Chapter 6

Whole Blood RNA Analysis, Aging and Disease

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Additional information is available at the end of the chapter

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1. Introduction

Microarray techniques allow to detect genome-wide perturbations during various treatments and to measure various responses by multitude of gene probes. Toxicogenomics, in which microarray techniques are specifically used in toxicology test, has been widely recognized as one of standard safety procedures for chemicals [1-3]. Gene expression microarrays have been used particularly for screening of genes involved in specific biological processes of interest, such as diseases or responses to environmental stimuli. Such experiments adopt the "healthy state" as a control, and identify highly expressed or suppressed genes. However, few studies deal with the features of gene expression and its variation at the "healthy state" to be influenced by species, age, sex, and individual variability. In measuring the state of disease and drug response, minimally invasive blood sampling, which allows for direct measurement of immune-responsive blood cells, excels other invasive biopsy techniques upon disease diagnostics and assessment of drug response, as well as health monitoring. Blood RNA contains an enormous amount of information on expression of messenger RNA and non coding functional RNA which remains without being translated into protein. Thus, blood RNA offers an opportunity to detect subtle change in physiological state. In this chapter, we discuss the potential of the RNA diagnosis using whole blood, showing a series of whole blood microarray experiments to evaluate variations of correlation among individuals and ages [4], dietary-induced hyperlipidemia, and other stresses using specific pathogen-free (SPF) miniature pigs.

2. The use of whole blood RNA analysis

Use of whole blood was intended on two accounts. First, RNA expression and degradation is susceptible to artificial manipulation such as cell separation and extraction. The whole blood manipulation avoids this risk, unlike dealing with extracted white blood cells. In addition, whole-blood RNA can be stabilized immediately by using RNA blood sampling

tube such as PAXgene. This avoids the cell separation process after sampling and minimizes the possibility of RNA denaturation. Usually, peripheral blood mononuclear cells (PBMCs) separation employs the difference of specific gravity between other blood components, which should be followed immediately after the blood sampling. Such manipulation requires a skilled operator to reduce the influence of separation procedures on gene expression. Second, the whole blood is a heterogeneous population of lymphocytes (monocytes, T-cells, and B-cells), granulocytes (neutrophils, eosinophils, and basophils), and platelets. One can expect that representative subpopulations in white blood cells may vary depending on the health condition of an individual. When a great alteration occurs in some subpopulations, the whole blood may also depart from the normal state of its age, because whole blood is a heterogeneous mixture of such subpopulations. Therefore, identification of gene expression characteristics and age-related variation in subpopulations in whole blood are essential issues.

3. The advantage of using miniature pigs

Pigs are a useful model animals of humans because they have similar anatomy and digestive physiology to human [5-6]. In particular, miniature pigs are easier to breed and handle than other nonprimates, making them an optimal species for preclinical test [7]. Moreover, blood samples can be taken repeatedly and human medical devices such as endoscopes and MRI and CT scanners are also applicable. These advantages increasingly allow miniature pigs for laboratory animals, with recent progress in upgraded supply systems. In spite of some large-scale microarray studies on pigs, only a limited amount of fundamental data is available for pigs compared to other laboratory species [8-9]. In September 2003, the Swine Genome Sequencing Consortium (SGSC) was formed by industry, government, and academia, to promote pig genome sequencing under international coordination [10]. In November 2009, since the announcement of completed swine genome map by members of the SGSC, its research environment has been enhanced [11].

4. Gene expression profiles change related to aging

It is particularly important to identify gene expression characteristics and variation of heterogeneous population of cells with age in whole blood.

Fractions of lymphocytes, monocytes, neutrophils, eosinophils, and basophils in white blood cells showed insignificant differences with age as a result of ANOVA analysis. This study attempted to identify characteristics of age-related gene expression by taking into account of change in the number of expressed genes by age and similarities of gene expression intensity between individuals.

4.1. Characteristics of study subjects

Five males and five females of 12 week old Clawn miniature pigs were housed individually in cages of 1.5 m^2 at the SPF facility of the breeder (Japan Farm Co., Ltd, Kagoshima, Japan)

for 18 weeks. Mean body weights of males and females at the beginning of the experiment were 7.0 kg and 6.9 kg respectively. During this period, all animals were fed with 450g/day standard dry feed (Kodakara73, Marubeni Nisshin Feed Co., Ltd., Tokyo Japan) with free access to water. Fetuses were taken out from their mothers on days 77 to the 84 days of the pregnancy by a Caesarean section. The unborn baby's sex was determined based on the shape of the vulva.

Sex	n	12 weeks	16 weeks	20 weeks	24 weeks	30 weeks	*P* †
Male	5	7.0 ± 0.6	10.7 ± 3.8	12.1 ± 2.6	15.0 ± 1.7	17.7 ± 1.7	< 0.001
Female	5	6.9 ± 0.5	7.9 ± 3.2	10.1 ± 2.6	13.5 ± 2.1	16.0 ± 2.6	< 0.001

Values are mean±SD. †*P* values were calculated using one-way factorial ANOVA.

Table 1. Subject body weight results
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All blood samples were collected from the superior vena cava at 12, 16, 20, 24, and 30 weeks of age. 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.

Body weight change and hematological variation during breeding period are shown Table 1 and Table 2, respectively. One-way ANOVA analysis for age-related variations in red blood cell count (RBC), hemoglobin concentration (HGB), and hematocrit value (HCT) showed significant differences for both males and females. However, the mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) remained unchanged. Differences in platelet count (PLT) and fibrinogen level (Fbg) were significant only for females. Any significant differences were not observed for both males and females for Prothrombin time (PT), activated partial thromboplastin time (ATPP), and the white blood cell count (WBC). Similarly to humans, the ratio of lymphocytes to white blood cells increased with maturation from 16 to 30 weeks of age. However, its difference was statistically insignificant according to ANOVA analysis. From 12 to 30 weeks of age, the ratios of granulocytes (neutrophils, eosinophils, and basophils), lymphocytes, and monocytes to white blood cells were unchanged, and differences were also insignificant.

4.2. Microarray gene expression profiles - Number of expressed genes

To characterize the age-related gene expression in whole blood from miniature pigs, RNA analysis was conducted on bloods sampled from fetal stage, 12, 20, and 30 weeks subjects. Each RNA sample was analyzed by an Agilent #G2519F#20109 Porcine Gene Expression Microarray (44K) consisting of 43603 oligonucleotide probes.

The change in the number of expressed genes to identify age-related characteristics was examined. Microarray gene expressions were divided into two groups; “absent” and

"present", using flag indicators given by the scanner. Background level was determined from spot intensities outside the gene probing area. "Absent" was assigned to the spots whose intensities were less than the background level, while the rests were marked as "present." Then each gene was judged as either "expressed" or "unexpressed" based on the number of "present" events. We defined a certain gene as "expressed" when "present" exceeds 75% out of replicated events. A threshold of 75% was chosen by considering experimental deviation.

Hematological analysis	Sex	n	12 weeks	16 weeks	20 weeks	24 weeks	30 weeks	*P* †
RBC, 10^4/μL	Male	5	742.7 ± 72.6	858.0 ± 97.7	894.8 ± 55.8	919.0 ± 21.0	866.2 ± 24.5	< .05
	Female	5	727.0 ± 20.2	886.6 ± 62.2	921.2 ± 64.5	901.4 ± 46.1	838.4 ± 44.2	< .001
HGB, g/dL	Male	5	14.9 ± 1.6	16.4 ± 1.2	17.3 ± 0.6	18.3 ± 0.4	17.7 ± 0.3	< .001
	Female	5	14.9 ± 0.4	17.5 ± 0.8	18.0 ± 0.9	18.4 ± 1.1	17.5 ± 0.6	< .001
HCT, %	Male	5	50.9 ± 5.1	53.6 ± 2.7	54.7 ± 2.1	58.4 ± 2.8	55.3 ± 1.2	< .05
	Female	5	49.0 ± 1.8	56.1 ± 2.2	57.8 ± 4.2	57.9 ± 3.0	54.8 ± 2.8	< .01
MCV, fL	Male	5	65.8 ± 1.0	66.3 ± 2.5	67.3 ± 2.9	65.1 ± 1.4	65.8 ± 2.2	NS
MCH, Pg	Male	5	19.8 ± 0.5	20.0 ± 1.1	20.1 ± 0.9	20.5 ± 0.6	20.6 ± 0.8	NS
CHC, %	Male	5	30.1 ± 0.4	30.2 ± 1.0	29.9 ± 0.9	31.5 ± 0.9	31.2 ± 0.8	NS
PLT, 10^4/μl	Male	5	21.3 ± 0.4	31.6 ± 10.8	18.1 ± 4.4	25.0 ± 8.6	24.9 ± 5.1	NS
	Female	5	34.5 ± 2.0	24.8 ± 5.5	19.0 ± 5.0	24.8 ± 8.9	19.7 ± 5.7	< .05
PT, sec	Male	5	13.8 ± 3.2	15.5 ± 0.3	16.5 ± 0.9	15.9 ± 0.7	16.1 ± 0.6	NS
	Female	5	-	15.8 ± 1.1	16.1 ± 0.5	16.4 ± 0.5	16.0 ± 0.7	NS
APTT, sec	Male	5	< 20	< 20	< 20	< 20	< 20	
	Female	5	< 20	< 20	< 20	< 20	< 20	
Fbg, mg/dl	Male	5	171.3 ±36.9	185.8 ± 93.8	169.4 ± 39.4	158.6 ± 9.0	147.8 ± 34.2	NS
	Female	5	-	160.2 ± 19.4	145.2 ± 16.3	176.5 ± 20.1	123.3 ± 27.5	< .05
WBC, 10^2/μL	Male	5	62.0 ± 18.7	86.6 ±12.7	78.8 ± 24.7	79.6± 24.0	71.8 ± 13.2	NS
	Female	5	66.0 ± 23.4	74.0 ± 13.7	78.0 ± 18.7	72.4 ± 10.4	61.8 ± 11.3	NS
Lymphocyte, %	Male	5	34.8 ± 12.1	45.2 ± 7.4	44.6 ± 9.3	36.8 ± 6.9	33.6 ± 7.6	NS
Neutrophil, %	Male	5	55.0 ± 10.9	43.1 ± 10.3	44.8 ± 7.4	52.2 ± 7.0	56.2 ± 9.2	NS
Eosinophil, %	Male	5	3.8 ± 2.2	3.1 ± 1.4	3.0 ± 1.9	5.0 ± 2.7	4.6 ± 1.7	NS
Basophil, %	Male	5	0.3 ± 0.5	0.3 ± 0.4	0.2 ± 0.4	0.0 ± 0.0	0.2 ± 0.4	NS
Monocyte, %	Male	5	6.3 ± 1.0	8.0 ± 3.2	7.4 ± 1.5	6.0 ± 2.1	5.4 ± 1.3	NS

Biochemical variables for miniature pigs during the experiment are shown. Values are mean±SD. RBC, red blood cell count; HGB, hemoglobin concentration; HCT, hematocrit value; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin ; MCHC, mean corpuscular hemoglobin concentration; PLT, blood platelet count; PT, prothrombin time; ATPP, activated partial thromboplastin time; Fbg, fibrinogen level; WBC, white blood cell count; and NS: not significant. †*P* values were calculated using one-way factorial ANOVA.

Table 2. Subject hematology results
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The number of expressed genes was less in fetal stage and infancy period but increased with age, reaching a steady state of gene expression after 20 weeks of age (Figure 1). Expressed genes for male and female were analyzed by one-way factorial ANOVA. Then Tukey-Kramer's method was applied only to significant groups. Differences between age groups (fetal stage, 12, 20, and 30 weeks of age) were significant for male, female, and mixed subjects of male and female. A Tukey-Kramer's multiple comparisons test revealed that differences between fetal stage and other age groups were statistically significant ($p<0.001$) for both male and female. Also, differences were significant ($P<0.05$) between 12 and 30 weeks females.

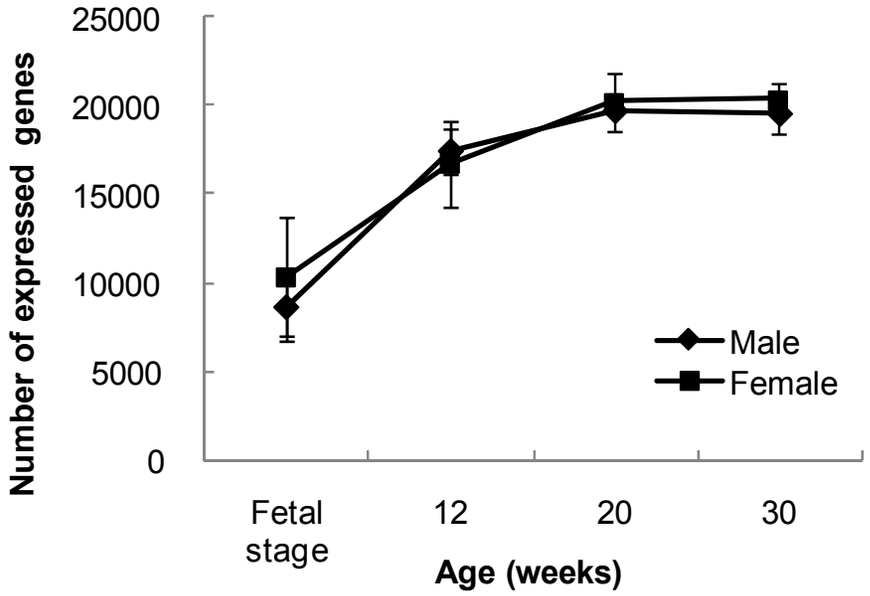

Figure 1. Number of genes expressed in whole blood of miniature pigs at different ages. In the graph, ◆ represents male and ■represents female. Values are means±SD.
doi:10.1371/journal.pone.0019761.g001

4.3. Microarray gene expression profiles – Correlation of gene expression

Variations in correlation coefficients among individuals of the same age and different age groups were evaluated. Pearson correlation coefficient was used for correlation analysis. Correlation coefficients for a total of 31 microarrays were obtained in normalized signals log-scale after excluding "absent" spots. A color-coded pairwise correlation matrix is shown in Figure 2. The color scale at the bottom indicates correlation strength.

The average correlation coefficient within the same age group is shown in Figure 3. Variations in gene expression were greater for younger subjects, but it diminished with age while generating resembling expression patterns. Correlation coefficient within 30 weeks age group was slightly smaller than that within 20 weeks age group. However, this difference is smaller than other distant age groups. Significant differences were observed between any age groups according to an ANOVA analysis using Fisher's Z-transform. The average correlation coefficient between different age groups is shown in Figure 4. Significant

differences were observed except between "fatal stage vs. 20 weeks" and "fatal stage vs. 30 weeks", and between "12 weeks vs. 20 weeks" and "12 weeks vs. 30 weeks" according to an ANOVA analysis using Fisher's Z-transform ($P < 0.05$). These results suggest that the variation in gene expression intensity within the same age was great in fetal stage and infancy period, but converged with age.

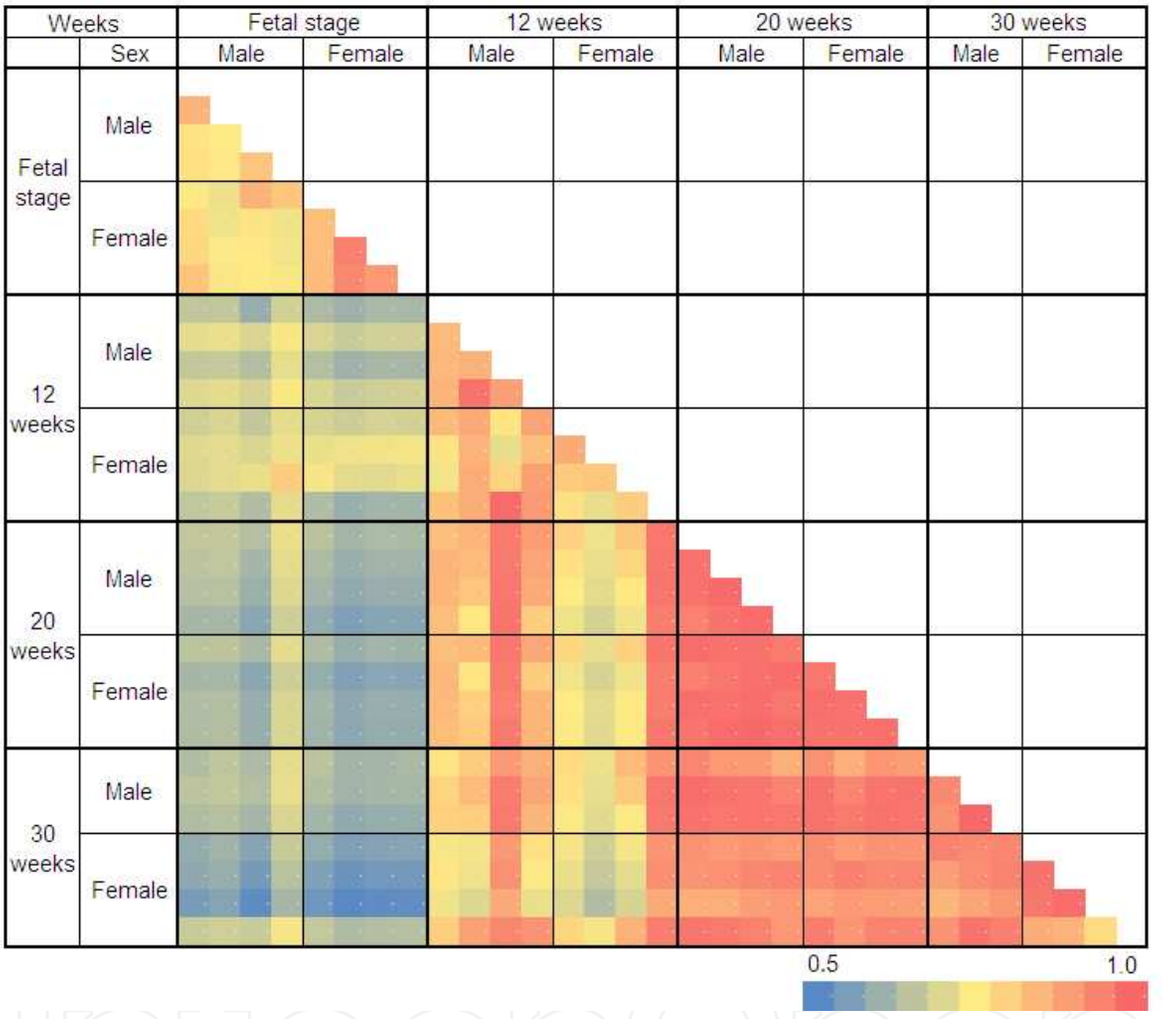

Figure 2. Correlation matrix of age-related gene expression. This color-coded correlation matrix illustrates pairwise correlations between the levels of gene expression in individuals. Probe sets with normalized signals (log-transformed and scaled) were used to calculate correlations between 31 arrays using Pearson correlation coefficient; signals flagged as "absent" were excluded.
doi:10.1371/journal.pone.0019761.g002

4.4. Classification of genes depending on the status of age-related expression

All spots on the microarray were divided into 16 categories as shown Table 3 after assigning "1" for expressed genes and "0" for unexpressed genes. Here, definitions of "expressed" and "unexpressed" are described in "Materials and methods." Category 1 consists of a total of 6,763 genes expressed in the fetal stage, 12, 20, and 30 weeks of age. Category 2 consists of a total of 7,564 genes expressed at 12, 20, and 30 weeks of age. Category 4 consists of a total

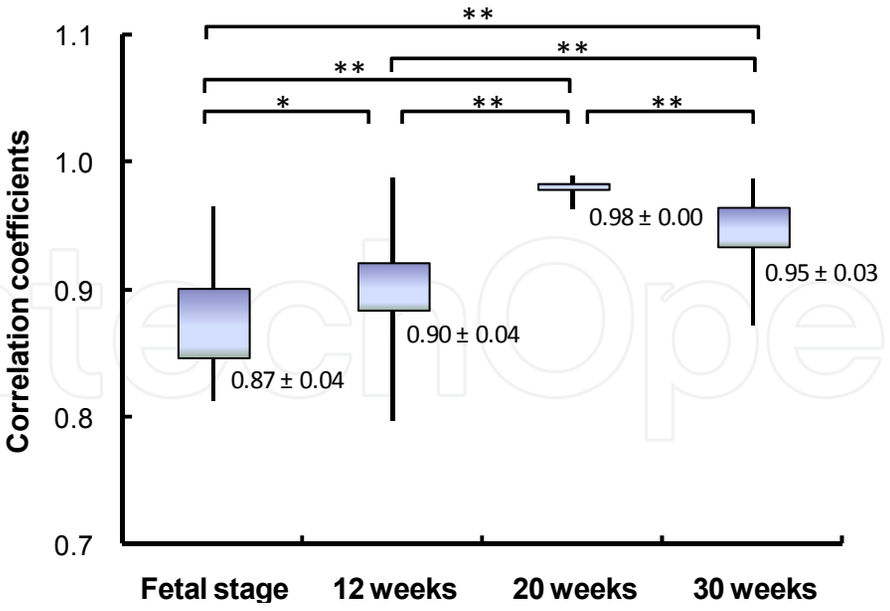

Figure 3. Age-related correlation coefficients within the same age groups. Correlation coefficients were calculated between individuals within the same age groups. The bottom and top of the boxes represent the 25th and 75th percentiles respectively. The lower and upper whiskers denote the minimum and maximum values of the data. Comparisons of the groups were made with the ANOVA test. * $p < 0.05$, ** $p < 0.01$.

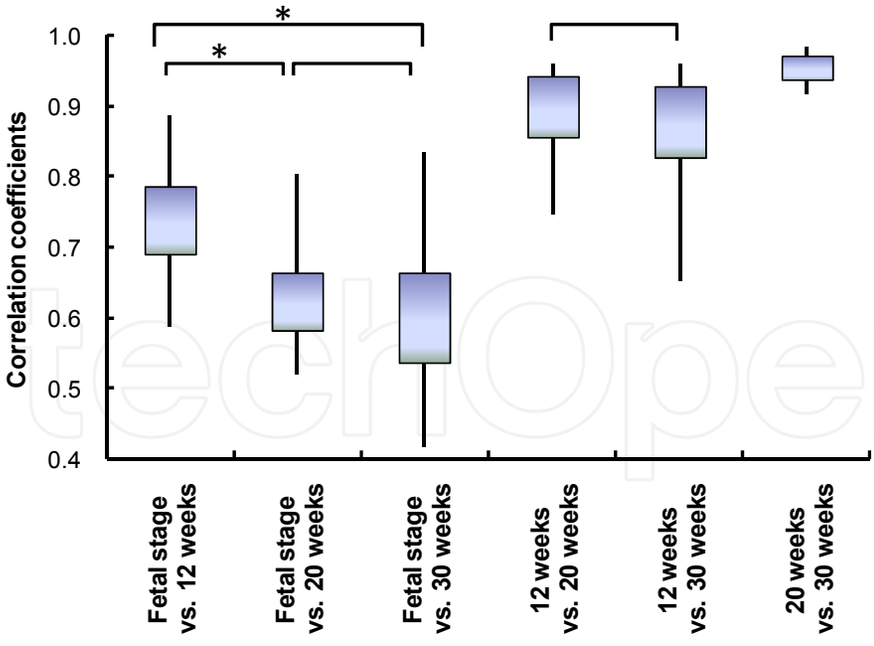

Figure 4. Age-related correlation coefficients between the different age groups. Correlation coefficients were calculated between the different age groups. The bottom and top of the boxes represent the 25th and 75th percentiles respectively. The lower and upper whiskers denote the minimum and maximum values of the data. Comparisons of the groups were made with the ANOVA test. * $p < 0.01$.

of 3,547 genes expressed after 20 weeks of age. Category 8 consists of a total of 827 genes expressed after 30 weeks of age. Sum of the genes expressed at certain age and those unexpressed (Categories 3, 5, 6, 7, 9, 10, 11, 12, 13, 14, and 15) was 1,051. Its fraction was 5.6% of 18,701 genes (Categories 1, 2, 4, and 8) expressing constantly once they appeared. Category 16 consists of genes unexpressed throughout the breeding period. Figure 5 shows the ratio of the genes belonging to each category.

Category	Fetal stage	12 weeks	20 weeks	30 weeks	Number of genes	Definition
1	1	1	1	1	6763	genes expressed from fetal stage to 30 weeks
2	0	1	1	1	7564	genes expressed from 12 to 30 weeks
3	1	0	1	1	49	
4	0	0	1	1	3547	genes expressed from 20 to 30 weeks
5	1	1	0	1	14	
6	0	1	0	1	80	
7	1	0	0	1	7	
8	0	0	0	1	827	genes expressed at 30 weeks
9	1	1	1	0	73	
10	0	1	1	0	124	
11	1	0	1	0	29	
12	0	0	1	0	428	genes expressed at 20 weeks
13	1	1	0	0	16	
14	0	1	0	0	147	genes expressed at 12 weeks
15	1	0	0	0	84	genes expressed in fetal stage
16	0	0	0	0	23851	genes not expressed from fetal stage to 30 weeks

Depending on the status of expression, all spots on the microarray can be divided into 16 categories.

Here, “1” represents an expressed gene and “0” represents an unexpressed gene.

Table 3. Genes classified into 16 categories according to the status of age-related expression
doi:10.1371/journal.pone.0019761.t005

To characterize gene expression in each category, TC Annotator List (Porcine version 14.0 3-11-10) was downloaded from the TIGR gene Indices. TC Annotator List includes the gene number and the GO terms. Out of 43,603 probes in the Agilent porcine microarray (#G2519F#20109), 6,019 genes bear GO annotation. Microarray cDNA probes were classified by GO terms of “biological processes”. Out of all genes, fraction in Categories 1, 2, 4, 8, and 16 were 31%, 20%, 8%, 2%, and 38% respectively.

Then the difference in gene expression between all spots and those in 4 categories (Categories 1, 2, 4, and 8) was examined. GO groups dominantly expressed in Category 1 relates to mitosis (GO:0000070, GO:0000022, GO:0007052, and GO:0007100) and to immune

(GO:0043161, GO:0045059, GO:0019886), while those highly expressed in Category 2 related to cellular defense and regulation.

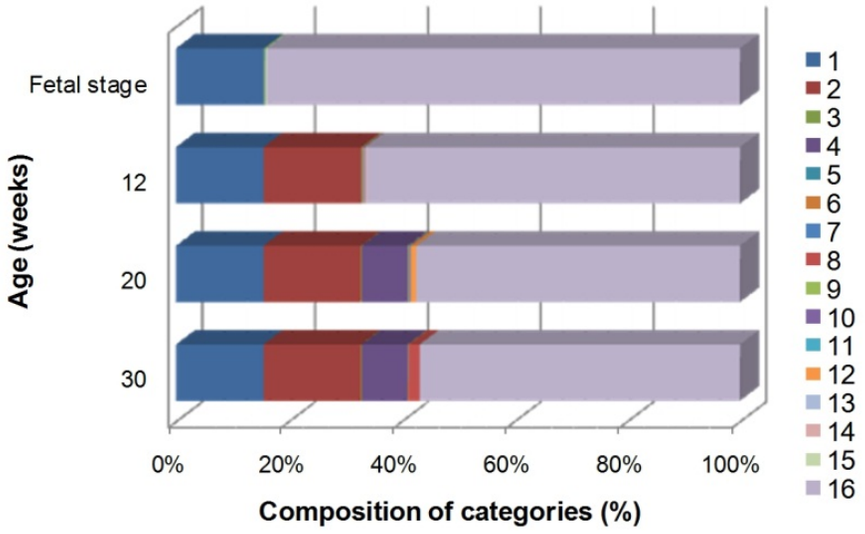

Figure 5. Ratios of categories for groups of the same age. The ratios of the genes in each category were calculated for groups in the fetal stage and at 12, 20, and 30 weeks of age. Categories are defined in Table 3.
doi:10.1371/journal.pone.0019761.g003

4.5. Age-related changes in gene expression levels for the immune system

Expression intensity of immunity gene was examined. Antigen processing and presentation (GO:0019882) and T cell selection (GO0045058) include the major histocompatibility complex (MHC) genes. By presenting antigens, MHC is involved in elimination of bacterial or viral pathogen, rejection of cancer cells, and rejective response on organ transplantation. Also MHC is indispensable in the immune system. Swine leukocyte antigens (SLA) are important immunogens for humoral responses and important mediators of the cellular immune responses through both direct and indirect presentation of peptides to T-cells [12]. SLA includes 6 of classical class I genes (SLA-1, SLA-2, SLA-3, SLA-6, SLA-7, and SLA-8) and 8 of classical class II genes (SLA-DMA, SLA-DMB, SLA-DOA, SLA-DOB1, SLA-DQA, SLA-DQB1, SLA-DRA, and SLA-DRB1) [13-14]. SLA class II lacks DPA1, DPB1, DRB3, DRB4, and DRB 5 in humans. On the Agilent porcine microarray, all of SLA genes except DOA are mounted on 28 spots. Among these, 11 SLA genes fell under Category 1, 1 fell under Category 2, and 1 fell under Category 8. Expression of SLA classical class I and class II genes are shown in Figure 6A and 6B, respectively. Both genes expressed in fatal stage, 12, 20, and 30 weeks in an increased manner by age.

The Agilent porcine microarray had 7 probes with 7 types of interferon and 7 probes for 4 types of interferon receptors. All of 7 interferon genes fell under Category 16. Normally these genes remain unexpressed but expressed upon necessity. In contrast, 1 type of interferon receptor gene fell under Category 1, 3 fell under Category 2, and were expressed

until 12 weeks of age. Their signal intensities stayed at constant levels after 12 weeks (Figure 6C).

Toll-like receptors (TLRs) are the principal pattern recognition receptors. With this innate immunity, the first immune response is mediated into reserved foreign patterns on recognition. TLRs recognize reserved molecular patterns, start rapid response to protect the host upon infection, and produce signals, such as cytokines and co-stimulatory molecules to activate the adaptive immune system [15-16]. Regulation of the TLR signaling cascade is important for inflammatory responses, innate host defense, and adaptive immune responses [17-18]. Most mammalian species are estimated to have between 10 and 15 types of TLRs. The Agilent porcine microarray has 10 types of TLRs probes. Among these TLRs, 5 of TLR genes fell under Category 2 (expressed until 12 weeks of age), 1 under Category 8, and 4 under Category 16. Their signal intensities remained constant after 12 weeks of age (Figure 6D).

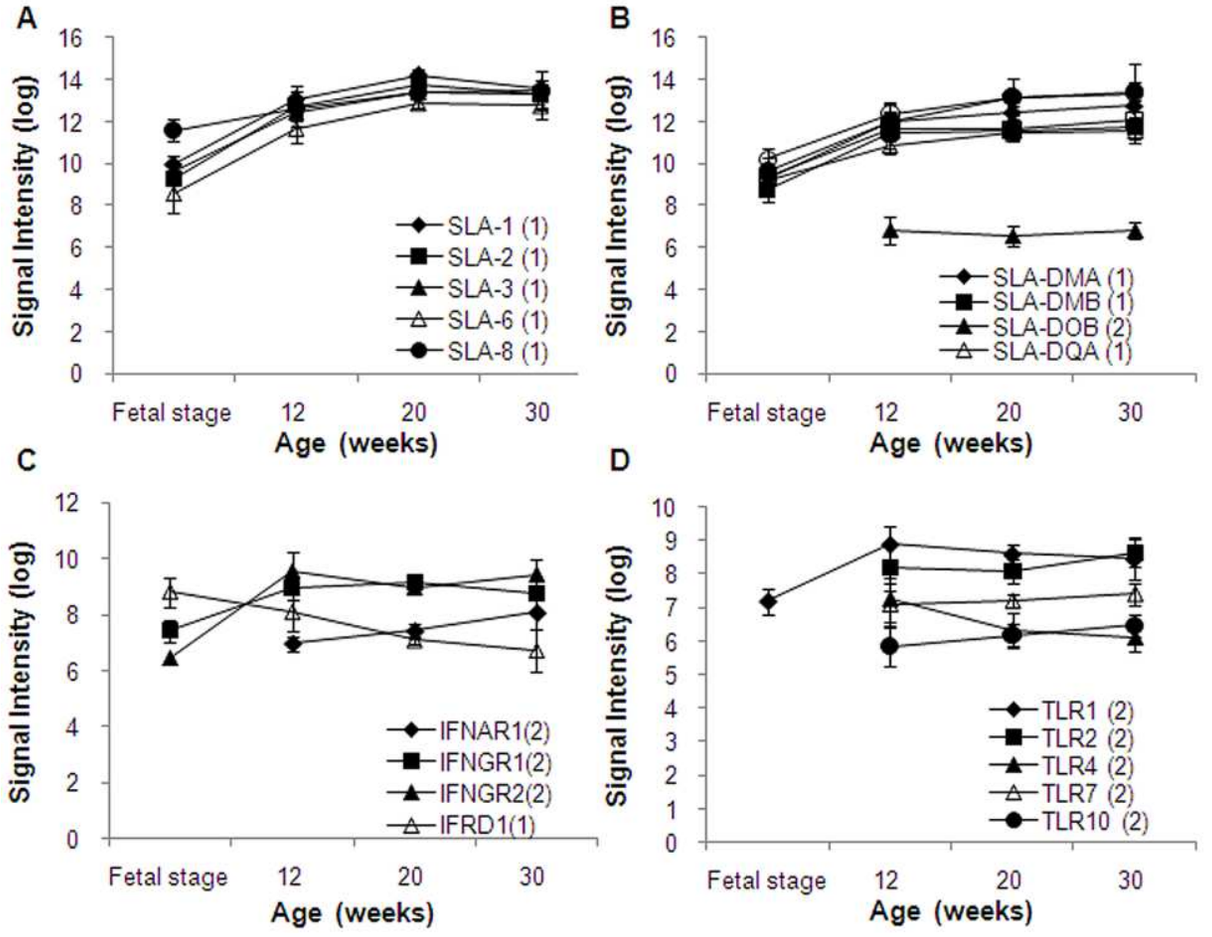

Figure 6. Signal intensity of major histocompatibility complex (MHC) genes. (A) Swine leukocyte antigens (SLA) classical class I genes. (B) Swine leukocyte antigens (SLA) classical class II genes. (C) Interferon receptor genes. (D) Toll-like receptor (TLR) genes. Signal intensities were normalized using quantile normalization and log-transformed after excluded signals flagged as "absent." The category numbers are shown in graph legends. Genes in Categories 1, 2, and 4 are shown in the graph.
doi:10.1371/journal.pone.0019761.g005

5. Gene expression profiles change related to hyperlipidemia

To examine the usage of whole blood RNA analysis for the early diagnosis of the disease, we showed transitions in dietary induced hyperlipidemia gene expression profiles of whole blood RNA in miniature pigs.

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 [19]. High-fat, high-cholesterol, and high-sugar diets have been shown to induce hyperlipidemia, obesity, and insulin resistance in humans and rodents [20-22]. Dietary-induced hyperlipidemia pig models have also been established [23-29].

A high-fat and high-cholesterol diet (HFCD) as a typical dietary treatment were used for dietary-induced hyperlipidemia miniature pig models, by using specific pathogen-free (SPF) Clawn miniature pigs.

Eight 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).

Almost no changes were observed in fasting plasma triglyceride levels. Fasting plasma total cholesterol concentrations had increased in the HFCD group by week 5 of the feeding period ($P<0.001$) and were maintained between 350 and 1150 mg/dL from weeks 10–27. Fasting plasma high-density lipoprotein cholesterol (HDL-C) concentrations increased and showed significant differences ($P < 0.001$) from weeks 10–27. Fasting plasma low-density lipoprotein cholesterol (LDL-C) concentrations also increased and showed significant differences from weeks 5–27. Fasting plasma glucose concentrations remained unchanged.

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

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 aporcine gene expression microarray consisting of 43603 oligonucleotide probes.

Variation in correlation coefficients among individuals on the same diet and between different diet groups was evaluated. Pearson correlation coefficients were used for the correlation analysis. Correlation coefficients for 23 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 7.

The correlation coefficients of whole blood expression profiles within the same diet groups were 0.97 (0.01) (mean (standard deviation; SD)), and 0.94 (0.05) for the control, HFCD whole blood at 10 weeks, 0.94 (0.03), and 0.93 (0.06) at 19 weeks, and 0.95 (0.02), and 0.95 (0.03) 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.

The whole blood correlation coefficients among the different diet groups were 0.95 (0.04) for control vs. HFCD at 10 weeks, 0.93 (0.03) at 19 weeks, and 0.95 (0.03) at 27 weeks, respectively.

5.2. Assigning known functions to gene expression - Gene ontology annotation

Up- and down-regulated genes were identified 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 ($p < 0.05$) and less than 0.5 ($p < 0.05$) were selected at 10, 19, and 27 weeks. As a result, the GO categories of many genes up-regulated at the end of the 19-week dietary period were related to nucleotide binding (GO: 0000166, GO: GO: 0005524, 0005525, GO: 0017076, GO: 0019001, GO: 00032553, GO: 00032555, GO: 0032561), and catabolic processes (GO: 0009057, GO: 0019941, GO: 0030163, GO: 0043632, GO: 0044257, GO: 0044265,). Many genes down-regulated after 27 weeks were in the GO categories related to biological adhesion (GO: 0007155, GO: 0022610).

5.3. Effect of white blood cells on whole blood gene expression profiles in dietary-induced hyperlipidemia

Microarray analyses were conducted from white blood cells at the end of the dietary period to evaluate the effect of white blood cells on whole blood gene expression profiles (Figure 8). The correlation coefficients of white blood cells expression profiles within the same dietary groups were 0.94 (0.05) and 0.95 (0.03) for the control and HFCD groups at 27 weeks. The white blood cells correlation coefficients was 0.94 (0.04) between control and HFCD. The average correlation coefficients between whole blood and white blood cells were 0.83 (0.04) and 0.79 (0.05) for control and HFCD. Using Fisher's Z-transformation to normalize the correlation distributions, no significant differences in correlation coefficients of white blood cells were observed between control and HFCD groups.

Up- and down-regulated genes were identified and classified these according to function using information from the Gene Ontology (GO) Database to understand the observed differences in white blood cells gene expression profiles for the different dietary groups, as the same as whole blood gene expression profiles. Top-ranked genes with fold changes in expression greater than 2.0 ($p < 0.05$) and less than 0.5 ($p < 0.05$) were selected at 27 weeks. As a result, many genes down-regulated related oxidation-reduction process (GO:0055114) and keg pathways of steroid biosynthesis.

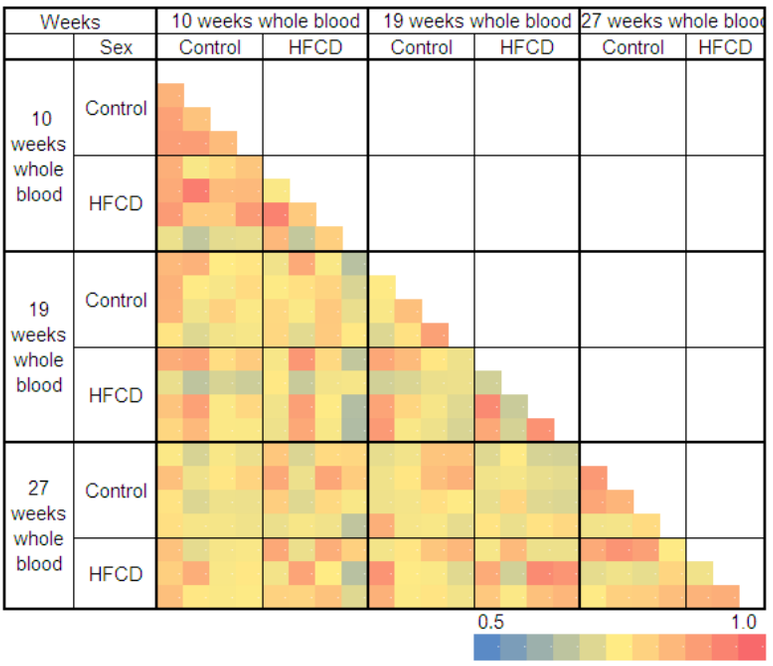

Figure 7. Correlation matrix of dietary-related gene expression profiles of whole blood. This color-coded correlation matrix illustrates pairwise correlations between the levels of gene expression in individuals. Probe sets with normalized signals (log-transformed and scaled) were used to calculate correlations between 23 arrays using Pearson correlation coefficient; signals flagged as "absent" were excluded. The color scale at the bottom indicates the strengths of the correlations.

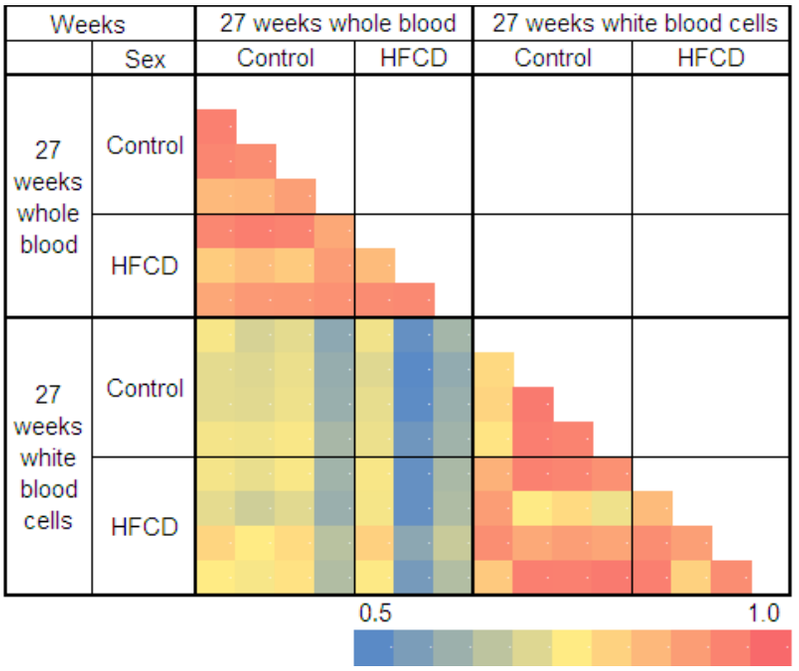

Figure 8. Correlation matrix of dietary-related gene expression profiles of whole blood and white blood cells. This color-coded correlation matrix illustrates pairwise correlations between the levels of gene expression in individual at feeding period at week 27. Probe sets with normalized signals (log-transformed and scaled) were used to calculate correlations between 15 arrays using Pearson correlation coefficient; signals flagged as "absent" were excluded. The color scale at the bottom indicates the strengths of the correlations.

6. Gene expression profiles change with other stresses

Furthermore, a possibility was shown that whole blood RNA analysis is applicable to evaluation of physiological state.

The degree of stress can be comparable according to the numbers of up-regulated and down-regulated genes, even if the stress is different in quality from the others.

Sodium azide was given orally to the miniature pigs over 20 weeks. There were no significant changes of hematological and biochemical properties for administrated dose of 300μg/kg, one hundredth of LD_{50}. On the other hand, gene expression profiles were obviously changed. Anesthesia group showed a slight degree, but the one week fasting group showed a significant difference. This can be clearly noticed when the contents of stress is classified by the function of up-regulated and down-regulated genes. Consequently, grade of the stress can be estimated according to the expression state of genes.

Stresses	P<0.05, Fold change>2		
	total	up regulation	down regulation
sodium azide 300μg/kg ; LD_{50} 1/100	893	339	554
blood removal（150ml）after 6 hours	1747	227	1520
Fasting a week	3136	1840	1296
anesthesia after 6 hours	160	87	73
non treatment (blood removal 20ml)	73	14	59

Table 4. Summery of gene expression condition of several types of stress
Number of genes

7. Effects of white blood cells on whole blood gene expression profiles

Whole blood contains a variety of cell types as red blood cells, granulocytes, lymphocytes, and platelets. Most of the nucleated cells in blood are white blood cells such as neutrophils, T-cells, B-cells, and monocytes. The number of white blood cells in humans is known to decrease steadily from infancy to adulthood, and its composition (i.e. lymphocytes, granulocytes) also changes with age [30]. In study of the gene expression profiles change related to aging, hematological data of the fetal stage was unavailable because the amount of collected blood was insufficient for the analysis. From 12 to 30 weeks of age, ANOVA analysis indicated no significant differences in the fractions of lymphocytes, neutrophils, eosinophils, basophils, and monocytes. In addition, these compositions were almost equal to those in human adults. The above result suggests that the gene expression profile change of age-related whole blood RNA is not due to the composition of white blood cell subpopulations.

The intraclass correlation between Staphylococcus enterotoxin B-stimulated and unstimulated blood from healthy subjects was significantly higher in leukocyte-derived samples the in whole blood, suggesting that the method of RNA isolation from whole blood

can be a critical step in blood RNA assay [31]. Although PBMCs do not contain neutrophils, eosinophils, basophils, nor platelets, Min et al. reported highly correlated results ($r^2 = 0.85$) for 8,273 genes expressed between the whole blood RNA, by using the PAX gene Blood RNA system, and peripheral blood mononuclear cell (PBMC) RNA samples isolated from healthy volunteers by using a Ficoll-Paque gradient and TRI Reagent (SIGMA) [32]. Other workers conducted a large scale genome-wide expression analysis of white blood cells subpopulations. This study indicates that correlation coefficients for T-cells and monocytes among different healthy subjects were 0.98±0.01 and 0.97±0.01, respectively. However, for the same subjects (n=5), correlation coefficients between T-cells and monocytes was 0.88±0.01, indicating varied correlation between white blood cells subpopulations. In addition, gene expression analysis were showed a varying dependence on the isolation method such as PAXgene, Buffy coat, and lysis. The correlation coefficients between isolation methods were 0.89±0.04, 0.91±0.04, 0.96±0.06, for PAXgene vs. lysis, PAXgene vs. Buffy coat, and Buffy coat vs. lysis, respectively [33]. In order to ensure the reliability for to clinical use of whole blood RNA diagnosis, the development of standard method and measurement standards needs to be sought.

The Gene Ontology (GO) Database was used to categorize gene expression profiles functionally to conduct the effects of white blood cells on whole blood gene expression profiles in our study of hyperlipidemia. As a result, the GO term, related to white blood cell function (GO: 0006954, 0007166), had a high correlation coefficient. 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), showed low correlation coefficients. We, therefore, conclude that the difference in the gene expression profiles between the whole blood and white blood cells are not only caused by differences in experimental protocols, but also by differences in RNA origin [34].

8. Conclusion

Whole blood RNA is easy to handle compared to isolated white blood cell RNA and can be used for health and disease monitoring and animal control. In addition, whole blood is a heterogeneous mixture of subpopulation cells. Once a great change occurs in composition and expressing condition of subpopulations, their associated change will be reflected on whole blood RNA.

Whole blood microarray analyses were conducted to evaluate variations of correlation among individuals and ages using specific pathogen-free (SPF) Clawn miniature pigs. The characteristics of age-related gene expression by taking into account of change in the number of expressed genes by age and similarities of gene expression intensity between individuals were identified. As a result, the number of expressed genes was less in fetal stage and infancy period but increased with age, reaching a steady state of gene expression after 20 weeks of age. Variation in gene expression intensity within the same age was great in fetal stage and infancy period, but converged with age. The variation between 20 and 30

weeks of age was comparable to that among 30 weeks individuals. These results indicate that uniformity of laboratory animals is expected for miniature pigs after 20 weeks of age.

In dietary-induced hyperlipidemia 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. Variation in whole blood gene expression intensity among individuals within the HFCD 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. Dietary-induced transitions of gene expression profiles for genes bearing GO terms were examined. Major changes included an induction of proteins involved in catabolic processes and protein metabolism after a 19-week dietary period, and a reduced expression of proteins involved in steroid metabolism and lipid biosynthesis after a 27-week dietary period.

In several kinds of stress study, the degree (extent) of stress can be comparable according to the gene number of up-regulate, or down-regulate, even if the stress is different in kind from the others.

A possibility was shown that whole blood RNA analysis is applicable to evaluation of physiological state. By considering variation in gene expression profiles of miniature pigs, whole blood RNA analyses can be used in practical applications. The blood RNA diagnostics under development may eventually be useful for monitoring human health.

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