Proteomics Analysis of Lungs from Somatic Cell Nuclear Transfer (SCNT) Bama Miniature Pig (Sus scrofa)

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#Equal Contribution

Abstract

This study aims to explore the influence of somatic cell nuclear transfer (SCNT) operation on the lung development of SCNT pigs using proteomics methods. The proteins from three adult SCNT and three normal reproductive Bama miniature pigs were extracted, separated and identified using two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS).

Thirty-one differentially expressed spots were identified, of which 23 spots were up-regulated, whereas 8 spots were down-regulated. The results also demonstrated that SCNT operation might result in abnormal expression of some important proteins in SCNT-derived lungs and affect the lung development in SCNT pigs survival.

Keywords: Pig; Somatic cell nuclear transfer (SCNT); Lung; Proteomics

Introduction

With the advent of transgenic pigs using the somatic cell nuclear transfer (SCNT), SCNT pig was thought to hold great promise in basic research, as well as in agricultural and biomedical applications, including xenotransplantation [1,2]. However, SCNT in pigs is limited by substantial problems with the current technologies, including extremely low efficiency, early embryonic and fetal mortality, postnatal mortality and abnormalities associated with SCNT [3,4]. Furthermore, many surviving cloned animals frequently have organ abnormalities including respiratory and metabolic deficiencies in their organs [5,6]. These abnormalities are likely caused by a lack of, or abnormal expression of developmentally important proteins which could affect the later development of surviving cloning animals [7,8].

Some recent studies have investigated the influence of SCNT on the normal development of cloned offspring using proteomics approaches [9,10]. The lung is one of the most important organs in the animal body. Lung abnormalities are also one of the reasons why cloned animals often die after birth [4]. In previous studies, SCNT-derived bovine and porcine placenta, and malformed umbilical cords from the early death of SCNT piglets were analyzed using the comparative proteomics approaches 2-DE and MS [11-13]. However, comparative proteomics analyses have not been used on the lungs of SCNT and normal reproductive pigs.

In order to explore the influence of SCNT on the porcine lung development, the lung proteins of SCNT and control pigs were subjected to a global comparative proteomics analysis using 2-DE, MALDI-TOF MS (Matrix-Assisted Laser Desorption Ionization/Time-of-Flight Mass Spectrometry) and western blot analysis.

Methods

The study was conducted following approval by the Ethics Committee of Shanghai Academy of Agricultural Sciences. All efforts were made to minimize animal suffering and the number of animals used. Donor fetal fibroblasts were obtained from a Bama miniature pig (male, 10-month-old) without heart disease. Three SCNT Bama miniature pigs from the same litter (male, 7-months-old) were bred as previously described [14,15]. Three normal reproductive pigs (7-months-old) of the same breed and sex derived from artificial insemination (AI) were used as control animals. The anatomical observation of SCNT-derived lungs and the controls were executed by senior veterinarian after slaughter.

Protein extraction

Frozen lung samples (about 300 mg) from three SCNT and three normal reproductive Bama miniature pigs were washed with PBS buffer (pH 7.0), pooled and manually homogenized (PRO 200 homogenizer, PRO Scientific, Monroe, CT, USA) in lysis buffer (8 M urea, 2 M thiourea, 40 mM Tris, 4% CHAPS, 65 mM DTT, 5 mM EDTA, 0.5% IPG buffer, and protease inhibitors) on ice, respectively.

The samples were heated in a boiling water bath (5 min), ultrasonicated and vortexed. The supernatant was aspirated after centrifugation at 12,000×g for 45 min (4°C), and the protein concentrations were determined using a BCA Protein Assay Reagent (Nanjing Jiancheng, China).

2-DE and protein staining

In this study, 100 μg of the extracted proteins were loaded onto the analytical and preparative 2-DE gels. The protein samples were mixed with rehydration solution (8 M urea, 2% CHAPS, 0.5% IPG buffer, 18 mM DTT, and a trace amount of bromophenol blue). An IPGphor IEF system (Amersham Pharmacia Biotech) and pH 3 to pH 10 nonlinear IPG strips (13 cm, nonlinear; Amersham) were used for isoelectric focusing (IEF). The IPG strips containing the protein samples were

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rehydrated for 12 h in 250 μL of rehydration buffer. IEF was performed at 500 V for 1 h, 1000 V for 1 h, and 8000 V for 5 h.

The gel strips were equilibrated for 15 min in equilibration buffer (50 mM Tris-HCl (pH 8.8), 6 M urea, 2% SDS, 30% glycerol, and 1% DTT). The strips were transferred onto 12.5% SDS-polyacrylamide gels. Electrophoresis was performed using a Hofer SE 600 system (Amersham) at 15 mA per gel for 30 min, followed by 30 mA until the bromophenol blue migrated to within 0.5 cm of the bottom of the gel. Three replicates were performed for each sample.

After 2-DE, the protein spots in the analytical gels were stained with ammoniacal silver nitrate and the preparative gels were stained with Coomassie Blue G-250. The resulting 2-D gels were scanned using a Bio-Rad GS710 scanner, and image analysis was accomplished using the Image Master Software (Amersham). Differences in the abundance of the differential protein spots were analyzed using a Student’s t test (p<0.005 was considered significant).

MALDI-TOF/TOF MS analysis

The protein spots of interest were excised from the preparative Coomassie blue-stained gels and destained for 20 min in 400 μL of 100 mmol/L NH₄HCO₃/30% CAN, washed in Millipore-Q water until the gels were destained, and then lyophilized. Each spot was digested for 24 h in 10.0 ng/mL trypsin (sequencing grade, Promega) at 37°C.

The peptides were extracted with 100 μL of 60% ACN/0.1% TFA for 15 min with sonication, and the supernatant was removed. The extraction was repeated three times, and the pellet was desalted and freeze-dried. The peptide mixtures were analyzed using a 4800 Plus MALDITOF/TOF™ Analyzer (Applied Biosystems, USA). The MS/MS spectra were searched with Mascot software version 2.2 (http://www.matrixscience.com) using the nr-NCBI mammalian genome database. The peptide mass tolerance was ± 100 ppm and the fragment mass tolerance was ± 0.8 Da.

The bioinformatics analysis tools used in our study included Data Explorer software (Applied Biosystems, USA), which provides lists of monoisotopic peaks, ProFound (http://prowl.rockefeller.edu/), and Mascot (http://www.matrixscience.com/search_form_select.html) web-based software packages.

Western blot analysis

As differentially expressed protein, Beta-actin (spot 8) was selected and validated using western blot analysis. Beta-actin rabbit monoclonal antibodies (monoclonal antibodies, mAb) was purchased from Cell Signaling Technology Inc. (Beverly, Massachusetts, USA) and GAPDH monoclonal antibodies for western blot analysis were purchased from Protein tech Group, Inc. (USA).

For the western blot analysis, 20 μg of the total protein was separated on 12% SDS-PAGE gels, and transferred onto a PVDF membrane (Millipore) using a semidry blotting apparatus for 1.5 h. The protein samples were incubated for 1 h with the blocking buffer containing 5% milk in Tris-buffered saline with 0.1% Tween-20 (TBST), and then incubated overnight at 4°C with the primary antibodies in blocking buffer. The antibodies used were Beta-actin rabbit mAb (1:5000) and GAPDH monoclonal antibodies (1:5000), which were used to correct differences in the amount of total loaded protein. After rinsing five times with TBST (for 15 min), the blots were incubated with the secondary antibodies (1:5000) for 1.5 h at room temperature, and rinsed with TBST (six times, each for 15 min). The proteins were detected using western blot detection reagents (Thermo Fisher Scientific).
A total of 31 differential proteins were classified into 8 groups based on the following molecular functions (Figure 2A): translation regulator activity (spot 4), molecular transducer activity (spot 13), catalytic activity (spots 3, 5, 14, 17, 20, 21, 22, 24, 26, and 31), enzyme regulator activity (spots 2, 10, 11, 12, and 31), antioxidant activity (spots 14, 19, 28, 30, and 31), binding (except for spot 3, 17, and 29), transporter activity (spots 7 and 28), and structural molecule activity (spots 10, 11, 12, 22, and 23). The 31 proteins in SCNT-derived lungs and control samples were localized to organelles (17%), extracellular regions (6%), macromolecular complexes (13%), cells (19%), extracellular region parts (5%), membrane-enclosed lumen (9%), cell part (19%), and organelle part (12%, Figure 2B).

As a differentially expressed protein, Beta-actin (Spot 8) from SCNT-derived and the control porcine lungs was validated by western blot analysis to confirm the validity of the LC–MS data in the present study. The results of western blot analysis showed that the level of Beta-actin in SCNT-derived porcine lungs was higher than that of the control porcine lungs (Figure 3).

Discussion

Somatic cell nuclear transfer (SCNT) pigs have been successfully produced in many countries in the past 10 years. Nevertheless, except for the low survival rate, cloned pigs often display organ abnormalities that might have adversely influence on postnatal life. In the previous

<table>
<thead>
<tr>
<th>Spot No. (NCBI Accession No.)</th>
<th>Protein Name</th>
<th>Protein MW(Da)/pI</th>
<th>Protein Score</th>
<th>Matched peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (gi</td>
<td>311250260)</td>
<td>B1 cell specific protein</td>
<td>21958.8/5.59</td>
<td>620</td>
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<tr>
<td>2 (gi</td>
<td>33359412)</td>
<td>Proliferating cell nuclear antigen (PCNA)</td>
<td>17968.8/4.57</td>
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<tr>
<td>3 (gi</td>
<td>148232214)</td>
<td>Ribose-5-phosphate isomerase</td>
<td>32845.9/8.42</td>
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<tr>
<td>4 (gi</td>
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<td>Poly(R)-binding protein 1 (PCBP1)</td>
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<tr>
<td>5 (gi</td>
<td>89167)</td>
<td>Ferritin heavy chain (FHC)</td>
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<td>6 (gi</td>
<td>2136514)</td>
<td>Ig gamma-4 chain C region (IGHG4)</td>
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<tr>
<td>7 (gi</td>
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<td>Hemoglobin subunit beta (HBB)</td>
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<tr>
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<td>75043336)</td>
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Table 1: Gel-based MS identification of 31 differentially regulated proteins from SNCT-derived and control lungs.

studies, comparative proteomics has emerged as a powerful tool for exploring the influence of nuclear transfer operation on SCNT animals [6,11,16]. Lung abnormalities are also one of the reasons leading to death in cloned animals [4]. For this reason, we investigated the protein profiling of SCNT-derived porcine lungs by 2-DE and MS.

In the present study, proteomic analysis revealed that SCNT operation causes the abnormal expression of some important proteins especially antioxidant activity-related proteins in SCNT-derived porcine lungs. Some important differentially expressed proteins in SCNT-derived porcine lungs were detailed categorically below.

**Antioxidant activity-related proteins**

The unbalance between production and elimination of reactive oxygen species (ROS) has severe consequences for cells. ROS accumulation in cells stimulates cell growth and proliferation, promotes the development of cancers, triggers apoptotic pathways, and leads to cell death [17]. As such, antioxidant activity-related proteins are important in proteomics studies. In the present paper, antioxidant activity-related proteins in the lungs of the cloned pigs were up-regulated (greater than 2-fold) except Prdx2 (spot 31).

As a mitochondrial antioxidant protein, superoxide dismutase 2 (SOD2 or Mn-SOD, spot 14), which is located in the mitochondria, is one of superoxide dismutases (SODs) are responsible for the detoxification of superoxide free radicals. SOD2 is a primary defense to oxidative stress and very responsive to oxidative stress [18]. In previous studies, SOD2 overexpression acts as a tumor suppressor, as observed in a wide variety of cancer cells, such as human lung tumors [19,20]. In this study, SOD2 upregulation in SCNT-derived porcine lungs may be an adaptive alteration for lung cells to protect against the increased oxygen stress.

Ferritin heavy chain (FHC, spot 5) is one of the two mammalian ferritins that act as major iron storage proteins in the cytoplasm [21,22]. FHC is responsible for iron sequestration, resulting in the suppression of apoptosis through ROS inhibition [23,24]. FHC is also a stress- and inflammation-regulated protein that exhibits the ferroxidase activity required for iron sequestration [25]. Evidences suggests that FHC upregulation may be an acute response to distress and may exert an anti-apoptotic effect against oxidative stress [24]. The overexpression of FHC in the present study is similar to those in other studies [24,25].

Cystatins, such as cystatin C (or cystatin-A8, spot 10) and cystatin B (CSTB, or stefin B, spot 12), which are endogenous inhibitors of lysosomal cysteine proteinases, are widely distributed among different tissues and cell types [26]. Cystatin C is a type II cystatin that occurs in most cell types of different organs, including the lung, and regulates the activity of lysosomal cysteine proteases, including cathepsins B, H, and L [27,28]. Cystatin C expression might be stimulated with oxidative stress, and it might also play a role in the inhibition of cathepsin B and the regulation of apoptosis elicited by oxidative stress [29]. Many studies also suggested that cystatin C overexpression may serve as a possible diagnostic biomarker for oxidative injury in tissues and cells from patients with cardiovascular diseases and kidney disorders [30,31]. Cystatin B, a type I cystatin first described as an inhibitor of the cysteine proteases, has a broad distribution in cells and tissues, and targets in vitro lysosomal cathepsins B, H, and L [32]. Cystatin B intracellularly controls the activity of lysosomal proteinases, which regulate protein turnover and are associated with cell biological behavior [33]. Increased levels of cystatin B has been found in a variety of human malignant tumors [34,35]. Our data suggest that both cystatin C and cystatin B are up-regulated proteins in SCNT-derived porcine lungs, which may be an adaptive alteration to antagonize increased oxygen stress.

Peroxisidoxins (PRDXs), a conserved family containing six antioxidant proteins widely expressed in different organisms, has been demonstrated to prevent hydrogen peroxide accumulation in cells to protect against oxidative damage and to regulate the signal transduction...
mediated by hydrogen peroxide in a large variety of organisms [6,36]. Prdx2 (spot 31) is a typical 2-Cysperoxidase and protects against H$_2$O$_2$-induced cell damage and apoptosis [37]. Whereas Prdx5 (spot 20) is an atypical 2-Cysperoxidase also an important antioxidant enzyme during oxidative stress that plays a vital role in reducing active oxygen from intracellular oxygen metabolism. In this paper, low Prdx2 expression and high Prdx5 expression in the SCNT-derived porcine lungs could be responses to the accumulation of hydrogen peroxide.

The expression changes of above-mentioned differential proteins may be an adaptive alteration to antagonize increased oxygen stress or responses to the accumulation of hydrogen peroxide.

Other related proteins

Other proteins of interest identified in this study have translation regulator activity, molecular transducer activity, catalytic activity, enzyme regulator activity, transporter activity, or structural molecule activity.

Proliferating cell nuclear antigen (PCNA, spot 2) is a critical protein required for DNA replication, repair, chromatin structure maintenance, chromosome segregation, and cell-cycle progression during cellular processes in proliferating cells, such as including cancer cells [38,39]. In the past, PCNA over-expression has been widely used as a tumor marker for human lung cancer [40,41]. We speculated that the up-regulation of PCNA in the lung of the cloned pigs could be also detrimental in this study.

Stathmin (also known as oncoprotein 18, spot 13) is a highly conserved cytosolic phosphoprotein that regulates microtubule dynamics [29,42]. Stathmin silencing using siRNA has also been shown to decrease the proliferation, viability, and invasion of cancer cells [43,44]. Overexpression of stathmin has been observed in various malignancies and serve as a biomarker in some human cancers [45,46]. In our study, the overexpression of stathmin might be one reason for lung abnormality in cloned pigs.

Ribosomal proteins (RPis) are organelle components that have a housekeeping role. Among RPs, especially 60S ribosomal protein L22 (spot 23, RPL22) have extraribosomal functions, including DNA replication, transcription and repair, cell growth and proliferation and regulation of apoptosis [47,48]. RPL22 has also been associated with genetic diseases in previous studies and its overexpression may suppress gene transcription [18]. In the present study, overexpression of RPL22 is speculated to be detrimental to the normal development and growth of porcine lungs.

Carbonic anhydrases (CAs) are widespread zinc enzymes that catalyze the equilibration between CO$_2$ and HCO$_3^-$ [49]. As cytosolic CAs, both CA II (spot 24) and CA I (spot 26) are ubiquitous in living organisms [50]. CA II is expressed in the alveolar epithelium and responsible for the interconversion of carbon dioxide and bicarbonate (CO$_2$ + H$_2$O ↔ HCO$_3^-$ + H$^+$) to maintain acid–base balance [51]. Low CA II expression is involved in muscular dystrophy which leads to muscle weakness in humans [52,53]. Significantly low expression of CA I and CA II is reported in lung cancer, which might lead to acidic cellular pH that promotes tumor cell motility, tumor growth, and metastasis [54].

Actins are an essential component of the cytoskeleton that are critical in a wide range of cellular processes, including cell migration, cell division, and the regulation of gene expression. Beta-actin (spot 8) which is encoded by an endogenous housekeeping gene, is a highly conserved, cytoskeleton-associated protein involved in cell motility, structure, and integrity [55]. However, recent studies have shown that Beta-actin expression changes in response to biochemical stimuli and disease states. Beta-actin regulates cellular structures and endogenous cytoarchitectural functions [56]. Its overexpression is also related to higher levels of protein oxidation in disease states [57].

In conclusion, the results of this study are generally consistent with previous studies. They also provide evidence that SCNT operation causes the abnormal expression of some important proteins in the lungs. Thus, SCNT operation can affect the lung development and survival of SCNT pigs. Many studies including the present study, have shown that nuclear transfer operation has adverse effects on the survival and development of clones, which should not be overlooked.

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Authors’ Contributions

Shu-shan Zhang and Cai-feng Wu are principal investigators, designed and conceived the experiments, and prepared the manuscript. Jian-jun, Dai and Ting-yu Zhang participated in two-dimensional gel electrophoresis and bioinformatics analysis. Prof. De-fu Zhang designed and conceived the experiments and prepared the manuscript.

Competing Interests

The authors declare that they have no competing interests. All authors read and approved the final manuscript.

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