Placenta 31 (2010) 853–859

Contents lists available at ScienceDirect

Placenta

journal homepage: www.elsevier.com/locate/placenta

Aberrant protein expression in the placenta of cloned mouse derived from embryonic stem cell

Hong Rye Kim [a], Rong Xun Han [a], Teruhiko Wakayama [b], Chang Sik Park [a], Dong Il Jin [a,*]

[a] Department of Animal Science & Biotechnology, Research Center for Transgenic Cloned Pig, Chungnam National University, 305-764 Daejeon, Republic of Korea
[b] Center for Developmental Biology, RIKEN Institute, Japan

ARTICLE INFO

Article history:
Accepted 13 July 2010

Keywords:
Mouse cloned placenta
Placentomegaly
TIMP-2
PBEF

ABSTRACT

Placentomegaly is a common phenotype in cloned mice. To assess differences in protein expression between placentae of cloned and uncloned mice, we used a proteomic approach involving 2-dimensional electrophoresis (DE) and MALDI-TOF MS. Proteins within isoelectric point range of 4–11 separately were analyzed in 2-DE with 3 replications of each sample. A total of approximately 3500 spots were detected in placental 2-DE stained with Coomassie blue. In the comparison of normal and cloned samples, a total of 41 spots were identified as differentially expressed proteins, of which 25 spots were up-regulated proteins such as TIMP-2, glutamate-ammonia, and esterase 10, while 16 spots were down-regulated proteins such as PBEF and annexin A1. The TIMP-2, which is related to extracellular matrix degradation and tissue remodeling processes, is an inhibitor of MMP-2. The PBEF is related to inhibition of apoptosis and induction of spontaneous labor. Western blot analysis confirmed increased TIMP-2 expression and decreased PBEF expression in cloned placentae compared with normal controls. Our results demonstrated composite profiles of key proteins involved in abnormal hypertrophic placenta derived from cloned mice and suggested that the reason for the placentomegaly may be due to abnormal gene expression in cloned mice.

1. Introduction

Somatic cell nuclear transfer (SCNT) in animals has the potential for use in a wide range of applications, including the generation of multiple copies of genetically superior farm animals, the preservation of endangered species or the production of transgenic animals. Improvements in the technology are highly worthwhile because the economic benefit associated with the improved technologies would have a favorable influence on both the biotechnology industry and basic research. Although mammalian species have been successfully cloned from SCNT, this cloning strategy suffers from unresolved technological limitations. For example, SCNT in animals is remarkably inefficient, with reported rates (number of live offspring/transferred cloned embryos) of 5% in mice, 3% in cattle, and 0.5% in pigs [1–5]. Furthermore, clones that develop to term tend to show a myriad of disorders, including large offspring syndrome, respiratory failure, and placental abnormalities [6–10]. The placenta is a highly specialized organ that supports normal fetal growth and development during pregnancy, and placental abnormalities have been associated with fetal losses and phenotypical abnormalities among cloned mammals [11–13]. Cloned mouse fetuses have been shown to develop to term and yield placental overgrowth regardless of the sex or source of donor nuclei [14–17], and placentomegaly in cloned mouse concepti has been associated with expansion of the spongiotrophoblast layer, increased numbers of glycogen cells, and enlargement of trophoblastic cells [18]. During placental development, the trophoblast cells develop into some of the extra-embryonic membranes and a large part of the placenta [19]. For proper growth of reconstructed embryos, the transferred somatic nuclei must be completely reprogrammed to allow normal development of the trophoblast cell lineage required for placental development. Thus, researchers have speculated that placentomegaly in cloned mice is likely to reflect epigenetic abnormalities that arise partly from inadequate nuclear reprogramming.

In support of this, recent studies have shown that the epigenetic reprogramming errors of somatic cell genome can result in dysregulated expression of developmentally imprinted genes in cloned embryos, fetuses, and placentae, leading to abnormalities in the resulting cloned animals [20–24]. Abnormal gene expression has been detected in term placentae of cloned mice [25,26], and large-scale dysregulation of normal gene expression patterns was

* Corresponding author. Tel.: +82 428215876.
E-mail address: dijin@cnu.ac.kr (Dong Il Jin).

0143-4004/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.placenta.2010.07.006

observed in placentomegaly of cloned mice using cDNA microarray [27]. Most of the previous investigations have focused on cDNA microarray detection of changes in mRNA levels and/or differences in the methylation patterns of imprinted genes, yielding a somewhat limited understanding of the molecular mechanisms underlying placentomegaly in cloned mice. We analyzed to identify altered protein profiles in abnormal placentae from SCNT bovine [28,29], but no previous study has examined protein expression abnormalities in neonatal mouse clones, particularly with an eye toward identifying proteins that may be involved in the complex biomolecular interactions underlying placentomegaly.

In an effort to identify proteins associated with placentomegaly, we used 2-DE in combination with MALDI-TOF MS, which is a technique commonly used to analyze complex cellular proteomes [30], to analyze differential protein expression patterns in the placentae of cloned mice versus normal mice. The novel identification of these differentially expressed proteins may provide important new insights into the molecular mechanisms underlying placentomegaly in cloned mice.

2. Materials and methods

2.1. Placental sample

Three cloned mice placentae and 4 normal placentae were provided from Dr. Wakayama (RIKEN Institute, Kobe, Japan). In brief, nuclei of B6D2F1 embryonic stem cells were injected into enucleated B6D2F1 oocytes, the reconstituted embryos were incubated for 72 h, and those that had developed to the morula or blastocyst stages were transferred to the uteri of 2.5 dpc (days postcoitum) pseudopregnant ICR females, generated as previously described [14]. Concepti were collected by Cesarean section at 19.5 dpc, and the placental samples were separated and frozen until use.

2.2. 2-DE and image analysis

For 2-DE, soluble proteins were extracted from placentae as previously described [30,31], with some modification. Briefly, placenta samples for resolution were mixed with an equal volume of lysis buffer A containing 1% SDS, 1 mM PMSF, protease inhibitor cocktail (Roche, Germany) and 100 mM Tris–HCl, pH 7.0. The samples were sonicated for 15 s and incubated on ice, and then mixed gently with shaking for 1 h with an equal volume of lysis buffer B containing 7 M urea, 2 M thiourea, 4% CHAPS, 0.1 M DTT, 1 mM PMSF, protease inhibitor and 40 mM Tris–HCl, pH 7.0. The samples were suspended by sonication for 15 s, and insoluble materials were removed by centrifugation at 15,000 g for 20 min at 4 °C. Protein samples were treated with 100 units/mL endonuclease (Sigma, MO) for 1 h at 30 °C, and then the solubilized protein extracts were quantified using a Bradford protein assay kit (Bio-Rad, CA). For resolution across pH 4–7 and pH 5.5–6.7 (acidic range), 1 mg protein was mixed with rehydration buffer (6 M urea, 2 M thiourea, 4% CHAPS, 0.4% DTT, 2% v/v IPG buffer pH 4–7/pH 5.5–6.7) to yield a final volume of 450 μL, and this mixture was loaded onto the appropriate IPG strip (pH 4–7/pH 5.5–6.7; 180 × 3 × 0.5 mm, GE Healthcare Bio-Sciences, Uppsala, Sweden). For the alkaline range (pH 6–9/pH 7–11), 2-DE was performed according to standard protocols [30,32,33]. Alkaline IPG strips (pH 6–9/pH 7–11; 180 × 3 × 0.5 mm) were rehydrated overnight in 300 μL modified rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 2.5% DTT, 10% isopropanol, 5% glycerol, 2% IPG buffer, pH 6–11). Rehydration of individual IPG strips was for two different ways performed in the swelling tray without sample. After rehydration, IEF was performed in a Multiphor II IEF system (GE Healthcare Bio-Sciences, Uppsala, Sweden), as follows. The prepared protein sample (1 mg) was mixed with modified rehydration buffer to a total volume of 110 μL, and this mixture was loaded into a cup on the anodic side. A “CleanGel” paper wick (GE Healthcare Bio-Sciences, Uppsala, Sweden) immersed in modified buffer containing 0.4% DTT was added to the cathodic side. First dimension IEF was performed on a Multiphor II IEF system (GE Healthcare Bio-Sciences, Uppsala, Sweden) and the sample proteins were resolved using the Ettan-DALT 2-DE system (GE Healthcare Bio-Sciences, Uppsala, Sweden). Automatic isoelectric focusing was carried out at 1.5 × 105 V/h. The voltage started at 100 V and was gradually increased to a final voltage of 8000 V. After the first dimensional IEF, in case of acidic range (pH 4–7, pH 5.5–6.7), each IPG gel strip was placed in an equilibration solution (6 M urea, 2% SDS, 50% glycerol, 2.5% acrylamide, 1.875 M Tris–HCl, pH 8.8) containing 5 mM TBP for 20 min with gentle shaking. The alkaline strips (pH 6–11) from the first dimension separation were equilibrated twice for 15 min each with gentle shaking in 10 mL each of equilibration solutions containing DTT (1%) for the first equilibration and iodoacetamide (4%) for the second equilibration [34,35]. Second dimension separation was performed on 8–16% linear gradient SDS polyacrylamide gels, using an Ettan-DALT system (GE Healthcare Bio-Sciences, Uppsala, Sweden). The gels (200 × 250 × 1.0 mm) were run overnight at 10–15 mA per gel, until the bromophenol blue marker dye ran off the bottom of the gel. After 2-DE, gels were stained using colloidal Coomassie brilliant blue (CBB) G-250. The stained gels were scanned at an optical resolution of 63.5 μm/pixel using a GS-710 calibrated densitometer (Bio-Rad, CA), and protein spots with differential intensities were identified using the MALANIE III software package (GE Healthcare Bio-Sciences, Uppsala, Sweden). Intensity (optical density) was measured by summing pixels within each spots boundary (spot volume) and recorded as a percentage of the total spot intensity on the gel: % vol = (spot volume/$\sum$ volumes of all spots resolved in the gel). Variations in abundance were calculated as the ratio of average values (% vol) between cloned and normal placentae. The mean value of percentage and S.E. were calculated for each spot in each class and then compared using the two side student's *t*-test. Protein spots whose expressions were found to be difference between the two classes at the significance level of $P < 0.05$ were selected for further analysis.

2.3. MALDI-TOFMS peptide mass fingerprinting

Coomassie blue-stained gel spots were excised and digested overnight with trypsin, and the peptide-containing supernatants were set aside. Residual peptides were extracted from the gel pieces by sonication for 20 min at 30 °C in a solution of 50% acetonitrile/0.5% TFA. The pairs of supernatants containing extracted peptides were combined and applied for MALDI-TOF MS analysis on a Voyager-DE STR MALDI-TOF MS (Applied BioSystems, CA). Approximately 1 μL of peptide solution extracted from a given spot was combined with 1 μL of matrix solution (10 mg/mL α-ciano-4-hydroxycinnamic acid, 0.1% TFA, and 50% acetonitrile), and loaded onto a 96-well MALDI sample plate for crystallization. Averages of 500 spectra were obtained for each sample, and scans were performed twice. The accelerated voltage was operated at 20 kV. All acquired spectra of samples were processed using Voyager™ 5.1 software (Applied Biosystems, CA) in a default mode. Parent mass peaks with mass range of 800–3500 Da and minimum signal to noise ratio of 20 were picked out for protein identification. Spectra were calibrated automatically upon acquisition using an external 3-point calibration. Peak assignment was performed manually using the DataExplorer™ software package (Applied BioSystems, CA), and spectra were saved as peak table files and used to search against non-redundant protein sequence databases available online [SWISS-PROT and/or NCBInr (2009/06/01) Data Bank]. The peptide mass fingerprinting (PMF) data were applied to ProFound and MASCOT search engines (http://prowl.rockefeller.edu/; http://www.matrixscience.com/search_form_select.html). Search parameters were other mammalia as taxonomy, trypsin as an enzyme, carbamidomethylation of cysteine as fixed modification and methionine oxidation as variable modification, and one missed cleavage site with a mass tolerance of less then 1 ppm (average) and 100 ppm (monoisotopic). To eliminate the redundancy of proteins that appeared in the database under different names and accession numbers, the single protein member belonging to the species *Mus musculus* or else with the highest protein score (top rank) depending on experimental molecular weight was singled out from the multiprotein family. The protein identification was further validated by Profound and MASCOT score. *Z* score in Profound is the distance to the population mean in unit of standard deviation. A *Z* score of 1.65 for a search means the 95th percentile range. Mowse scores greater than 67 in MASCOT are significant ($P < 0.05$).

2.4. Western blot analysis

Briefly, protein lysates (30 μg) were resolved by 12% SDS-PAGE, electro-transferred to PVDF membranes (Bio-Rad, CA) and blocked for 1 h at room temperature in TBS containing 5% skin milk. The membranes were then incubated for 1 h with anti-mouse TIMP-2 monoclonal antibody (diluted 1:1000; Cat No. ab1828, Abcam, MA), anti-rabbit beta-actin polyclonal antibody (diluted 1:5000; Cat No. ab8227, Abcam, MA) or anti-rabbit PBEF polyclonal antibody (diluted 1:10000; Bethyl, TX). Each blot was washed six times for 10 min each with TBS-T buffer, and then incubated with HRP-conjugated anti-mouse antibodies diluted 1:10000 for TIMP-2 or HRP-conjugated anti-rabbit antibodies diluted 1:5000 (for anti-actin) or 1:10000 (for anti-PBEF). The results were visualized using an ECL kit (GE Healthcare Bio-Sciences, Uppsala, Sweden) and diagnostic film (Sigma, MO).

2.5. Statistical analysis

Significant differences among samples were determined by Duncan's multiple range tests following ANOVA analysis using GLM of SAS (SAS Institute Inc., Cary, NC). Statistically significant data should have a *p*-value lower than 0.05.

3. Results

3.1. Detection of differential protein expression in the placentae of cloned versus normal mice

We used 2-DE to generate differential protein expression maps of placentae from normal mice and cloned mice derived by nuclear