Theriogenology 81 (2014) 901–905

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Comparative proteomic analysis of hearts of adult SCNT Bama miniature pigs (*Sus scrofa*)

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ARTICLE INFO

Article history:
Received 25 July 2013
Received in revised form 7 January 2014
Accepted 7 January 2014

Keywords:
Pig
SCNT
Heart
Comparative proteomics

ABSTRACT

This study aims to determine the effects of SCNT on cardiac development of SCNT pigs through proteomic methods. Heart proteins from three adult SCNTs and two normal reproductive Bama miniature pigs were extracted, separated, and identified via comparative proteomic methods, including two-dimensional gel electrophoresis, mass spectrometry, and Western blot. Eleven differentially expressed spots were identified as differentially expressed proteins, of which five spots were upregulated proteins such as cardiac myosin heavy chain, cathepsin D, and heat shock protein beta-1 (HSP27). By contrast, six spots were downregulated proteins such as alpha skeletal muscle and actin. The results also demonstrated that nuclear transfer might result in abnormal expression of some important proteins in hearts from SCNT pigs, and affect the cardiac development in SCNT pigs' survival.

1. Introduction

Somatic cell nuclear transfer is a useful and promising technique for pig breeding and human medicine [1]. In the last several years, many cloned pigs have been produced in different countries using SCNT. However, SCNT pigs are still susceptible to early embryonic and fetal deaths, stillbirths, and abnormalities [1–3]. Molecular studies have confirmed that incomplete genetic reprogramming during SCNT causes these abnormalities [4,5]. However, messenger RNA abundance is not always consistent with the protein levels because of posttranslational modifications of proteins [6]. Therefore, information about the differences in protein expression between SCNT and normal reproductive animals would help elucidate the mechanisms underlying the development of SCNT animals.

In previous studies, SCNT-derived placenta from bovines and pigs, and malformed umbilical cords from early death of SCNT piglets were analyzed by comparative proteomic approach composing of 2-DE (two-dimensional gel electrophoresis) and MS (mass spectrometry) [1,2,7,8]. There are no relevant reports about comparative proteomics of heart from adult SCNT pig.

In the present study, to investigate the differences in protein expression that may contribute to the functional differences of hearts between SCNT and normal reproductive adult pigs derived from artificial insemination, we conducted a global comparative proteomic analysis of heart proteins between both 2-DE, matrix-assisted laser desorption ionization/time of flight mass spectrometry (MALDI-TOF MS) and Western blot.

2. Materials and methods

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-month old) were produced as described previously [9,10]. Two normal reproductive pigs (7-month old) of the same breed and sex derived from artificial insemination used as

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http://dx.doi.org/10.1016/j.theriogenology.2014.01.006

control animals. The SCNT pigs were dissected and diagnosed with cardiac hypertrophy by senior veterinarian, and the hearts from the control pigs develop normally. All pigs were maintained according to the principles of the Shanghai guide for the care and use of laboratory animals. The samples were obtained after slaughter, and stored in liquid nitrogen until use.

2.1. Protein extraction

Frozen heart samples (about 300 mg) were washed with PBS buffer (pH 7.0), cut into 1 × 1 mm pieces, and homogenized (PRO 200 homogenizer; PRO Scientific, Monroe, CT, USA) in lysis buffer (4% SDS, 1 mM DTT, 150 mM Tris-HCl, pH 8.0) on ice. The samples were heated in a boiling water bath (5 minutes), ultrasonicated, and vortexed. The supernatant was aspirated after centrifugation at 12,000× *g* for 45 minutes (4 °C), and the concentration of proteins were determined with a bicinchoninic acid protein assay reagent (Nanjing Jiancheng Province, China).

2.2. Two-dimensional gel electrophoresis

In this study, 100 and 500 μg heart proteins were loaded onto analytical and preparative gels of 2-DE, respectively. Protein samples were mixed with a rehydration solution (containing 8 M urea, 2% 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate, 0.5% immobilized pH gradients (IPG) buffer, 18 mM DL-Dithiothreitol (DTT), and a trace of bromophenol blue). The IPGphor isoelectric focusing (IEF) system (Amersham Pharmacia Biotech) and pH 3 to 10 nonlinear IPG strips (13 cm, nonlinear; Amersham) were used for IEF. The IPG strips with the protein samples were rehydrated for 12 hours in 250 μL of rehydration buffer. Then, IEF was performed at 500 V for 1 hour, 1000 V for 1 hour, and 8000 V for 5 hours.

The gel strips were equilibrated for 15 minutes 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 the Hofer SE 600 system (Amersham) at 15 mA per gel for 30 minutes, followed by 30 mA until the bromophenol blue migrated to the bottom of the gel. Three replicates were performed for each sample.

After 2-DE, protein spots in analytical gels and preparative gels were stained with ammoniacal silver nitrate and Coomassie Blue G-250, respectively. The 2-D gels were scanned using a Bio-Rad GS710 scanner, and image analysis was accomplished using Image master 2D Elite (Amersham). The difference in the abundance of differential protein spots was analyzed with the Student's *t*-test ($P < 0.05$ was considered significant).

2.3. MALDI-TOF/TOF MS analysis and data analysis

Protein spots of interest cut from the preparative Coomassie blue–stained gels were destained for 20 minutes in 400 μL of 100 mmol/L NH_4HCO_3 per 30% cellulose acetate nitrate, washed in Millipore-Q water until the gels were destained, and then lyophilized. Each spot was digested for 24 hours in 10.0 ng/mL trypsin (sequencing grade; Promega) at 37 °C.

Peptides were extracted with 100 μL of 60% ACN per 0.1% TFA for 15 minutes with sonication, and the supernatant was removed. The extraction was repeated three times, desalted, and freeze-dried. Peptide mixtures were then analyzed using a 4800 Plus MALDI TOF/TOF Analyzer (Applied Biosystems, Foster City, CA, USA). The MS/MS spectra were searched by using Mascot software version 2.2 (http://www.matrixscience.com) using the mammalian genome database (non-redundant National Center for Biotechnology Information). Peptide and fragment mass tolerance were ±100 ppm and ±0.8 Da.

2.4. Western blot analysis

One differentially expressed protein was selected and validated using Western blot analysis. The antibody (anti-heavy chain cardiac Myosin antibody) was purchased from Santa Cruz Biotechnology (USA).

For Western blot analysis, 20 μg of total protein was separated on 12% SDS-PAGE gels, followed by transferring to a Poly(vinylidene fluoride) membrane (Millipore) using a semidry blotting apparatus for 1.5 hours. They were incubated for 1 hour with the blocking buffer containing 5% milk powder in Tris-buffered saline with 0.1% Tween-20 (TBST), and then incubated overnight at 4 °C with primary antibodies in block buffer. We used the following antibodies: Anti-heavy chain cardiac Myosin antibody (Abcam, 1:2000) and β-actin (Cell signaling, 1:2000), which was used to correct differences in the amount of total loaded protein. After rinsing with TBST (five times, each for 15 minutes), the blots were incubated with the secondary antibodies for 1.5 hours at room temperature, rinsed with TBST (six times, each for 15 minutes), and proteins were detected using Western blotting detection reagents (Thermo Fisher Scientific).

3. Results

3.1. Separation of differentially expressed proteins

Approximately 600 spots were found across the range of pH 3 to 10 in SCNT-derived and control hearts, and 11 differential proteins at least 2.0-fold differences including five upregulated and six downregulated proteins were further selected for MALDI-TOF MS (Fig. 1).

3.2. Identification of differentially expressed proteins

Differentially expressed proteins were determined by MALDI-TOF MS (Table 1). The results were evaluated based on the number of peptides matched to the candidate proteins, the difference in the number of matched peptides between the candidate protein and the next best fit, and the coverage of the candidate protein's sequence by the matching peptides. The bioinformatics analysis tools of our study included Data Explorer software (Applied Biosystems), which provided lists of monoisotopic peaks, ProFound (http://prowl.rockefeller.edu/), and Mascot (http://www.matrixscience.com/search_form_select.html) web-based software packages.