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Proteomic identification of abnormally expressed proteins in early-stage placenta derived from cloned cat embryos

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ABSTRACT

It is unknown whether gene expression in cloned placenta during pre- and post-implantation is associated with early pregnancy failure in the cat. In this study, protein expression patterns were examined in early-stage (21-day-old) domestic cat placentas of fetuses derived from AI (CP; N = 4) and cloned embryo transfer (CEP; N = 2). Differentially expressed proteins were analyzed by two-dimensional gel electrophoresis and matrix-assisted laser desorption/ionization time-of-flight (TOF) mass spectrometry (MS). A total of 21 proteins were aberrantly expressed ($P < 0.05$) by >1.5-fold in CEP compared with CP. Compared with CP, 12 proteins were upregulated in CEP (peptidyl-prolyl cis-trans isomerase A, annexin A2, protein DJ-1, adenylate kinase isoenzyme 1, protein disulfide-isomerase A3, actin cytoplasmic 1, serum albumin, protein disulfide-isomerase A6, and triosephosphate isomerase), and nine proteins were downregulated (triosephosphate isomerase; heterogeneous nuclear ribonucleoprotein H; tropomyosin alpha-4; triosephosphate isomerase 1; 60 kDa heat shock protein, mitochondrial; serum albumin; calumenin; keratin type 1; and prohibitin). The identities of the differentially expressed proteins were validated by peptide mass fingerprinting using matrix-assisted laser desorption/ionization-TOF/TOF MS/MS. The abnormally expressed proteins identified in this study might be associated with impaired development and dysfunction of CEP during early pregnancy. Abnormal protein expression might also induce fetal loss and contribute to failure to maintain pregnancy to term.

1. Introduction

Since the cloned sheep Dolly [1] was produced in 1997, various species have been cloned by SCNT [2,3], and cloning has become a useful technique for production of embryos for embryonic stem cell research, animal models of disease, drug development, xenotransplantation, and the restoration of endangered species. Unfortunately, the success rate in producing cloned animals has been very low [1–3]. Most fetal loss occurs during early gestation because of chromosomal defects, endocrine disease, infection, immunologic factors, chemical agents, hereditary disorders, trauma, maternal disease, psychological factors, and abnormal placentation [4].

The mammalian placenta is a crucial organ with various roles, including nutrient uptake, respiratory gas exchange, waste elimination, and hormone synthesis necessary for fetal growth and survival during gestation. In addition, the placenta acts as an immunologic barrier. The mammalian placenta can be classified according to its shape and contact points; classifications include zonary (cat and dog), diffuse

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(horse and pig), cotyledonary (ruminants), and discoid (primates and rodents) placenta [5–7]. In the cat, the zonary placenta is separated into lamellar and junction zones after implantation [6]. The trophoblast lamellae are similar to the labyrinth, which covers the fetal mesenchyme and contains fetal blood vessels and some connective tissue cells [8].

During early pregnancy, normal embryos implant in the endometrium of surrogates. At this time, proper gene expression is important for pregnancy maintenance and fetal survival [9–12]. Disruption of the implantation process can lead to implantation failure, early abortion, abnormal fetal development, and pregnancy failure. Our laboratory successfully produced a cloned cat in 2005 [13] and the first red fluorescence protein transgenic cloned cat in 2008 [14]. However, most cloned embryos failed to maintain pregnancy through the first trimester; many factors are likely to be involved in early pregnancy loss. Recent reports indicate that abnormal protein expression in the placenta is associated with fetal mortality and morbidity in pigs [9] and cattle [10]. Differential expression of various proteins can lead to abnormal development and function of cloned term placenta, which are associated with morbidity and mortality of the cloned fetus [9–12]. Abnormal expression of different proteins in 26-day-old SCNT porcine extraembryonic tissue [9], SCNT porcine term placenta [11], cloned bovine placenta [10], and cloned term feline placenta [12] have been suggested to be associated with failure of pregnancy to reach term. These abnormally expressed proteins affected various physiologic processes during placentation, including cellular apoptosis, extracellular matrix remodeling during pregnancy, cell senescence, and mitochondrial malfunction, which, in turn, might contribute to increased fetal mortality and morbidity. Failure of early pregnancy and loss of the cloned fetus are frequently observed in research cats [12]; however, molecular mechanisms underlying early pregnancy loss during cat cloning have yet to be identified. Considering the previous findings on placental protein expression, the present study was initiated to explore the hypothesis that abnormal expression of functionally important proteins in the early-stage placenta might be associated with early loss of clone pregnancy. The aim of the present study was to investigate whether the pattern of protein expression in the early-stage placenta of the cloned feline fetus differed from that of the fetus derived from AI.

2. Materials and methods

2.1. Animals

Female cats (*Felis catus*, 1 to 3 years of age) were used as oocyte donors and recipients. Females were induced to superovulate by treatment with 200 IU pregnant mare serum gonadotropin (Daesung Inc., Republic of Korea), followed 72 hours later by 150 IU hCG (Daesung Inc.). Twenty-two hours after hCG treatment, *in vivo*-matured oocytes were collected into tyrode's lactate-HEPES buffer. Cumulus cells were removed from oocytes by gentle pipetting in medium 199 (TCM199, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 0.1% hyaluronidase. Denuded oocytes were washed and maintained until use in TCM199 supplemented with 0.3% BSA at 37 °C in an atmosphere of 5% CO_2 and 95% air. Care and use of animals was conducted in accordance with the Gyeongsang National University Guidelines for the use of laboratory animals (approval no. GAR-110502-X0018).

2.2. AI

Artificial insemination (AI) was carried out as described previously [15], with minor modifications. Fresh semen was obtained from male cats (≤5 years of age) using an artificial vagina. Semen was diluted in 20 mM Tris buffer, pH 6.5, supplemented with 20% egg yolk, and 1 to 5 × 10^6 motile sperm were surgically inserted into the uterus of the ovulating recipient with a disposable Pasteur pipette.

2.3. SCNT

The SCNT procedure was performed as described previously [13]. Briefly, denuded oocytes were cultured in TCM199 containing 0.2 μg/mL demecolcine for 1 hour at 38 °C in an atmosphere of 5% CO_2 and 95% air, and replaced in TCM199 supplemented with 5 μg/mL cytochalasin B and 0.2 μg/mL demecolcine. The first polar body with a chromatin plate was removed using the enucleation micropipette (Sutter Instrument Co., Novato, CA, USA) on a micromanipulator (Narishige, Tokyo, Japan) connected to an Olympus microscope. Somatic cells, isolated from a small piece of domestic cat skin and cultured in a dish (NUNC, Thermo Fisher Scientific Inc., Rochester, NY, USA), were used as donor cells. The donor cells ranged from passage 1 to 9 and were dissociated with 0.05% trypsin-EDTA (Gibco, Carlsbad, CA, USA) at 90% confluence. Trypsinized cells were washed three times with Ca^{2+}- and Mg^{2+}-free Dulbecco's-PBS containing 0.3% BSA. The 20- to 25-μm diameter donor nuclei were injected into the perivitelline space of enucleated oocytes. The reconstructed oocytes were fused in 0.3 M mannitol solution supplemented with 0.1 mM Mg^{2+} and 0.1 mM Ca^{2+} using electrostimulation with two direct current (DC) pulses of 2.0 kV/cm for 20 μs from an Electro Cell Fusion Generator (Nepagene, Chiba, Japan). Fused embryos were then activated in 0.3 M mannitol solution supplemented with 0.1 mM Mg^{2+} and 0.1 mM Ca^{2+} by electrostimulation with DC pulses of 1.0 kV/cm for 20 μs in a fusion chamber. Embryos were then incubated in TCM199 supplemented with 2 mM 6-dimethylaminopurine at 38 °C in a humidified atmosphere of 5% CO_2 and 95% air for 4 hours. Single-cell stage embryos were surgically transferred into the oviducts of synchronized recipients through the infundibulum using a disposable Pasteur pipette. A total of 325 cloned embryos were transferred into the oviducts of 15 surrogates.

2.4. Protein preparation

To determine the reason for early pregnancy loss, proteomics analysis was used to systemically important gene expression pattern in placentas from control (N = 4) and cloned (N = 2) fetuses obtained from individual surrogate mothers at 21 days by cesarean delivery. Proteins were