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SLA Genetic Polymorphism and Large Scale Gene Expression Profiling of Cloned SNU Miniature Pigs Derived from Same Cell Line

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ABSTRACT

In order to investigate genetic stability and gene expression profile after cloning procedure, two groups of cloned pigs were used for swine leukocyte antigen (SLA) gene nucleotide alteration and microarray analyses. Each group was consist of cloned pigs derived from same cell line (n=3 and 4, respectively). Six SLA loci were analyzed for cDNA sequences and protein translations. In total, 16 SLA alleles were identified and there were no evidence of SLA nucleotide alteration. All SLA sequences and protein translations were identical among the each pig in the same group. On the other hand, microarray assay was performed for profiling gene expression of the cloned pigs. In total, 43,603 genes were analyzed and 2,150～4,300 reliably hybridized spots on the each chip were selected for further analysis. Even though the cloned pigs in the same group had identical genetic background, 18.6～47.3% of analyzed genes were differentially expressed in between each cloned pigs. Furthermore, on gene clustering analysis, some cloned pigs showed abnormal physiological phenotypes such as inflammation, cancer or cardiomyopathy. We assumed that individual environmental adaption, sociality and rank in the pen might have induced these different phenotypes. In conclusion, the results of the present study indicate that SLA locus genes appear to be stable following SCNT. However, gene expressions and phenotypes between cloned pigs derived from the same cell line were not identical even under the same rearing conditions.

(Key words : Somatic cell nuclear transfer, SLA, Microarray, SNU Miniature pig)

INTRODUCTION

In order to produce transgenic and knock-out pigs, the somatic cell nuclear transfer (SCNT) technique is primarily used for relatively high efficiency and possibility of gene targeting. However, early lethality or aberrant development of cloned pigs was also reported previously. This abnormalities might be arise from genetic alteration including genomic damage in donor cells (Humpherys *et al*., 2002), and/or abnormal chromosome distribution at the two-cell stage (Kawasumi *et al*., 2007) or insufficient epigenetic reprogramming of the somatic cell nucleus by the oocyte (Ogonuki *et al*., 2002). On the contrary, cloned pigs that survive to reproductive age seem to be normal in reproduction characteristics (Polejaeva *et al*., 2000; Tian *et al*., 2009). Thus it will be important to analyze genetic stability and gene expression profile of the cloned pig especially for further use of the animal in clinical applications.

Leukocyte antigen is an important gene for immunologic rejection. Swine leukocyte antigen (SLA) class I antigens (*SLA-1*, *SLA-2* and *SLA-3*) are expressed on the surfaces of most nucleated cells, and SLA class II antigens (*SLA-DQA*, *DQB1*, *DRA* and *DRA1*) are expressed on antigen-presenting cells. SLA type can be obtained by comparing coding sequences (CDS) and protein translations (Smith *et al*., 2005a; Smith *et al*., 2005b). Previous reports showed that SLA is correlated with disease resistance such as melanoma initiation and bacte-

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rial phagocytosis (Tissot *et al*., 1987; Lacey *et al*., 1989), and has a potential association with inflammation (Nino-Soto *et al*., 2008). Type of SLA will be one of important considerations for xenotransplantation research because rejection is one of major obstacles in xenotransplantation. Diversity of SLA might result in various case of immune reaction. Thus, SLA homozygote pig lines are valuable research animals for xenotransplantation and other immunologic researches. For these purposes several SLA homozygote lines have already been established (Ho *et al*., 2009).

Various transgenic and/or knockout pigs are produced by SCNT technique for xenotransplantation or research model purposes. However, information about SLA type and large scale gene expression profile of the SCNT derived cloned pig is still very limited. Therefore, the present study was performed to analyze genetic stability and functional gene expression in cloned pigs derived from same cell line. Two groups of cloned pigs were produced by SCNT using 2 different fibroblast cell lines for each group. Then we compared complete SLA coding sequences and performed microarray analysis.

MATERIALS AND METHODS

Animal Source

This study was conducted with two group of cloned pigs derived from different fetal fibroblast cell lines. All the cloned pigs were produced using the SCNT technique described previously (McElroy *et al*., 2008; Koo *et al*., 2010). Both cell lines used in the study were isolated from day-30-fetuses of Seoul National University (SNU) miniature pig (Lee *et al*., 2006). The SNU miniature pigs were originated at the University of Minnesota in 1949. In 1973, they were delivered via hysterectomy and were maintained as an SPF-closed colony (Setcavage and Kim, 1976). All cloned pigs of this study were maintained in the same specific pathogen-free (SPF) animal room.

There were three male cloned pigs in group I and four female cloned pigs in group II, and they were all born from different surrogate mother pigs except 9–4 and 9–6 pigs in group I. All cloned pigs used for analysis in this study were older than 30 months without any congenital defects or physical problems. Information of the animals used in the study was summarized in Table 1. This study was approved as an animal use protocol by the Institutional Animal Care and Use Committee of Seoul National University.

Confirmation of Cloned Animal

To certify that the cloned pigs were derived from the same pig fibroblast cell line, we performed a microsatellite test using 13 markers and conducted *mtDNA* sequence analysis.

For microsatellite analysis, 13 microsatellite markers, each from a different autosome (SW935, SW951, SW787, S00090, S0025, SW122, SW857, S0005, SW72, S0155, S0225, SW24 and SW632) were labeled using one of the fluorescent dyes 5-FAM, HEX, NED or PET. Length variations were assayed via PCR amplification of genomic DNA with fluorescently labeled locus-specific primers and PAGE on an automated genetic analyzer using fragment length determination (ABI 3130xl; Applied Biosystems, USA). All tests were conducted in duplicate.

To obtain *mtDNA* D-loop sequences, amplification via PCR with specific primers (Kim *et al*., 2002) {Kim, 2002 #1} and cloning and sequencing with universal primers were conducted. For the sequence-based *mtDNA* analysis, a neighbor-joining alignment tree of the

Table 1. Cloned miniature pigs used in this study

	[a]Group	ID	Gender	Birth date	Body weight (kg)	Age at blood sampling (day)
1	Group I (MP5 cell line)	9~4	Male	2004. 8. 2.	112	1,043
2		9~6	Male	2004. 8. 2.	150	1,078
3		9~10	Male	2004. 8. 10.	90	1,063
4	Group II (MP1 cell line)	9~20	Female	2004. 3. 6.	95	1,374
5		9~21	Female	2004. 3. 9.	97	1,372
6		9~23	Female	2004. 4. 21.	86	1,335
7		9~24	Female	2004. 5. 4.	100	1,350

[a] Groups were divided according to the SNU miniature pig fibroblast cell line used for SCNT.

mitochondrial DNA D-loop region was constructed for unrelated SNU miniature pigs (SNU-pig1-3; same strain as the cloned pig), cloned SNU miniature pigs (GI-pig-1-3 for group I and GII-pig1-4 for group II) and domestic pigs (Domestic 1-3; surrogate mother pigs).

Sample Preparation

Fresh bloods were sampled from the jugular vein under anesthesia on day of sacrifice. Sampling age and body weight of each pig were not same in this study (summarized in Table 1). These differences influence on the rearing density, but other environmental changes such as temperature and humidity did not happen.

RNA was extracted from peripheral blood mononuclear cells (PBMCs) using a QIAmp RNA blood mini kit (Qiagen, USA), and cDNA was reverse-transcribed from 5 μg of total mRNA using a First Strand cDNA synthesis kit (Takara, Japan) according to the manufacturer's instructions. The cDNAs were stored at -20℃ prior to use.

Sequence-based SLA Typing and Analysis

Swine leukocyte antigen (SLA) sequence-based typing was conducted in seven cloned SNU miniature pigs. Briefly, the complete coding sequences of six SLA loci were acquired through RT-PCR, cloned and sequenced according to the method of a previous study with minor modifications (Lee *et al*., 2005; Yeom *et al*., 2010). On the basis of sequence and protein translation comparisons with the IPD-MHC database (http://www.ebi.ac.uk/ipd/mhc/sla/index.html), two SLA class I genes (*SLA-1* and *SLA-2*) and four SLA class II genes (*DRA, DRB1, DQA* and *DQB1*) were analyzed (Smith *et al*., 2005a; Smith *et al*., 2005b). CLC DNA workbench version 3.6.5 (CLCbio, Denmark) was used for joining and analyzing the sequences and protein translation. Complete SLA CDSs and protein translation were compared across all same cell-derived cloned pigs.

Microarray Analysis

The expression levels of 43,603 mRNAs in the PBMCs were compared. Briefly, for control and test RNAs, target cRNA probes synthesis and hybridization were performed using Agilent's Low RNA Input Linear Amplification kit (Agilent Technology, USA) according to the manufacturer's instructions. The hybridized images were scanned using Agilent's DNA microarray scanner and were quantified with Feature Extraction Software (Agilent Technology, USA). All data normalization and selection of fold-changed genes were performed using GeneSpringGX 7.3 (Agilent Technology, USA). Following normalization to the 50th percentile of the measurement taken, genes scored less than 0.1 were excluded from the data analysis. The normalized data which designated as flag-P in all pigs were analyzed. Due to single tests for each pig was conducted, multiple testing could not be performed. However, based on the previously reported study, more than 2-fold changes were considered as differently-regulated (Yang *et al*., 2002; Watanabe *et al*., 2006; Seo *et al*., 2007). Functional allocation analysis with official gene symbols of Entrez, 0.5 similarity threshold, 0.5 multiple linkage threshold and a *Sus scrofa* background were conducted using the DAVID 6.7 program (http://david.abcc.ncifcrf.gov/) (Huang da *et al*., 2009). Gene clustering analysis was conduct using the GOTERM BP FAT and KEGG algorithm.

RESULTS

Confirmation of Cloned Pigs

Cloned pigs in the same group showed identical short tandem repeats (Table 2). The *mtDNA* of cloned pigs was significantly different between the each cloned pig used in this study, except between group II pigs 3 and 4 (Fig. 1). We assumed that oocytes from

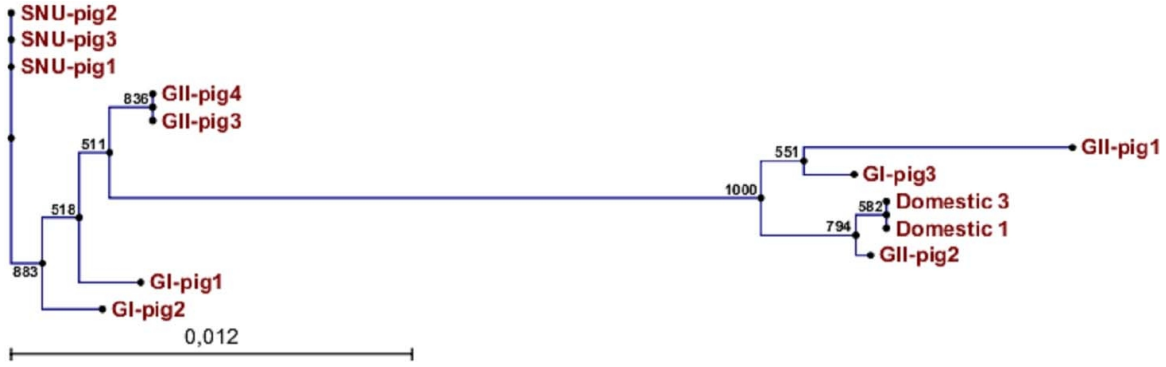

Fig. 1. ***mtDNA*** **sequence analysis of same fibroblast cell-derived cloned pigs.**

Table 2. Results of microsatellite testing for cloned SNU miniature pigs

		Microsatellite DNA marker												
STR		SW935	SW950	SW786	SW00089	SW0025	SW121	SW856	S0004	SW71	S0154	S0224	SW23	SW631
Dye		5-FAM	5-FAM	5-FAM	5-FAM	HEX	HEX	HEX	HEX	NED	NED	NED	PET	PET
size (bp)		75～120	123～142	145～178	228～258	85～113	273～341	140～170	206～282	96～130	144～169	171～204	92～142	146～190
Cloned pig Group I	1	102/112	127/129	161/163	247/247	106/106	129/131	155/155	211/211	105/105	161/163	192/194	104/106	168/176
	2	102/112	127/129	161/163	247/247	106/106	129/131	155/155	211/211	105/105	161/163	192/194	104/106	168/176
	3	102/112	127/129	161/163	247/247	106/106	129/131	155/155	211/211	105/105	161/163	192/194	104/106	168/176
Cloned pig Group II	1	102/116	127/127	161/163	247/247	106/106	131/131	155/155	211/211	105/105	161/163	192/194	104/124	167/169
	2	102/116	127/127	161/163	247/247	106/106	131/131	155/155	211/211	105/105	161/163	192/194	104/124	167/169
	3	102/116	127/127	161/163	247/247	106/106	131/131	155/155	211/211	105/105	161/163	192/194	104/124	167/169
	4	102/116	127/127	161/163	247/247	106/106	131/131	155/155	211/211	105/105	161/163	192/194	104/124	167/169
Unrelated SNU miniature pig	1	102/112	127/127	159/163	247/251	100/106	127/131	155/155	211/211	105/105	161/163	186/192	104/106	176/176
	2	102/112	127/127	163/163	247/249	106/106	127/131	155/155	211/211	105/105	163/163	192/192	106/106	176/176
	3	102/112	127/129	159/163	247/247	100/106	123/123	155/155	211/239	105/105	161/161	192/192	106/124	168/176
Surrogate mother	1	102/102	127/129	159/159	247/247	106/106	123/123	155/159	223/251	105/113	163/165	186/192	124/124	168/178
	2	116/118	127/127	157/159	247/251	100/106	123/129	155/155	243/243	115/115	153/165	174/192	106/112	168/170
	3	100/102	127/127	157/163	247/254	100/106	123/131	147/155	223/243	105/115	153/165	192/192	118/124	168/178

the same origin were used for SCNT in this case. The results indicate that all the pigs used in this study are cloned ones and pigs in the same group were derived from the identical fibroblast cell line.

Sequence-Based SLA Typing

In six SLA loci, 16 alleles were detected: *SLA-1*0201/0701, SLA-1*0601, SLA-2*0201, SLA-2*0301, SLA-2*0601, DQA*0102, DQA*0201, DQA*0301, DQB1*0210, DQB1*0301, DRA*010101, DRA*0201, DRB1*0201, DRB1*0301* and *DRB1*0401*. *SLA-1*0201* and *SLA-1*0701* were duplicated alleles (Smith *et al*., 2005c). The local designations of SLA alleles for each cloned pig group are summarized in Table 3. Group I cloned pigs were homozygous for three SLA loci, and group II cloned pigs were homozygous for six SLA loci. No SLA alleles were shared between two cloned pig groups. All SLA nucleotide sequences and protein translations among the cloned SNU miniature pigs in the same group were identical, and there were no SLA sequences for which a new SLA nomenclature was assigned.

Table 3. Defined SLA alleles of each cloned pig group

Class	Loci	Size (base pairs)	Cloned pigs group 1 (n=3)		Cloned pigs group 2 (n=4)	
			Designation	[a]Similarity(%)	Designation	Similarity(%)
Class I	*SLA-1*	1,563	*SLA-1*0601*	100	*SLA-1*0201/0701*	100
	SLA-2	1,217	*SLA-2*0301, SLA-2*0601*	100	*SLA-2*0201*	100
Class II	*DQA*	805	*DQA*0102, DQA*0301*	100	*DQA*0201*	100
	DQB1	909	*DQB1*0301*	100	*DQB1*0201*	100
	DRA	830	*DRA*0201*	100	*DRA*10101*	100
	DRB1	1,105	*DRB1*0301, DRB1*0401*	100	*DRB1*0201*	100

[a] Similarity represents the percentage of similar residues in alignment positions to overlapping alignment positions in the sequences.

Table 4. Differences in mRNA expression among cloned pigs

		No. of genes(ratio)			
		[a] Total selected gene	Differentially expressed genes (%)	[b] Up-regulated genes (%)	Down-regulated genes (%)
Clone group I	Pig 1 vs. pig 2	2,194	643 (29.3)	318 (14.5)	324 (14.8)
	Pig 1 vs. pig 3	2,150	719 (32.8)	442 (20.1)	277 (12.6)
	Pig 2 vs. pig 3	2,574	849 (38.7)	332 (15.1)	517 (23.6)
Clone group II	Pig 1 vs. pig 2	4,223	1,039 (24.6)	406 (9.6)	633 (15.0)
	Pig 1 vs. pig 3	4,364	2,062 (47.3)	503 (11.5)	1,559 (35.7)
	Pig 1 vs. pig 4	4,265	1,184 (27.8)	409 (9.6)	775 (18.2)
	Pig 2 vs. pig 3	4,330	1,746 (40.3)	491 (11.3)	1,255 (29.0)
	Pig 2 vs. pig 4	4,278	797 (18.6)	373 (8.7)	424 (9.9)
	Pig 3 vs. pig 4	4,399	1,739 (39.5)	1,224 (27.8)	514 (11.7)

[a] Among the 43,603 of total analyzed genes, 2,150～4,399 reliable ones were selected for calculation.
[b] The ratios were calculated based on the formerly shown pig.

Table 5. Remarkably upregulated gene cluster of cloned pigs

		Phenotype	No. of genes	*p*-value	Algorithm
Group I	Pig 1	Inflammation	16	0.005	GOTERM BP FAT
	Pig 2				
	Pig 3	Cancer	32	0.008	GOTERM BP FAT
Group II	Pig 1				
	Pig 2	Apoptosis	30	0.003	GOTERM BP FAT
	Pig 3	Cardiomyopathy	24	0.005	KEGG
	Pig 4				

Microarray Analysis

In total, 43,603 genes were analyzed in this study. In group I, 2,150～2,574 genes were selected, and 29.3～38.7% of them showed differential expressions compared to each other. In group II, 4,223～4,399 genes were selected, of which 18.6～47.3% of genes showed differential expressions (Table 4).

On gene clustering analysis, some cloned pigs showed abnormal physiological phenotypes (Table 5). In group I, pig 1 showed an mRNA expression pattern of inflammation and pig 3 showed high expressions of cancer-related genes. In group II, pig 3 showed high expressions of cardiomyopathy-related genes. In addition, common differentially expressed functional clusters were also observed for binding-related genes involved in cation binding, ion binding, metal binding and transition metal ion binding (data not shown).

DISCUSSION

The SCNT technique is widely used to produce transgenic and knockout pigs (Polejaeva, 2001). Many factors, e.g., micromanipulation, artificial activation of reconstructed embryos, various chemical additives for *in vitro* culture, and transfection procedure, may contribute to genomic damage. Verifying genetic stability of cloned pigs is very important for using the animals as organ donor for xenotransplantation or valuable laboratory animal. The genetic stabilities and phenotypes of cloned pigs have previously been studied (Kishigami *et al*., 2008) but reports about SLA types of cloned pigs were still very limited. In this study, we found stability of SLA type in the cloned pigs derived from same donor cell line, however, gene expression patterns and phe-

notypes are not identical.

In order to minimize error for generalization, two groups of cloned pigs derived from 2 different fibroblast cell lines were used in present study. They were housed in the SPF rooms of the same animal facility, and it was an suitable environment for assessing the physiologic phenotypes of cloned pigs. As results of microsatellite, SNU miniature pigs were not the inbred state, and there were 5 STR differences among 2 cloned pig lines. There were no nucleotide alterations among six SLA loci and all SLA alleles and SLA types were identical among the cloned pigs in the same group. This finding indicates that same cell-derived cloned pigs might have immunological homogeneity. This SLA genetic stability is particularly important with respect to the production and selection of cloned animals as laboratory models or xenograft donors. Since only seven cloned pigs and six SLA loci were examined in this study, the possibility of genomic damage or mutation in entire genome could not be ruled out completely. However, the results indicate that SLA locus genes appear to be stable following SCNT, consistent with results from a previous report demonstrating that abnormalities in cloned pigs resulted primarily from epigenetic reprogramming rather than genomic damage (Cho *et al*., 2007).

In microarray analysis, overall 30% of the selected genes showed significantly different expressions. Several pigs showed the possibility of cancer or cardiomyopathy. This phenotypes might be related with high differential gene expression of these pigs (32.8~47.3%) compared to those in other pigs. These pigs also showed relatively lower body weight (90 and 86 kg). In addition, ion binding-related genes such as those involved in cation binding, ion binding, metal ion binding and transition metal ion binding were differentially expressed in each pig. However these binding-related genes might not induce a significantly different physical phenotype because they are commonly found to be differentially expressed in PBMCs (van Leeuwen *et al*., 2005; Korkor *et al*., 2011). Genomic imprinting could be used for analysis of fetal growth and reprogramming (Wilkins and Haig, 2003), and some imprinting genes also showed different expressions, although no specific functional clustering was observed (data not shown). Since all cloned pigs were housed in the same animal room and maintained under same SPF conditions, the influence of infectious disease could be eliminated, so we assumed that individual environmental adaption, sociality and rank in the pen might have had unknown effects on phenotype.

Previous study for monozygotic human twin about disease discordant, there were no genetic and epigenetic transcriptome differences which related disease discordance, but about 40% of the expression quantitative trait loci (eQTL) represented different aspect (Baranzini *et al*., 2010), and it was similar with this study. To conduct further systemic analysis for entire genetic background and difference of gene expression, additional SNP and eQTL analysis might be needed for more detailed analysis of cloned pigs.

In summary, the 3~4 year-old adult cloned pigs in this study could provide valuable data on genetic stability and phenotype expression in cloned pigs derived from same cell line origin. Although only SLA genetic stability was examined, the SLA locus gene appears to be stable following SCNT. Under SPF environment, cloned pigs showed about 30% differential gene expression, and some pigs showed abnormal phenotypes such as cardiomyopathy, cancer, apoptosis and inflammation. Our results provide information regarding the genomic stabilities and phenotypic expressions of cloned pigs produced by using SCNT technique.

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