0.0	0.0±0.0	0.0±0.0	NS
23	0.2±0.4	0.0±0.0	0.0±0.0	NS
27	0.1±0.2	0.5±0.5	0.4±0.5	NS

Values are the mean ± SD. NS; not significant.
$^{\dagger}P$ values were calculated using a one-way factorial ANOVA.

Table 10. The ratio of eosinophils to white blood cells (%).

Time point (weeks)	Control	HFCD	HFCSD	$P^{\dagger}$
0	2.8±2.6	2.8±1.5	2.0±2.2	NS
5	3.6±1.9	3.0±1.0	3.0±1.4	NS
10	3.1±1.4	3.2±1.3	4.1±1.7	NS
14	3.0±1.9	3.3±1.3	2.4±1.7	NS
19	5.0±2.7	4.1±0.7	5.6±1.8	NS
23	4.6±1.7	6.2±2.7	4.4±0.9	NS
27	2.8±0.8	3.1±1.9	3.4±1.1	NS

Values are the mean ± SD. NS; not significant.
$^{\dagger}P$ values were calculated using a one-way factorial ANOVA.

Table 11. The ratio of neutrophils to white blood cells (%).

Time point (weeks)	Control	HFCD	HFCSD	$P^{\dagger}$
0	52.8±16.0	59.6±1.8	54.0±15.5	NS
5	53.8±9.8	55.0±1.0	60.2±3.8	NS
10	43.1±10.3	41.2±6.3	45.5±2.6	NS
14	44.8±7.4	53.0±11.9	51.4±6.1	NS
19	52.2±7.0	48.1±5.4	44.8±4.1	NS
23	56.2±9.2	51.6±3.0	50.6±9.9	NS
27	58.8±13.8	60.1±4.4	48.0±18.5	NS

Values are the mean ± SD. NS; not significant.
$^{\dagger}P$ values were calculated using a one-way factorial ANOVA.

Table 12. The ratio of lymphocytes to white blood cells (%).

Time point (weeks)	Control	HFCD	HFCSD	$P^{\dagger}$
0	37.5±11.0	31.1±4.0	36.8±13.1	NS
5	34.8±10.5	36.3±3.5	30.4±2.9	NS
10	45.2±7.4	45.8±6.0	44.7±3.0	NS
14	44.6±9.3	36.2±10.7	39.2±6.4	NS
19	36.9±6.9	42.0±4.9	43.4±3.8	NS
23	33.6±7.6	34.0±2.9	39.6±10.2	NS
27	32.3±13.5	29.5±5.0	41.2±19,5	NS

Values are the mean ± SD. NS; not significant.
$^{\dagger}P$ values were calculated using a one-way factorial ANOVA.

Table 13. The ratio of monocytes to white blood cells (%).

Time point (weeks)	Control	HFCD	HFCSD	$P^{\dagger}$
0	7.0±3.4	6.3±1.5	7.3±3.9	NS
5	7.6±3.1	5.7±2.5	6.2±1.8	NS
10	8.0±3.2	9.6±1.8	5.4±2.1	NS
14	7.4±1.5	7.5±0.9	6.6±2.3	NS
19	6.0±2.1	5.8±1.1	6.2±1.8	NS
23	5.4±1.3	8.2±1.9	5.2±2.1	N <.05
27	6.0±1.9	6.8±1.0	7.0±1.2	NS

Values are mean ± SD. NS; not significant.
$^{\dagger}P$ values were calculated using a one-way factorial ANOVA.

Table 14. Effect of diet on organ weight of miniature pigs (g).

Organ	Control	HFCD	HFCSD	$P^{\dagger}$
Heart	149.7±5.5	163.3±4.2	153.7±22.9	NS
Liver	328.0±33.2	667.7±80.9	682.3±21.6	<.001
Kidney	86.3±7.5	99.0±12.5	97.7±4.9	NS
Stomach	169.3±15.2	181.7±13.8	180.0±26.5	NS
Spleen	36.3±3.1	72.0±12.2	74.7±13.3	<.01

Values are mean ± SD. NS; not significant.
$^{\dagger}P$ values were calculated using a one-way factorial ANOVA.

Table 15. Top 10 significantly up-regulated genes in HFCD.

Whole blood at 10 weeks			Whole blood at 19 weeks			Whole blood at 27 weeks			White blood cells at 27 weeks		
TIGR or Unigene ID	Gene Symbol	Fold Change	TIGR or Unigene ID	Gene Symbol	Fold Change	TIGR or Unigene ID	Gene Symbol	Fold Change	TIGR or Unigene ID	Gene Symbol	Fold Change
TC440907		5.8	TC440907		7.7	TC440907		8.5	TC410790	IRG6	5.9
TC438339		5.7	TC506587		6.2	TC487165	CXCL10	7.3	TC448587	ABCA1	5.3
TC448587	ABCA1	5.3	TC448587	ABCA1	4.8	TC441966	UPK2	5.9	TC440907		5.1
TC414205		3.8	TC407415	MX2	4.7	TC410790	IRG6	5.5	TC447991		4.3
TC407338	MX1	3.4	Ssc.51683	ABCB4	4.3	TC448587	ABCA1	5.3	TC438339		4.0
TC432467		3.1	TC426495		4.3	TC438339		5.1	TC453638		4.0
TC407865		2.9	TC438339		4.0	TC490662	ROMO1	4.7	TC450749		3.5
TC448870		2.9	TC450749		3.9	TC506587		4.4	TC407748	PCD1B	3.4
TC467477	SERPING1	2.7	TC425970	LGALS9	3.8	TC427843		3.6	TC426495		3.3
TC445197		2.6	TC450410		3.8	TC453638		3.6	Ssc.48813		3.2

Table 16. Top 10 significantly up-regulated genes in HFCSD.

Whole blood at 10 weeks			Whole blood at 19 weeks			Whole blood at 27 weeks			White blood cells at 27 weeks		
TIGR or Unigene ID	Gene Symbol	Fold Change	TIGR or Unigene ID	Gene Symbol	Fold Change	TIGR or Unigene ID	Gene Symbol	Fold Change	TIGR or Unigene ID	Gene Symbol	Fold Change
TC448587	ABCA1	4.0	TC487165	CXCL10	15.7	NP411727	GRK7	17.9	Ssc.9135		20.1
TC438339		4.0	TC440907		8.4	TC490662	ROMO1	16.9	TC434357		11.6
TC440907		3.8	TC509090		5.1	TC417023		15.6	TC511398		8.5
TC411549		3.1	TC448587	ABCA1	5.0	TC480907		13.5	TC473927		7.1
TC472367		2.4	TC450410		4.9	TC444971		13.3	Ssc.24568		6.7
TC433245		2.1	TC438339		4.5	TC511837		12.7	TC448587	ABCA1	5.7
			TC470851		4.5	TC423285		12.2	TC438339		4.9
			TC480583		4.4	Ssc.46601		11.8	TC440907		4.8
			TC410790	IRG6	4.3	Ssc.56049		11.5	TC430783		4.4
			Ssc.5472		4.1	Ssc.35808		11.2	TC512775		4.3

Table 17. Top 10 significantly down-regulated genes in HFCD.

Whole blood at 10 weeks			Whole blood at 19 weeks			Whole blood at 27 weeks			White blood cells at 27 weeks		
TIGR or Unigene ID	Gene Symbol	Fold Change	TIGR or Unigene ID	Gene Symbol	Fold Change	TIGR or Unigene ID	Gene Symbol	Fold Change	TIGR or Unigene ID	Gene Symbol	Fold Change
TC431538		0.2	TC418697		0.1	TC427825		0.2	TC415077		0.2
TC409256		0.2	TC502826		0.1	TC416132		0.2	TC417273		0.2
TC437880		0.2	TC407798	WNT10B	0.2	TC463385		0.2	Ssc.25428		0.3
Ssc.31818		0.2	TC433727		0.2	Ssc.50105		0.3	Ssc.56656	SQLE	0.3
TC434967		0.3	TC495620	IL18	0.2	Ssc.67286		0.3	TC436654		0.4
TC423396		0.3	TC503806		0.2	TC462549		0.3	TC491413		0.4
TC440884		0.3	TC468870		0.2	TC409660		0.3	TC407223	MYO7A	0.4
TC443308		0.3	TC410474		0.2	TC414897	CNN1	0.3	TC478649	PFKFB1	0.4
Ssc.46725		0.3	TC466297	MGP	0.2	TC423618		0.3	TC473047		0.4
Ssc.38146		0.3	TC449799		0.2	TC494014		0.3	TC446195		0.4

Table 18. Top 10 significantly down-regulated genes in HFCSD.

Whole blood at 10 weeks			Whole blood at 19 weeks			Whole blood at 27 weeks			White blood cells at 27 weeks		
TIGR or Unigene ID	Gene Symbol	Fold Change	TIGR or Unigene ID	Gene Symbol	Fold Change	TIGR or Unigene ID	Gene Symbol	Fold Change	TIGR or Unigene ID	Gene Symbol	Fold Change
Ssc.56656	SQLE	0.3	TC491388	CCL3L1	0.2	TC442299		0.2	TC491413		0.2
TC426935		0.4	TC407798	WNT10B	0.2	TC407798	WNT10B	0.2	TC414897	CNN1	0.2
Ssc.47297		0.4	TC486323		0.3	Ssc.56656	SQLE	0.2	TC443738		0.2
Ssc.58815		0.4	Ssc.36434		0.3	TC471905		0.3	TC421743		0.2
Ssc.32174		0.4	TC452410	MAOA	0.3	TC429709		0.3	TC412754		0.2
TC452974		0.4	Ssc.56656	SQLE	0.3	TC472114		0.3	TC463385		0.3
TC477452		0.5	TC413430		0.3	TC423826		0.3	TC477521		0.3
TC418062		0.5	TC410474		0.3	TC458674		0.3	NP7655660		0.3
TC491240		0.5	TC509046		0.3	TC417970		0.3	TC442860	TPM1	0.3
TC495442		0.5	Ssc.61167		0.3	TC437991		0.3	TC417854		0.3

Table 19. Functional classes of up- or down-regulated genes between HFCD and control.

Weeks	Induction/Repression	Category	Accession	Term	%	P-value
10	Induction	GO MF	GO:0003924	GTPase activity	28.6	1.9e−02
10	Induction	KEGG		complement and coagulation cascades	28.6	2.2e−02
19	Induction	GO MF	GO:0032555	purine ribonucleotide binding	13.6	3.9e−03
19	Induction	GO MF	GO:0032553	ribonucleotide binding	13.6	3.9e−03
19	Induction	GO MF	GO:0000166	nucleotide binding	15.3	4.0e−03
19	Induction	GO MF	GO:0003924	GTPase activity	5.1	4.7e−03
19	Induction	GO MF	GO:0017076	purine nucleotide binding	13.6	6.2e−03
19	Induction	GO BP	GO:0019941	modification-dependent protein catabolic process	5.1	1.9e−02
19	Induction	GO BP	GO:0043632	modification-dependent macromolecule catabolic process	5.1	1.9e−02
19	Induction	GO BP	GO:0044257	cellular protein catabolic process	5.1	2.5e−02
19	Induction	GO BP	GO:0051603	proteolysis involved in cellular protein catabolic process	5.1	2.5e−02
19	Induction	GO MF	GO:0032561	guanyl ribonucleotide binding	6.8	2.6e−02
19	Induction	GO MF	GO:0019001	guanyl nucleotide binding	6.8	2.6e−02
19	Induction	GO MF	GO:0005525	GTP binding	6.8	2.6e−02
19	Induction	GO BP	GO:0030163	protein catabolic process	5.1	2.7e−02
19	Induction	GO BP	GO:0044265	cellular macromolecule catabolic process	5.1	3.5e−02
19	Induction	GO BP	GO:0009057	macromolecule catabolic process	5.1	4.9e−02
27	Induction	KEGG		Toll-like receptor signaling pathway	14.3	1.4e−02
10	Repression	GO MF	GO:0004857	enzyme inhibitor activity	11.1	1.6e−02
10	Repression	GO MF	GO:0004866	endopeptidase inhibitor activity	8.3	4.8e−02
19	Repression	GO BP	GO:0002684	positive regulation of immune system process	7.9	1.3e−02
27	Repression	GO BP	GO:0007155	cell adhesion	11.1	9.6e−03
27	Repression	GO BP	GO:0022610	biological adhesion	11.1	9.6e−03
27	Repression	KEGG		steroid biosynthesis	5.6	4.0e−02

GO MF; GO molecular function, GO BP; GO biological process, Kegg; Kegg pathway.

Table 20. Functional classes of up- or down-regulated genes between HFCSD and control.

Weeks	Induction/Repression	Category	Accession	Term	%	P-Value
19	Induction	GO MF	GO:0000166	nucleotide binding	13	3.7e−04
19	Induction	GO MF	GO:0032555	purine ribonucleotide binding	9.8	5.1e−03
19	Induction	GO MF	GO:0032553	ribonucleotide binding	9.8	5.1e−03
19	Induction	GO MF	GO:0017076	purine nucleotide binding	9.8	8.2e−03
19	Induction	GO MF	GO:0005524	ATP binding	6.5	4.8e−02
27	Repression	GO BP	GO:0016125	sterol metabolic process	8.5	4.9e−04
27	Repression	KEGG		steroid biosynthesis	6.4	1.7e−03
27	Repression	GO BP	GO:0008202	steroid metabolic process	8.5	2.3e−03
27	Repression	GO BP	GO:0016126	sterol biosynthetic process	6.4	2.4e−03
27	Repression	KEGG		ECM-receptor interaction	8.5	4.0e−03
27	Repression	GO BP	GO:0008203	cholesterol metabolic process	6.4	9.9e−03
27	Repression	GO BP	GO:0006694	steroid biosynthetic process	6.4	1.1e−02
27	Repression	GO BP	GO:0055114	oxidation reduction	12.8	1.5e−02
27	Repression	GO BP	GO:0007155	cell adhesion	8.5	1.7e−02
27	Repression	GO BP	GO:0022610	biological adhesion	8.5	1.7e−02
27	Repression	GO BP	GO:0008610	lipid biosynthetic process	6.4	4.8e−02

GO MF; GO molecular function, GO BP; GO biological process, Kegg; Kegg pathway.

Table 21. Predominant GO terms for which the ratio changed in HFCD and HFCSD.

			Induction/Repressions		Correlation coefficients between whole blood and white blood cells at 27 weeks		
Accession	GO term	Number of genes	HFCD	HFCSD	Control	HFCD	HFCSD
GO:0006954	inflammatory response	155	Repression	Induction/Repression	0.92±0.03	0.97±0.02	0.95±0.02
GO:0000003	reproduction	310	Induction	Induction/Repression	0.91±0.02	0.93±0.03	0.88±0.03

Values are mean ± SD.

Table 22. Predominant GO terms for which the ratio changed in HFCD.

				Correlation coefficients between whole blood and white blood cells at 27 weeks		
Accession	GO term	Number of genes	Induction/Repression	Control	HFCD	HFCSD
GO:0006936	muscle contraction	212	Induction/Repression	0.91±0.03	0.91±0.02	0.91±0.02
GO:0007626	locomotory behavior	207	Induction/Repression	0.88±0.04	0.92±0.05	0.86±0.04
GO:0007517	muscle organ development	199	Repression	0.94±0.05	0.95±0.02	0.94±0.02
GO:0008152	metabolic process	145	Repression	0.93±0.02	0.92±0.05	0.96±0.03

Values are mean ± SD.

Table 23. Predominant GO terms for which the ratio changed in HFCSD.

				Correlation coefficients between whole blood and white blood cells at 27 weeks		
Accession	GO term	Number of genes		Control	HFCD	HFCSD
GO:0006412	Translation	253	Induction/Repression	0.90±0.02	0.92±0.03	0.87±0.03
GO:0009792	embryonic development ending in birth or egg hatching	432	Induction/Repression	0.92±0.02	0.93±0.03	0.90±0.02
GO:0006118	electron transport	200	Induction/Repression	0.82±0.07	0.77±0.09	0.83±0.11
GO:0006366	transcription from RNA polymerase II promoter	209	Induction/Repression	0.96±0.02	0.98±0.01	0.97±0.01
GO:0040010	positive regulation of growth rate	207	Induction	0.92±0.02	0.92±0.04	0.86±0.04
GO:0007166	cell surface receptor linked signaling pathway	185	Repression	0.97±0.01	0.93±0.06	0.97±0.01
GO:0002119	nematode larval development	374	Induction	0.91±0.02	0.93±0.03	0.88±0.03
GO:0006886	intracellular protein transport	191	Induction	0.91±0.06	0.86±0.08	0.86±0.07
GO:0001666	response to hypoxia	189	Repression	0.95±0.02	0.95±0.03	0.92±0.02
GO:0040007	Growth	332	Induction	0.91±0.02	0.93±0.03	0.88±0.03

Values are mean ± SD.

Table 24. Predominant GO terms for which the ratio unchanged in HFCD or HFCSD.

Accession	GO term	Number of genes	Correlation coefficients between whole blood and white blood cells at 27 weeks		
			Control	HFCD	HFCSD
GO:0007165	signal transduction	527	0.89±0.04	0.89±0.07	0.80±0.05
GO:0008283	cell proliferation	353	0.91±0.03	0.87±0.07	0.83±0.05
GO:0007267	cell-cell signaling	321	0.94±0.02	0.95±0.03	0.92±0.02
GO:0008285	negative regulation of cell proliferation	288	0.95±0.03	0.87±0.08	0.83±0.06
GO:0006468	protein amino acid phosphorylation	266	0.95±0.01	0.94±0.02	0.90±0.03
GO:0008284	positive regulation of cell proliferation	262	0.92±0.04	0.96±0.02	0.90±0.03
GO:0006916	anti-apoptosis	224	0.91±0.03	0.93±0.03	0.89±0.02
GO:0042493	response to drug	218	0.93±0.03	0.93±0.05	0.85±0.04
GO:0007399	nervous system development	210	0.82±0.06	0.82±0.08	0.74±0.04
GO:0006508	Proteolysis	210	0.90±0.04	0.93±0.03	0.94±0.04
GO:0006470	protein amino acid dephosphorylation	198	0.91±0.07	0.90±0.09	0.84±0.06
GO:0000122	negative regulation of transcription from RNA polymerase II promoter	193	0.93±0.03	0.91±0.04	0.89±0.03
GO:0015031	protein transport	190	0.95±0.03	0.96±0.02	0.94±0.03
GO:0009887	organ morphogenesis	186	0.93±0.03	0.93±0.05	0.92±0.01
GO:0006357	regulation of transcription from RNA polymerase II promoter	179	0.93±0.04	0.94±0.04	0.89±0.03
GO:0001764	neuron migration	174	0.96±0.01	0.95±0.02	0.93±0.01
GO:0007264	small GTPase mediated signal transduction	157	0.97±0.01	0.94±0.05	0.99±0.00
GO:0007275	multicellular organismal development	151	0.95±0.03	0.93±0.04	0.91±0.02
GO:0007420	brain development	150	0.89±0.10	0.89±0.09	0.88±0.09
GO:0043066	negative regulation of apoptosis	145	0.94±0.04	0.95±0.02	0.93±0.01

Values are mean ± SD.

CHAPTER III

Evaluation of the physiology of miniature pig fed Shochu distillery waste using mRNA expression profiling

1 Introduction

Shochu is a traditional Japanese distilled spirit made from sweet potato, rice, barley, buckwheat, and sugar cane. The consumption of *imo-Shochu,* which is made from sweet potato, has rapidly increased in recent years. In the production process, large amounts of distillery waste remain after fermentation. In 1996, sea dumping of Shochu distillery waste (SDW) was prohibited by the London treaty. Therefore, development of a procedure for the disposal of this industrial waste has become a serious issue. One potential method that many researchers are addressing is to use SDW as a feed for livestock because of the abundance of useful nutrients and functional ingredients (Kawaida *et al*., 1989, 1990, 1991; Mahfudz *et al*., 1996; Kamizono *et al*., 2010; Hayashi, 2012).

The miniature pig is a useful animal model for documentation of vital reactions and molecular mechanisms because 1) it can be used in long-term experiments owing to its long life-span and 2) its size and short reproduction cycle allow for easier breeding and handling (Vodicka et al., 2005; Gutierrez *et al*., 2015). Furthermore, the Swine Genome Sequencing Consortium (SGSC) was organized in 2003 to sequence the pig genome (Schook *et al*., 2005), and pig genomics research was further enhanced because of the

completion of the pig genome map by members of the SGSC in 2009 (Archibald *et al*., 2010). One miniature pig strain, the Clawn miniature pig, was established in 1978 by Nakanishi (Nakanishi *et al*., 1991). It was bred and maintained in a specific closed group as an inbred laboratory animal; therefore, it has limited inter-individual differences in its physiology (Nakanishi *et al*., 1991). In addition, our study revealed that gene expression patterns of miniature pigs become uniform after 20 weeks of age; therefore, after this age, they are physiological stable (Takahashi *et al*., 2011). Variation in gene expression among individuals is lost after 20 weeks of age, and high reproducibility of experimental results is obtained with the Clawn miniature pig model.

Microarray analysis is a useful tool for rapidly obtaining a large amount of gene expression information. This technique allows for the identification of genes that vary in expression in response to various stresses. In toxicogenomic studies, it is one of the standard procedures for the evaluation of biological responses to stimuli, and the elucidation of the mechanisms behind these responses (Tong *et al*., 2003; Pennie *et al*., 2004; Williams-Devane *et al*., 2009). Especially for animal models in long-term studies, microarray analysis of whole blood is an important tool for the evaluation of physiological state because of its availability and the non-invasive nature of sample collection. Whole blood is an important tissue, because it plays key roles in immune

responses, metabolism, and communication between cells and the extracellular matrix in almost all tissues and organs. Moreover, because blood samples can be collected repeatedly from the same individual, it can be used for temporal assessments. Blood RNA contains substantial amounts of information regarding the expression of messenger RNA and non-coding functional RNA molecules. Numerous studies have revealed the function of blood as a biomarker of pathological changes occurring in other tissues, and for characterizing these pathological changes (Liew *et al*., 2006; Bushel *et al*., 2007; Staratschek-Jox *et al*., 2009; Umbright *et al*., 2010; Fricano *et al*., 2011).

In this study, we performed a feeding experiment using the SDW diet and a pig model, and evaluated the effects of the diet on pig physiology.

2 Materials and methods

Animals and diet.

Eight female Clawn miniature pigs, aged 8–9 months, were housed individually in 1.5 m^2-cages at the conventional facility of the breeder (Japan Farm Co., Ltd., Kagoshima, Japan). During this period, all animals were fed 450 g/day standard dry feed (Kodakara73, Marubeni Nisshin Feed Co., Ltd., Tokyo, Japan), with free access to

water. The pigs were divided into two experimental groups: the control group (n = 4) was fed standard dry feed and the experimental group (n = 4) was fed the SDW feed. All pigs were provided with 450 g/day of either standard dry feed or SDW feed (composed of standard dry feed:water:SDW in a 1:1:1 ratio). The diet was administered to the pigs during an experimental period of three months. Fresh SDW was obtained from Okuchi Liquor Co., Ltd., (Kagoshima).

Body measurement.

Body weight and length were measured at the beginning and end of the feeding period. Body length (from the base of the neck to the base of the tail) was measured using a measuring tape.

Hematology and clinical chemistries.

All blood samples were collected from the superior vena cava at the end of the experimental period. Blood (prevented from clotting with ethylenediaminetetraacetic acid [EDTA]), plasma (with EDTA), and serum samples were collected for hematology and biochemical tests. Hematology and biochemical tests were conducted by the

Clinical Pathology Laboratory, Inc. (http://www.patho.co.jp/index.html) (Kagoshima, Japan) using standard clinical methods.

Preparation of samples and RNA extraction.

For microarray analysis, 16–20 mL of blood was collected from each subject, placed in two PAXgene™ tubes (Qiagen/ BD GmbH, Crawley, U.K.), incubated at 18–25°C for 4 h for RNA stabilization, and stored at -80°C. Total RNA was extracted from whole blood using the PAXgene™ Blood RNA System Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's guidelines. Quantification of extracted RNA was performed with a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Quality of the purified RNA was confirmed on an Agilent® 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA).

Microarray assay.

Labeled cDNA with fluorescent Cyanine 3-CTP was used for hybridization onto porcine gene expression microarray template slides (44K) (#G2519F#20109, Agilent Technologies) using the *in situ* Hybridization Kit Plus (Agilent Technologies). Array slides were incubated at 65°C for 17 h in microarray hybridization chambers (Agilent

Technologies). After hybridization, microarray slides were washed following the manufacturer's protocol, and were scanned with an Agilent DNA Microarray Scanner (#G2565BA, Agilent Technologies) at 5-µm resolution. The scanned images were analyzed using Agilent Feature Extraction Software, version 9.5.3.1 (Agilent Technologies).

Analysis of microarray data.

The signal intensity of each gene was globally normalized using quantile normalization. Background levels were defined by the spots outside of the gene probing area. Using the Agilent microarray slides, spots of each probe were divided into two groups: "absent" and "present" using the flag values provided by the scanner. "Absent" spots had a signal intensity that was less than that of the background level, whereas the rest of the spots were designated as "present". Only data for "present" spots were used for the analysis. The data were analyzed using Pearson's correlation coefficient. Correlation data were converted using the z-Fisher transformation, and were tested for significant differences using a one-way analysis of variance (ANOVA) followed by the Tukey's *post-hoc* test among all groups, and Welch's *t*-tests between two groups. A probability of a false positive of $p < 0.05$ was considered statistically significant. The expression data were

logarithm-transformed and grouped using hierarchical cluster analysis in the Gene Cluster 3.0 program (de Hoon *et al*., 2004) available from: http://bonsai.hgc.jp/~mdehoon/software/cluster/. To create the hierarchical clustering map, we used the Java Treeview program (Saldanha, 2004) available from: http://jtreeview.sourceforge.net/.

Ethical considerations.

All experimental protocols were approved by the Committee for the Care and Use of Experimental Animals at AIST (Permit Number: 2016-055).

3 Results

Physical examination.

There was no difference in preference between the control and SDW diets; thus, the same volume of feed was consumed by both groups. Temporal changes in mean body length and weight for the two dietary groups are shown in Figure. 9. Body length and weight did not differ according to the dietary treatment at any time, and the variables

increased smoothly in both groups during the feeding period. Paired *t*-test analysis for dietary-related variation revealed no significant difference at any time.

Hematological analysis.

Table 25 lists the results of the hematological analysis. Hematopoietic parameters are some of the most sensitive markers to assess toxicity in humans and non-human animals (Liju *et al*., 2013). Very few differences were observed in the complete blood count test (white blood cell count, red blood cell count, hemoglobin concentration, mean corpuscular volume, and platelet count) between the two groups. Based on the blood biochemical analysis, no phenotypic parameter changes, such as hepatic dysfunction (total bilirubin, aspartate transaminase, alanine transaminase, alkaline phosphatase, and γ-glutamyltransferase), renal dysfunction (urea nitrogen, creatinine, and uric acid), or pancreatic dysfunction (serum amylase) were caused by toxicity of the SDW diet. Additionally, the parameters indicating the nutritional state of the animals (e.g., total protein, total cholesterol, and albumin) were normal in the SDW diet-treated pigs. There was also no abnormality in lipid metabolism (triglycerides), glycometabolism (blood glucose), clotting function (prothrombin time, activated partial thromboplastin time, and fibrinogen level), or electrolyte concentration (Na, Cl, K, and Ca) in the SDW diet-fed

group. Statistically significant differences, according to *t*-test analysis, between the values from the control and SDW groups were not observed for any item.

Microarray analysis of gene expression profiles.

RNA analyses were conducted with blood samples obtained at the end of the feeding period to characterize the dietary effects on gene expression profiles in the whole blood of miniature pigs. Each RNA sample was analyzed by a porcine gene expression microarray consisting of 43,603 oligonucleotide probes.

We compared the Pearson's correlation coefficients for gene expression profiles between the control and SDW groups. High correlations were found not only within the same group, but also between the two groups. Figure. 10 shows a scatterplot of the microarray intensities of the two groups. The resulting data showed a high degree of correlation ($r = 0.9748$). Therefore, DNA microarray analysis indicated that the global gene-expression patterns were similar between the control and SDW groups.

Reproducibility and variation of mRNA expression among stress feeding factors.

To compare other feeding stress factors, we evaluated microarray data of five groups: control; SDW; high-fat and high-cholesterol diet (HFCD) (Takahashi *et al*., 2012);

high-fat, high-cholesterol, and high-sucrose diet (HFCSD) (Takahashi *et al*., 2012); and sodium azide (AZIDE). The data were already deposited in the Gene Expression Omnibus (GEO) database, accession number GSE78769. AZIDE was administered orally to the pigs over 20 weeks. There were no changes in hematological or biochemical parameters for the administered dose of 300 μg/kg, one hundredth of the LD50.

A color-coded pairwise correlation matrix is displayed in Figure. 11. Figure. 12 shows the Pearson's correlation coefficients for gene expression profiles among individuals within the same dietary group; this figure displays the inter-individual differences in gene expression profiles under the same dietary conditions during the dietary period. Correlation coefficients varied from 0.91 to 0.99, with an average of 0.96 and an S.D. of 0.02. No significant differences in correlation coefficients within dietary groups were observed for any gene product after the treatments (Figures. 11, 12). This indicated uniformity in the dietary experiences within each group, and it was possible to compare the gene expression profiles among the groups.

Next, we analyzed expression profile correlations among the different diet groups (Figure. 13). First, we confirmed the high correlation between the control and SDW group individuals; the correlation factors varied from 0.93 to 1.00, with an average of

0.97 and an S.D. of 0.02. The values indicated similarity between the control and SDW groups, and suggested that the SDW diet did not change the RNA expression profile of the pigs.

The correlation factors between the control and the two types of hyperlipidemia-induced diet groups ranged from 0.87 to 0.96, with an average of 0.93 and S.D. of 0.03 (control vs. HFCD), and 0.83 to 0.96, with an average of 0.91 and S.D. of 0.04 (control vs. HFCSD). In the comparison of the control vs. SDW, correlations for whole blood expression profiles were statistically significant according to Tukey's *post-hoc* tests. A low correlation coefficient was obtained for the control vs. hyperlipidemia-induced diet groups ($p < 0.01$).

For a toxicity evaluation of the SDW diet, we investigated the correlation coefficient between the control and AZIDE groups. The whole blood correlation coefficients between the control and AZIDE group was 0.82 to 0.88, with an average of 0.85 and an S.D. of 0.02.

Significant differences were observed between the control vs. SDW and the control vs. AZIDE comparisons, according to Tukey's *post-hoc* test ($p < 0.01$), and this indicates that the gene expression profiles of the SDW group and AZIDE groups were strikingly different.

To compare with the various food stress factors (i.e., HFCD, HFCSD, and AZIDE), we conducted a cluster analysis of the microarray expression data. As shown in Figure. 14, the data were roughly divided into two groups: the control and SDW data were classified in the same cluster, which was separated from the AZIDE cluster.

4 Discussion

In this study, we investigated the biochemical characterization and the gene expression profile of blood from pigs fed an SDW diet for the evaluation of their overall health status. There were no differences in growth or blood biochemistry between the control and SDW group pigs, indicating that the SDW diet maintained sufficient nutritional and good physiological condition of pigs.

In the assessment of gene expression, we conducted a microarray analysis with whole blood. For gene expression levels between the two groups (control and SDW), we found high correlations. Interestingly, in the SDW groups, the number of genes with fold changes in expression greater than 2.0 ($p < 0.05$) or less than 0.5 ($p < 0.05$) was only 56 (47 up-regulated and nine down-regulated genes) compared to the control group. This result indicated that the gene expression pattern of the SDW group was nearly identical

to that of the control group, suggesting that the SDW diet did not affect the gene expression of the pigs. For toxicity evaluation of the SDW diet, we compared the gene profile to that of the AZIDE group. AZIDE is an environmental pollutant. Because AZIDE is a readily biodegradable material and has no discharge regulation, some researchers have used AZIDE for toxicity examination (Rippen *et al*., 1996; Berndt *et al*., 2001; Massie *et al*., 2003). Through microarray analyses, we found that 4,521 genes (2,312 up-regulated and 2,209 down-regulated genes) were significantly altered in the AZIDE group compared to the control group; yet, very few genes were altered in the SDW group. Pistol *et al.* (2015) revealed differentially expressed genes as an effect of a zearalenone mycotoxin-contaminated diet in pigs. Similarly, we detected differentially expressed genes, including *TLR7* (toll-like receptor 7) and *ID2* (inhibitor of DNA binding 2), in the AZIDE-treated group; in contrast, these genes were not altered in the SDW group. Because these characteristic biomarker genes for toxic stress were unchanged in the SDW group, the SDW diet did not appear to be toxic or stressful for pigs.

The results of our cluster analysis also clearly demonstrated that the SDW-fed pigs had an expression pattern similar to that by normal feeding and, by contrast, the hyperlipidemia-inducing diets and the toxic AZIDE diet led to different gene expression

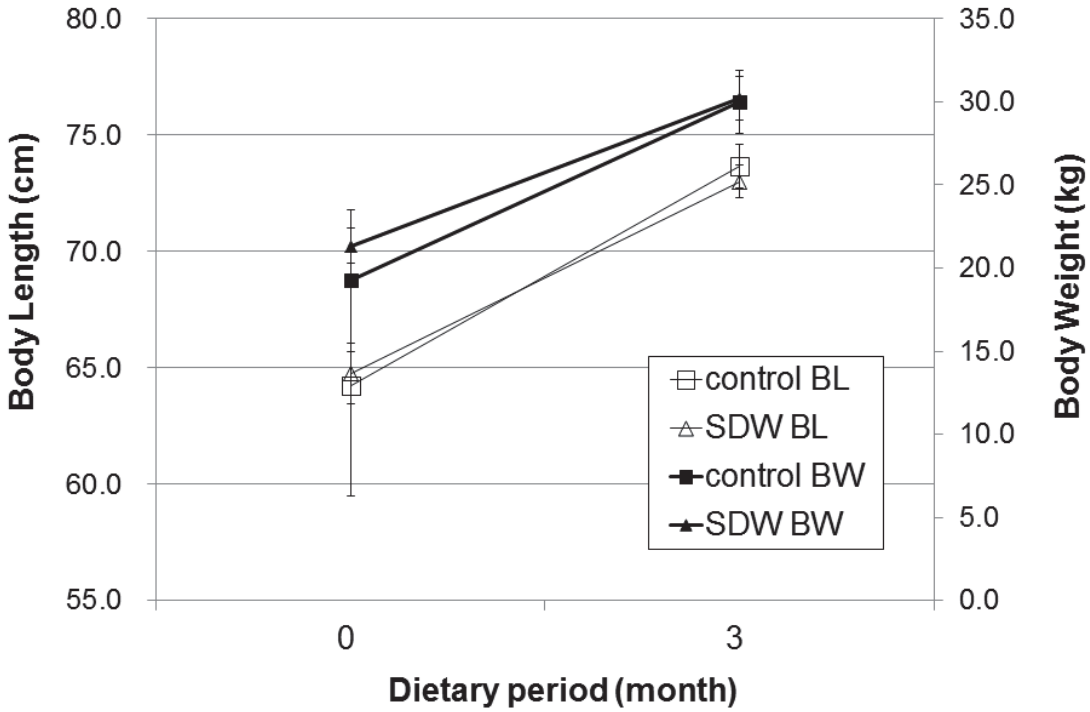
patterns. Many researchers have established the evaluation method for physiology and health of pigs through gene expression profiling by microarray analysis (da Costa *et al*., 2004; Moser *et al*., 2004; Niewold *et al*., 2005; Pena *et al*., 2014). We previously revealed the uniformity of the gene expression profile in miniature pigs older than 20 weeks of age (Takahashi *et al*., 2011), and reported the effectiveness of the microarray in the evaluation of physiological changes caused by the diet of the pigs (Takahashi *et al*., 2012). Our results support the efficacy of whole blood RNA microarray analysis for generating information concerning physiological changes resulting from the diet of Clawn miniature pigs. Furthermore, they indicate that whole blood RNA microarray analysis could be a useful tool for discriminating safe feed.

The pig populace in the Kagoshima Prefecture, Japan's top pig farming prefecture, was 1,332,000 in 2015 (Ministry of Agriculture, Forestry and Fisheries reference: http://www.maff.go.jp/). The standard dry feed amount required per pig throughout the fattening period is approximately 295 kg (Kawaida *et al*., 2007). When we substitute the SDW diet (feed intake: 266 kg/pig) for standard dry feed throughout the fattening period, we could use approximately 350,000 tons of SDW as a biomass resource. In this study, we fed SDW to animals for 3 months. The length of this period is equivalent to the fattening period. Even if we completely substituted standard dry feed with

SDW-containing feed, we could efficiently feed with SDW. The use of SDW-treated feed as a substitute feed could thus become one of the best solutions to the handling of the industrial waste, SDW. To establish the safety of SDW made from other sources, such as rice and barley, further investigation would be necessary.

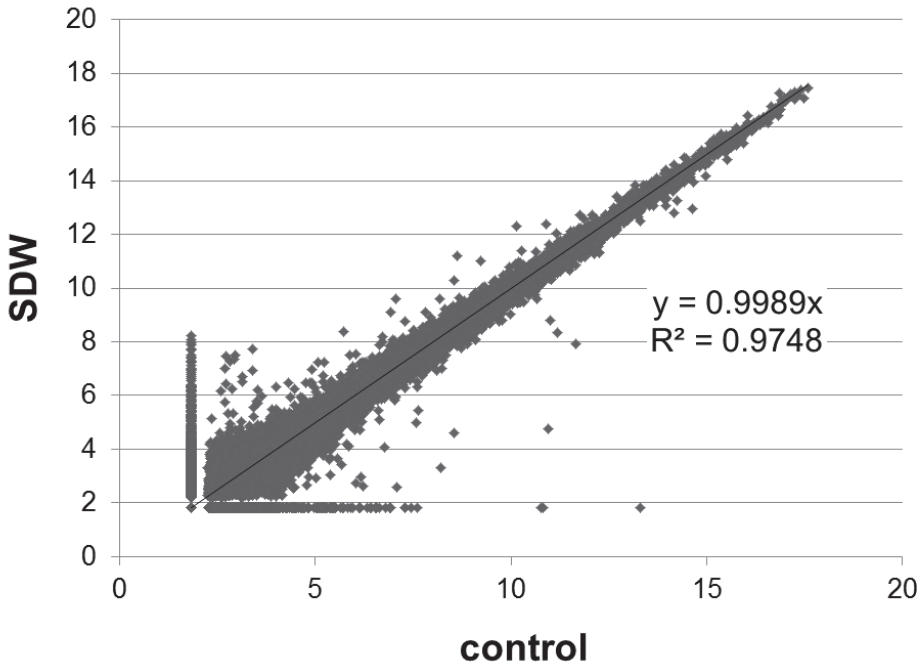
In conclusion, our study clarified the physiological invariance of pigs fed the SDW diet, and showed that the SDW diet is an available and safe feed substitute for standard dry feed.

Figure. 9 Subject body lengths and weights



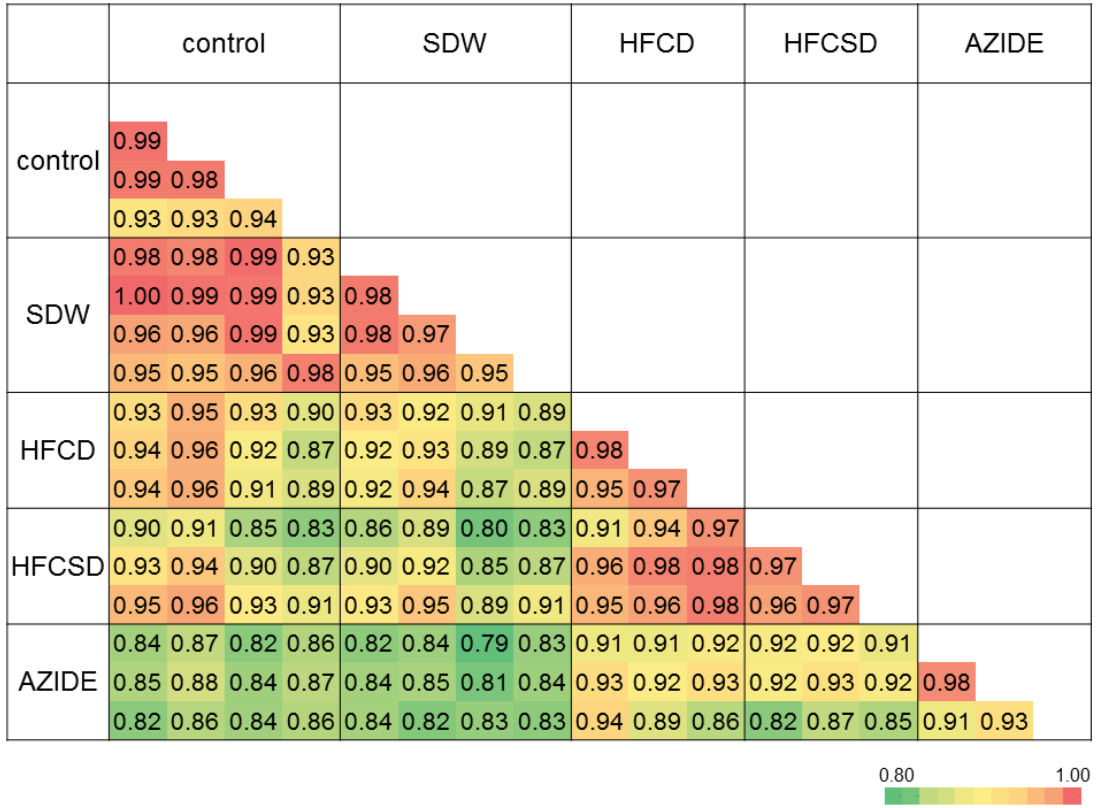
□ control body length, ▵SDW body length, ■ control body weight, ▲SDW body weight.

Figure. 10 Scatter plot of gene expression profiles of whole blood



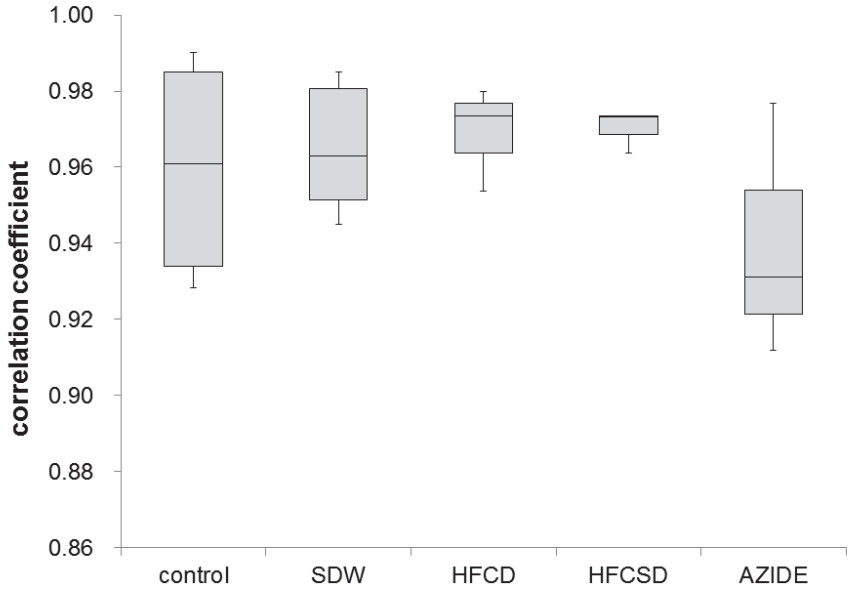
The global gene-expression patterns were compared between the control and SDW groups with oligonucleotide DNA microarrays. High correlations were found between the two groups.

Figure. 11 Correlation matrix of gene expression profiles of whole blood



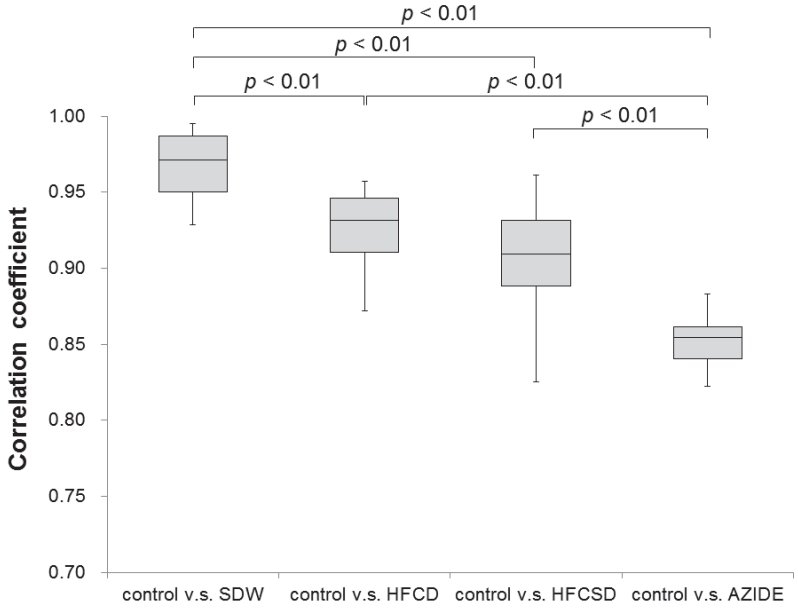
Triangular heat map representing the pairwise correlation coefficients. Correlation coefficients increases from green to red.

Figure. 12 Summary of correlation coefficients within the same diet group



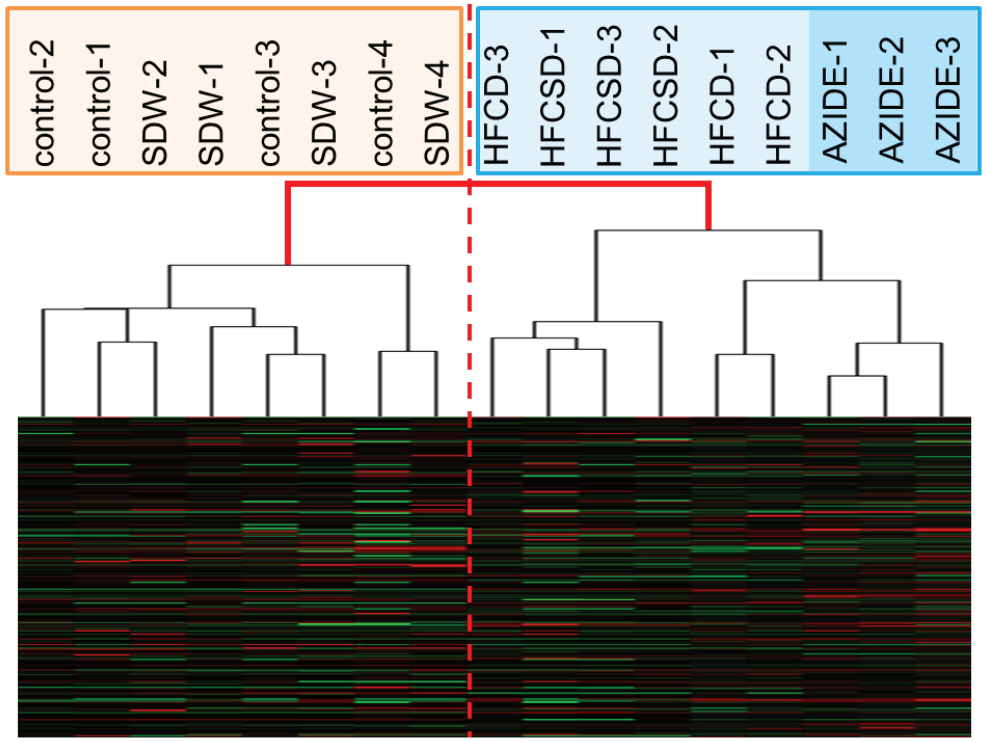
The top and bottom of the boxes denote the 75th and 25th percentiles, respectively. The lower and upper whiskers indicate the minimum and maximum values of the data. SDW, Shochu distillery waste; HFCD, high-fat and high-cholesterol diet; HFCSD, high-fat, high-cholesterol and high-sucrose diet; AZIDE, sodium azide.

Figure. 13 Summary of dietary-related correlation coefficients among different diet groups



The top and bottom of the boxes denote the 75th and 25th percentiles, respectively. The lower and upper whiskers indicate the minimum and maximum values of the data. SDW, Shochu distillery waste; HFCD, high-fat and high-cholesterol diet; HFCSD, high-fat, high-cholesterol and high-sucrose diet; AZIDE, sodium azide.

Figure. 14 Results of clustering analysis of gene expression data



The cluster analysis was performed using a hierarchical approach with the complete linkage-method. SDW, Shochu distillery waste; HFCD, high-fat and high-cholesterol diet; HFCSD, high-fat, high-cholesterol and high-sucrose diet; AZIDE, sodium azide.

Table 25. Results of hematological analysis.

test item			control (average ± S.D.)	SDW (average ± S.D.)	*p*-value
complete blood count	WBC, $10^2/\mu L$	white blood cell count	107.0 ± 35.6	107.0 ± 13.2	NS
	RBC, $10^2/\mu L$	red blood cell count	700 .0± 30.3	637.0 ± 42.8	NS
	HGB, g/dL	hemoglobin concentration	15.2 ± 1.5	15.1 ± 0.7	NS
	MCV, fL	mean corpuscular volume	50.0 ± 4.0	50.0 ± 3.0	NS
	PLT, $10^4/\mu l$	platelet count	31.8 ± 1.4	27.2 ± 9.8	NS
liver function	total bilirubin, mg/dL		0.2 ± 0.1	0.2 ± 0.0	NS
	AST(GOT), IU/L	aspartate transaminase	47.0 ± 23.7	54.0 ± 49.7	NS
	ALT(GPT), IU/L	alanine transaminase	30.0 ± 8.1	30.0 ± 19.2	NS
	ALP, IU/L	alkaline phosphatase	461.0 ± 337.0	758.0 ± 559.9	NS
	g-GTP, IU/L	γ-glutamyltransferase	71.0 ± 7.4	86.0 ± 30.0	NS
pancreatic function	serum amylase, IU/L		5130.0 ± 774.7	5701.0 ± 877.3	NS
renal function	BUN, mg/dL	urea nitrogen	22.6 ± 1.5	21.2 ± 3.0	NS
	creatinine, mg/dL		1.0 ± 0.2	1.0 ± 0.3	NS
	uric acid, mg/dL		0.2 ± 0.1	0.3 ± 0.1	NS
nourishment state	total protein, g/dL		7.8 ± 0.9	8.1 ± 1.5	NS
	total cholesterol, mg/dL		77.0 ± 8.6	96.0 ± 35.3	NS
	albumin, g/dL		5.0 ± 0.4	5.0 ± 0.7	NS
lipid metabolism	TG, mg/dL	triglyceride	17.0 ± 7.1	47.0 ± 38.7	NS
glycometabolism	blood glucose, mg/dL		77.0 ± 3.5	73.0 ± 8.2	NS
clotting function	PT, sec	prothrombin time	17.0 ± 1.0	17.0 ± 1.7	NS
	APTT, sec	activated partial thromboplastin time	20.0 ± 0.0	20.0 ± 0.0	NS
	Fbg, mg/dl	fibrinogen level	191.0 ± 24.4	213.0 ± 60.9	NS
electrolyte	Na, mEq/L		142.0 ± 1.0	144.0 ± 3.1	NS
	Cl, mEq/L		98.0 ± 1.5	100.0 ± 3.5	NS
	K, mEq/L		6.2 ± 1.2	6.7 ± 3.3	NS
	Ca, mg/dL		11.5 ± 0.2	11.2 ± 0.8	NS

Values are the average ± S.D. NS; not significant. *p*-values were calculated using Welch's *t*-tests.

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