Comparative Proteomic Analysis of Liver Mitochondrial Proteins Derived from Cloned Adult Pigs Reconstructed with Meishan Pig Fibroblast Cells and European Pig Enucleated Oocytes

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Abstract. Somatic cell nuclear transfer (SCNT) has been exploited in efforts to clone and propagate valuable animal lineages. However, in many instances, recipient oocytes are obtained from sources independent of donor cell populations. As such, influences of potential nuclear-cytoplasmic incompatibility, post SCNT, are largely unknown. In the present study, alterations in mitochondrial protein levels were investigated in adult SCNT pigs produced by microinjection of Meishan pig fetus fibroblast cells into enucleated matured oocytes (maternal Landrace genetic background). Mitochondrial fractions were prepared from liver samples by mechanical homogenization and differential centrifugation. Liver mitochondria were then subjected to two-dimensional difference gel electrophoresis (2-D DIGE). Protein expression changes were confirmed with a volume ratio greater than 2 fold (P < 0.05). 2-D DIGE analysis further revealed differential expression of three proteins between the Meishan (n=3) and Landrace (n=3) breeds. Differential expression patterns of 16 proteins were detected in SCNT pig liver tissue (n=3) when compared with Meishan control samples. However, none of the 16 proteins correlated with the three differentially expressed Meishan and Landrace liver mitochondrial proteins. In summary, alteration of mitochondrial protein expression levels was observed in adult SCNT pigs that did not reflect the breed difference of the recipient oocytes. Comparative proteomic analysis represents an important tool for further studies on SCNT animals.

Key words: 2-D DIGE, Liver, Mitochondrial protein, Pig, Somatic cell nuclear transfer

Somatic cell cloning by nuclear transfer (SCNT) has potential agricultural applications for replicating food animals with desired genetic merits, animal transgenesis, and in conservation of endangered species. Even though cloned offspring have been successfully produced in a variety of species, SCNT is struggling with an extremely low efficiency. Pre- and post-natal development is often compromised, and a variable proportion of SCNT offspring show aberrant developmental patterns and increased pre- and perinatal mortality [1]. Aberrant reprogramming of donor somatic cell nuclei may result in many severe problems in animal cloning [2, 3]. From a mitochondrial biology perspective, the inability to establish functional interactions between the donor nucleus and recipient mitochondria is also likely responsible for such a developmental deficiency [4–6].

Mitochondria are involved in ATP synthesis, reactive oxygen species production, calcium signaling and apoptosis [7, 8]. Cloning of pigs was achieved via nuclear transfer, in which a donor cell is microinjected or electrically fused with an enucleated oocyte [9, 10]. In the process of nuclear transfer, the transfer of donor cell mitochondria with nuclei into recipient oocytes has resulted in mitochondrial heteroplasmy and recipient oocyte homoplasmy [11]. Where heteroplasmy was observed, the majority of mitochondrial DNA (mtDNA) did originate from the recipient oocyte. Previous reports also demonstrated that the oocyte mtDNA haplotype influenced SCNT efficiency [12–14]. Indeed, the interaction between the cytoplasm and nuclear genomes is of critical importance in interspecies SCNT (iSCNT), as most of the mRNAs, proteins, organelles and other cytoplasmic components are contributed by the oocyte. The species-specific nature of mitochondrial biogenesis and function makes it particularly significant for iSCNT. However, Gómez et al. [15] reported that production of African wild cat cloned offspring by iSCNT did not impart negative effects on the health
of the offspring. SCNT pigs derived from Chinese pig fibroblast cells (Meishan breed) microinjected into European (Landrace breed) pig oocytes [9] demonstrated normal developmental and reproductive parameters [16]. However, it is still unknown whether different mtDNA haplotypes in SCNT offspring may impair mitochondrial function.

Two-dimensional (2-D) polyacrylamide gel electrophoresis (PAGE) has been used extensively for detection of a number of biological targets ranging from developmental characteristics to identifying various biomarkers in complex protein samples. Recently, global comparative proteomic analysis was performed to identify the proteins that cause problems in animal cloning [17–20]. Different expression levels in disease-relevant proteins and/or in apoptosis were observed in piglets, fetuses and placenta of offspring derived from SCNT pigs [17–20]. Yet, the conventional proteomic methodology was hampered by methodological errors in quantification due to large gel-to-gel variations. A 2-D difference gel electrophoresis (2-D DIGE) system was employed with the inclusion of an internal standard that greatly reduces data variation [21–23].

Here, we study differences of protein expression profiles of liver mitochondria in adult SCNT pigs derived from Chinese pig (Meishan breed) fibroblasts microinjected into European pig oocytes (maternal Landrace breed) using a 2-D DIGE system.

Materials and Methods

Animals

SCNT pigs were originally produced by piezo-actuated microinjection (Prime-Tech, Tsuchiura, Japan) of Meishan fetal fibroblast nuclei into enucleated oocytes (maternal Landrace background: Landrace or Landrace × Large White gilts crossed with Duroc boars) as reported [9, 16, 24]. Meishan or Landrace controls were derived by breeding of the respective parental stocks. Meishan pig mtDNA sequences showed 28 amino acid differences compared with published Landrace sequences (Accession No. AB292606). The mtDNA genotypes of the SCNT derived pigs showed heteroplasmacy, with more than 99% of the recipient oocyte (Landrace) and 0.1–1.0% of the donor mtDNA (Meishan) [25]. SCNT pigs (females, n=3: 9, 7 and 3 years of age; Z1-3) were slaughtered because of astasia/abasia. Liver samples were collected from SCNT, Meishan (females, n=3: 7, 6, and 6 years of age; M1-3) and Landrace pigs (females, n=3: 2 years of age; L1-3) and kept on ice until use (1–2 h).

All procedures and experiments were performed according to protocols approved by the NILGS Laboratory Animal Experimental Guides and the Japanese Prime Minister’s Office of Laboratory Animal Welfare (No. 6, 1980).

Extraction of solubilized protein from liver mitochondria

Mitochondrial fractions from liver samples were prepared by mechanical homogenization and differential centrifugation at 4°C as previously described [26]. Resulting mitochondrial pellets were solubilized in lysis buffer (pH 8.5) consisting 7 M urea, 2 M thiourea, 4% CHAPS, and 30 mM Tris base by vortexing for 1 min and sonication for 30 sec at ambient temperature. The mitochondrial solution was then centrifuged at 15,000 g for 10 min. A 1000-fold dilution of supernatant was taken for determination of the protein concentration using a Quick Start™ Bradford Protein Assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

2-D DIGE gel analysis

A technique was used that allowed for multiplexing using the 2-D DIGE with Ettan™ DIGE technology (Amersham Biosciences, GE Healthcare, Piscataway, NJ, USA). Briefly, sample proteins (50 µg each) were labeled with CyDye™ DIGE Fluor minimal dyes (Cy2, Cy3, Cy5). Each mixture contained two samples differentially labeled using Cy3 and Cy5. An internal standard (pool of all the samples) labeled with Cy2 was loaded onto an IPG strip (pH 3–10 NL; 13 cm). The first-dimension isoelectric focusing (IEF) was performed using an Ettan™ IEFphor™ 3 IEF System. To prevent nonspecific oxidation, a DeStreak™ reagent was added in a rehydration solution in place of dithiothreitol. The second-dimensional separation was carried out using 10% SDS polyacrylamide gels (16 × 16 × 0.5 cm) in an SE600 Ruby system (GE Healthcare). After 2-D PAGE, gels were scanned with an Ettan™ DIGE Imager laser scanner, and double images (Cy3 and Cy5) of each sample were analyzed with the ImageMaster™ 2D Platinum 6.0 software (GE Healthcare). Quantitative comparisons of protein between samples were made based on the relative change of each protein spot compared with its own in-gel internal standard. The significance level was set to 0.05.

Protein identification

Two-dimensional PAGE was performed according to the protocol provided by the manufacturer. Gels were stained with Coomassie G-250 (SimplyBlue™ SafeStain, Invitrogen) to visualize proteins. Target protein spots were excised from the gels, and selected spots were subjected to a commercial proteomic analysis service (Theravalues, Osaka, Japan) using nano-ESI (electrospray ionization)-MS/MS (tandem mass spectrometry) or LC (liquid chromatography)-MALDI-MS/MS. Proteins with significant protein scores (P<0.05) were identified using Mascot (Matrix Science, London, UK) searches through the NCBI database.

Results

Comparative proteomic analysis between Meishan and Landrace breeds

In total, 420 spots were automatically matched among the 2-D gels, and then Meishan (M1-3) and Landrace (L1-3) samples were subjected to ImageMaster™ 2D Platinum analysis. The matched spots were useful in analysis of protein expression changes across gels, and three spots were identified as different with a volume ratio greater than 2.00 in liver mitochondria (P < 0.05) (Fig. 1A, Table 1). All of the studied proteins were downregulated (2.48 to 4.42-fold decreases) in Landrace pigs compared with Meishan pigs. Additionally, differences were observed in four proteins between the Meishan and Landrace breeds, with volume ratios greater than 1.50 (P < 0.05) (Fig. 2).

Comparative proteomic analysis between SCNT and Meishan

In total, 410 spots were automatically matched among the 2-D gels by ImageMaster™ 2D Platinum software. Differential expression patterns were observed in 16 proteins in SCNT pigs (Z1-3) compared...
Fig. 1. Comparison of 2-D patterns of liver mitochondria proteins resolved by Ettan-DIGE. Indicated spots with matching numbers were identified with differential expression concentrations with a volume ratio greater than 2.00 (P < 0.05). Protein numbers refer to the identified proteins presented in Tables 1 and 2 (Spot ID). Proteins were separated by isoelectric point (pI), from 3–10 non-linear along the horizontal axis, and by molecular weight (Mr), from 10 to 100 kD, vertically. A) Comparison between Landrace and Meishan pigs. Downregulated proteins in Landrace pigs compared with Meishan pigs (P < 0.05) are indicated in orange. B) Comparison between SCNT and Meishan pigs. Downregulated proteins (orange) and upregulated proteins (violet) in SCNT pigs compared with Meishan (P < 0.05) are indicated.

Table 1. Comparison of proteomic expression levels between Landrace (L) and Meishan (M) pigs

<table>
<thead>
<tr>
<th>Spot ID</th>
<th>Ratio (L/M)</th>
<th>L</th>
<th>M</th>
<th>pI, Mr</th>
</tr>
</thead>
<tbody>
<tr>
<td>720</td>
<td>−2.48</td>
<td>0.47 ± 0.10</td>
<td>1.16 ± 0.20</td>
<td>7.80, 47</td>
</tr>
<tr>
<td>627</td>
<td>−4.10</td>
<td>0.36 ± 0.16</td>
<td>1.47 ± 0.82</td>
<td>5.65, 53</td>
</tr>
<tr>
<td>1191</td>
<td>−4.42</td>
<td>0.29 ± 0.18</td>
<td>1.27 ± 0.31</td>
<td>7.69, 20</td>
</tr>
</tbody>
</table>

a Experimental isoelectric point. b Experimental molecular weight (kDa).

Fig. 2. Protein expression changes with a P-value < 0.05 and a fold change > 1.50 among SCNT pigs. Upregulated (> 1.5) and downregulated spots (−1.5) in all or each individual SCNT pig (Z1-3) and in the Landrace group (L) were calculated versus the mean of the control Meishan group by the ImageMasterTM Platinum 6.0 software. The color scales range from dark violet to bright red, which correspond respectively up- or downregulated proteins as indicated. ID: spot ID for calculation by software; corresponds to the IDs in Tables 1 and 2 and Fig. 1.
with Meishan pigs (M1-3) with a volume ratio greater than 2.00 (P < 0.05) (Fig. 1B, Table 2). Five proteins were upregulated (2.15 to 3.45-fold increases), and 11 were downregulated (2.08 to 2.63-fold decreases). Three of the upregulated proteins were identified: two of the three were hydroxymethylglutaryl-CoA synthase mitochondrial precursor (HMG-CoA synthase) and nonspecific lipid-transfer protein (Table 3, 1–3). Additionally, two of the downregulated proteins were identified: aldehyde dehydrogenase 7 family, member A1 (*Bos taurus*), and alanine-glyoxylate aminotransferase 2, mitochondrial (*Bos taurus*) (Table 3, 4–5). Lastly, differences were observed in 19 proteins in SCNT pigs when compared with Meishan pigs with a volume ratio greater than 1.50 (P < 0.05) (Fig. 2).

**Discussion**

The present study investigated differences in mitochondrial protein expression in 2-D gel analyses in pigs obtained by SCNT using Chinese pig (Meishan) fibroblast cells and European (Landrace) pig oocytes. All the SCNT adult pig samples showed differential protein expression in liver mitochondria when compared with Meishan pigment controls. Differential protein expression levels were observed in SCNT pigs, which did not reflect the expression patterning observed in Landrace (mtDNA genotype) and Meishan (nuclear genotype) control samples. A total of 209 nucleotide substitutions, including 26 amino acid substitutions, were detected

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**Table 2.** Comparison of proteomic expression levels between SCNT (Z) and Meishan (M) pigs

<table>
<thead>
<tr>
<th>Spot ID</th>
<th>Ratio (Z/M)</th>
<th>Z</th>
<th>M</th>
<th>pM, M</th>
<th>Pr. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>517</td>
<td>3.45</td>
<td>2.21 ± 1.10</td>
<td>0.64 ± 0.26</td>
<td>8.3, 61</td>
<td>1</td>
</tr>
<tr>
<td>663</td>
<td>3.25</td>
<td>1.90 ± 0.31</td>
<td>0.59 ± 0.29</td>
<td>5.91, 51</td>
<td>2</td>
</tr>
<tr>
<td>664</td>
<td>2.78</td>
<td>1.83 ± 0.34</td>
<td>0.66 ± 0.28</td>
<td>6.13, 50</td>
<td></td>
</tr>
<tr>
<td>518</td>
<td>2.43</td>
<td>1.81 ± 0.80</td>
<td>0.75 ± 0.19</td>
<td>8.0, 60</td>
<td></td>
</tr>
<tr>
<td>660</td>
<td>2.15</td>
<td>1.53 ± 0.26</td>
<td>0.72 ± 0.26</td>
<td>5.72, 51</td>
<td>3</td>
</tr>
<tr>
<td>604</td>
<td>−2.08</td>
<td>0.65 ± 0.12</td>
<td>1.34 ± 0.13</td>
<td>6.79, 56</td>
<td>4</td>
</tr>
<tr>
<td>699</td>
<td>−2.14</td>
<td>0.59 ± 0.17</td>
<td>1.26 ± 0.29</td>
<td>6.51, 49</td>
<td></td>
</tr>
<tr>
<td>678</td>
<td>−2.16</td>
<td>0.60 ± 0.18</td>
<td>1.30 ± 0.30</td>
<td>6.43, 50</td>
<td></td>
</tr>
<tr>
<td>255</td>
<td>−2.25</td>
<td>0.57 ± 0.19</td>
<td>1.28 ± 0.38</td>
<td>6.58, 89</td>
<td></td>
</tr>
<tr>
<td>248</td>
<td>−2.44</td>
<td>0.52 ± 0.15</td>
<td>1.28 ± 0.39</td>
<td>6.12, 89</td>
<td></td>
</tr>
<tr>
<td>647</td>
<td>−2.48</td>
<td>0.54 ± 0.18</td>
<td>1.34 ± 0.23</td>
<td>6.25, 52</td>
<td></td>
</tr>
<tr>
<td>684</td>
<td>−2.50</td>
<td>0.54 ± 0.15</td>
<td>1.35 ± 0.29</td>
<td>6.83, 49</td>
<td></td>
</tr>
<tr>
<td>262</td>
<td>−2.51</td>
<td>0.52 ± 0.19</td>
<td>1.31 ± 0.40</td>
<td>6.24, 88</td>
<td></td>
</tr>
<tr>
<td>263</td>
<td>−2.59</td>
<td>0.50 ± 0.25</td>
<td>1.30 ± 0.47</td>
<td>6.4, 89</td>
<td></td>
</tr>
<tr>
<td>653</td>
<td>−2.63</td>
<td>0.50 ± 0.28</td>
<td>1.32 ± 0.24</td>
<td>6.66, 52</td>
<td></td>
</tr>
<tr>
<td>656</td>
<td>−2.63</td>
<td>0.48 ± 0.27</td>
<td>1.27 ± 0.24</td>
<td>6.84, 51</td>
<td>5</td>
</tr>
</tbody>
</table>

* Experimental isoelectric point. ** Experimental molecular weight (kDa). † Protein numbers are refer to those in Table 3.

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**Table 3.** Identified proteins in porcine liver mitochondria

<table>
<thead>
<tr>
<th>Pr. No.</th>
<th>Identified protein</th>
<th>Organism</th>
<th>NCBI accession No.</th>
<th>Score</th>
<th>Peptide matched</th>
<th>Cov. (%)a</th>
<th>Theoretical Mr (Da)</th>
<th>Theoretical pI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Non-specific lipid-transfer protein</td>
<td>Sus scrofa</td>
<td>gi</td>
<td>270289750</td>
<td>913</td>
<td>18</td>
<td>30</td>
<td>58483</td>
</tr>
<tr>
<td>2</td>
<td>Hydroxymethylglutaryl-CoA synthase, mitochondrial precursor</td>
<td>Sus scrofa</td>
<td>gi</td>
<td>47523816</td>
<td>186</td>
<td>4</td>
<td>6</td>
<td>56898</td>
</tr>
<tr>
<td>3</td>
<td>Hydroxymethylglutaryl-CoA synthase, mitochondrial precursor</td>
<td>Sus scrofa</td>
<td>gi</td>
<td>47523816</td>
<td>143</td>
<td>4</td>
<td>6</td>
<td>56898</td>
</tr>
<tr>
<td>4</td>
<td>Aldehyde dehydrogenase 7 family, member A1</td>
<td>Bos taurus</td>
<td>gi</td>
<td>86823839</td>
<td>902</td>
<td>18</td>
<td>14</td>
<td>55344</td>
</tr>
<tr>
<td>5</td>
<td>Predicted: alanine--glyoxylate aminotransferase 2, mitochondrial-like</td>
<td>Sus scrofa</td>
<td>gi</td>
<td>311273569</td>
<td>224</td>
<td>4</td>
<td>7</td>
<td>56820</td>
</tr>
</tbody>
</table>

a Sequence coverage.
in comparisons of mtDNA sequences in the Landrace and Meishan breeds (Accession No. NC_000845, D16483.1, AY237530 and AB292606.1). However, 2-D DIGE analysis did not detect protein expression differences between Landrace and Meishan pigs reflective of the amino acid substitutions identified in these breeds. The components of functional mitochondria is encoded primarily by nuclear genes; mtDNA includes only 13 polypeptide genes, all of which encode essential components of oxidative phosphorylation (in addition to tRNA and rRNA genes [reviewed in 7]). Using 2-D PAGE in rodent liver tissue, the mitochondrial protein concentration varied in an age-dependent manner [27, 28]. The majority of mitochondrial proteins that changed significantly with age (6–7 months vs. 24–25 months) were found in liver tissue [27]. Here, we analyzed pigs with different ages (2–9 years old). It is possible that the differences between the Landrace (2 years old) and Meishan pigs (6–7 years old) reflected age-related phenomena.

While the mammalian mitochondrial genome is functionally stable, many sequence differences have been observed in vertebrate species [7, 29]. The species-specific nature of this organelle’s biogenesis and utility makes it particularly relevant to functional iSCNT [30]. In concert with mitochondrial dysfunction, abnormal nuclear reprogramming is a major factor in failure of iSCNT to conserve endangered species [31]. Previous reports provided evidence for oocyte mtDNA haplotype influences on SCNT efficiency in cattle [12, 13, 32]. Yan et al. [14, 33] demonstrated that donor-host mtDNA haplotype compatibility improved bovine SCNT efficiency; thus, homotypic SCNT blastocysts possess a more successful epigenetic symmetry pattern than heterotypic SCNT blastocysts. It is unclear in the present study whether the differential protein expression patterning was reflective of a donor-host mtDNA incompatibility.

One SCNT pig sample (Z3) showed the greatest profiling disparity compared with the other clones. Accordingly, the source and handling of recipient oocytes was then questioned. Z3 was derived from SCNT embryos using in vitro matured oocyte and encapsulation methods with sodium alginate [24]. The oocyte environment during development could readily influence epigenetic changes in SCNT animals [32]. Interestingly, in a similar study in cattle, mitochondrial protein profiles varied among adult clones produced from the same cell line as used for the donor cells [26]. One resultant SCNT cow (CA1) was produced using an oocyte obtained from an ovary cultured overnight in PBS at 15 C. This animal showed the greatest profiling disparity compared with the other adult clones. One possible explanation for this variation is that epigenetic differences led to the discordant outcomes in generating viable cloned animals. This hypothesis, however, needs further examination in SCNT animals using comparative proteomic analysis.

Mitochondria play a variety of essential roles in cellular metabolism, including in the production of ATP through oxidative phosphorylation and the initiation of the signal cascade leading to apoptosis. Influences on the propagation of mitochondria and mtDNA could be of great importance in determining the early developmental potential of SCNT embryos [5, 34]. Accordingly, many abnormalities observed in SCNT fetuses and offspring may be influenced by deficiencies in mitochondrial function. Recently, comparative proteomic analyses of SCNT piglets and placentae from SCNT pigs were reported [18–20, 35]. Two-dimensional gel electrophoresis analysis revealed changes in the responses of several detoxification-related proteins to stress and inflammation in sudden death observed in SCNT piglets with extensive hepatopneumonic apoptosis [17]. Lee et al. [18] assessed the global protein expression profiles in term placentas from SCNT pigs and found that the proteins closely involved in the apoptotic signaling pathway were different in comparison with controls. In malformed umbilical cords of SCNT piglets, proteins prevention of oxidative stress were downregulated, while molecules involved in apoptosis were upregulated [20]. In the present study, two novel proteins including HMG-CoA synthase were identified as common differences in the SCNT pigs. HMG-CoA synthase is a potential regulatory site in the pathway converting acetyl-CoA to ketone bodies. The HMG-CoA synthase family is a mitochondrial enzyme that catalyzes the first reaction of ketogenesis, a metabolic pathway that provides lipid-derived energy for various organs during times of carbohydrate deprivation, such as fasting. The enzyme activity of HMG-CoA synthase increases 27-fold during starvation in piglets [36]. In our study, none of the SCNT pigs showed morphological abnormalities, and they all had with normal reproductive ability. However, they were euthanized due to astasia/abasia complications coupled with anorexia. The increases in HMG-CoA synthase levels in the SCNT pigs likely reflected the observed anorexic condition. In addition, two of the proteins that were elevated in SCNT pigs were involved in ketogenesis, which would be consistent with pigs exhibiting liver disease.

Two-dimensional DIGE using the Ettan™ DIGE system enabled multiple protein extractions on a single 2-D gel. Additionally, the inclusion of an internal standard greatly reduced the variation among gels [23]. For further analysis, the DeCyder™ 2-D Differential Analysis Software v7.0 (GE Healthcare) including a multivariate statistics module provided a powerful tool for characterization and classification of biological samples based on protein expression data [23]. Yet, a difficulty in using this system is that all samples must be analyzed at the same time, as calculations are based on labile internal standards. Nonetheless, the Ettan™ DIGE system will be a powerful tool in identifying candidate proteins from large pools. While the number of samples was limited in the present study, our results demonstrate important considerations concerning uniformity and normal health of SCNT animals when using different breeds for recipient oocytes.

In conclusion, alteration of mitochondrial protein expression levels was observed in adult SCNT pigs and not reflective of breed differences associated with recipient oocytes. This study further demonstrates that comparative proteomic analysis represents an important tool for studies of SCNT-derived animals and in animal development.

Acknowledgments

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