Proteomic Analysis of the Extraembryonic Tissue from Cloned Porcine Embryos*§

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Cloned animals developed from somatic cell nuclear transfer (SCNT) embryos are useful resources for agricultural and medical applications (1–3). Although various species of cloned animals have been successfully produced, a very low birth rate by the SCNT embryos has been observed (4). Embryonic, fetal, and postnatal death and other developmental abnormalities have been reported in the SCNT animals (5, 6). In addition, survived cloned neonates frequently show abnormal phenotypes like large offspring syndrome (7, 8).

The right number of cells in blastocysts is required for normal embryo development. However, the total number of cells in the blastocysts from the porcine SCNT embryos is smaller than the total of control blastocysts. SCNT embryos have a lower developmental competence to the blastocyst stage than control embryos (9). During embryo development, DNA methylation plays an important role for regulating gene expression. Genome wide demethylation occurs just after fertilization followed by de novo methylation as soon as differentiation occurs (10–12). According to a recent report, relatively normal demethylation occurs in the inner cell mass, not in the trophectoderm of the cloned blastocysts (13). This suggests that placenta development will be abnormal because trophectoderm produces extraembryonic tissue including placenta.

Oxidative stress occurs because of the production of reactive oxygen species (ROS), and antioxidant enzymes reduce oxidative stress by scavenging ROS. These ROS are free radicals and induce cellular damages (14). Pregnancy increases oxidative stress by increasing the metabolic activity in placental mitochondria and reduces the scavenging power of antioxidants (15).

Apoptosis is an important process during animal development and reproduction (16, 17). During pregnancy, apoptosis is physiologically important for normal placental growth and development (18, 19). Apoptotic stimuli induce the release of cytochrome c from mitochondria; the cytochrome c binds to Apaf1 (apoptotic protease-activating factor 1) for the formation of apoptosomes. Then apoptosomes activate Caspase 8, which cleaves pro-Caspase 3. An apoptotic process is also mediated via mitochondria-independent pathways that converge to the proteolytic activation of Caspase 3 (20). Antiapoptotic Bcl-2 plays an important role for preventing apoptosis in the syncytiotrophoblasts of placenta (21).

To investigate the reasons why the birth rate in the animals cloned by SCNT is so low, we examined the 26-day-old SCNT porcine fetus and extraembryonic tissue at which the sono-
gram can first detect pregnancy. We focused on the extraembryonic tissue, which is developed from trophoblasts, because of abnormal epigenetic reprogramming in the trophoblasts of the SCNT blastoderm embryos. The size of extraembryonic tissue from SCNT embryos at the 26th day was abnormally small compared with the wild type control. To find the differences at the molecular level in the extraembryonic tissue, proteomic analysis was performed. In the extraembryonic tissue from SCNT embryos, 12 protein spots were up-regulated, and 27 protein spots were down-regulated. In the Western blot analysis, antioxidant enzymes were down-regulated, and apoptosis marker proteins were up-regulated in the extraembryonic tissue from SCNT embryos. In the terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assay, apoptotic trophoblasts were observed in the placenta from SCNT embryos. These results indicate that apoptosis occurred in the extraembryonic tissue from SCNT embryos during early pregnancy. It may explain the low birth rate of the animals cloned by SCNT.

EXPERIMENTAL PROCEDURES

Extraembryonic Tissue—In vitro maturation, nuclear transfer, and embryo culture were performed according to previous reports (22, 23). Somatic cells were the fetal fibroblasts isolated from the F1 fetus produced by the crossing of inbred Duroc male and Landrace female pigs. Approximately 200 SCNT embryos were transferred to the oviducts of each synchronized recipient. Recipients were prepared as described previously (24). Three extraembryonic tissues from SCNT embryos and three control tissues at the 26th day of pregnancy were prepared.

Fig. 1. Normal and SCNT fetuses and extraembryonic tissue from 26th day of pregnancy. A, normal fetus and extraembryonic tissue from 26th day of pregnancy. B, SCNT fetus and extraembryonic tissue from 26th day of pregnancy. a and a', fetus; b and b', amnionic sac; c and c', placenta; d and d', extraembryonic tissue. The SCNT extraembryonic tissue was abnormally small compared with the control, whereas the size and shape of the fetus and amnionic sac look similar to each other. Bar, 5 mm.

Fig. 2. Two-dimensional gel electrophoresis. Proteins were isolated from normal and SCNT extraembryonic tissues, and 500 µg of total protein were loaded to the 2-DE gel. The first dimension was 18-cm pH 3–11 nonlinear IPG, and the second dimension was 10 and 12% gels (A and C, controls; B and D, SCNT extraembryonic tissue). The proteins were visualized by the silver staining. Among 2000 spots, 39 proteins were identified. In Tables I and II, 12 up-regulated and 27 down-regulated proteins are listed.
Silver staining kit (Amersham Biosciences) was used for 2-DE gels. The gels were fixed in 40% ethanol and destained in 10% acetic acid for 30 min and sensitized in ethanol glutardialdehyde. The peptides for MALDI-TOF and MALDI-TOF/TOF Calibration—

### Table I

**Up-regulated proteins in the SCNT extraembryonic tissue**

| Spot no. | Protein name | NCBI accession no. | Swiss-Prot accession no. | Method of ID | PMF (MS) Score Peptides matched | Peptides obtained | Sequence coverage % | Experimental Molecular mass | pl | Theoretical Molecular mass | pl |
|----------|--------------|---------------------|--------------------------|--------------|-------------------------------|------------------|-----------------|----------------------|-----|------------------------|-----|
| 1        | LMNB1 protein (fragment) | Q6DC98            | A                        |              | 109                           | 10               | 25              | 29                   | 38,262 | 6.25                   | 38,289 | 5.37                  |
| 2        | Annexin A2# | AAU85387           | Q5Y2C7                   | A            | 180                           | 18               | 45              | 45                   | 38,012 | 7.43                   | 38,795 | 6.49                  |
| 3        | Heterogeneous nuclear ribonucleoprotein H1 | CAI24000           | Q8117L                   | A            | 80                            | 12               | 65              | 32                   | 50,243 | 6.66                   | 49,454 | 5.89                  |
| 4        | Annexin A1 (Annexin I) (Lipopartin I) (Calpain II) (Chromobindin-9) (p35) (phospholipase A2-inhibitory protein) | P19619            | A                        |              | 77                            | 11               | 52              | 36                   | 38,669 | 6.75                   | 39,020 | 6.43                  |
| 5        | ABO44390 NID (aldose 1-epimerase) | BAB18973           | Q9GKX6                   | A            | 71                            | 7                | 25              | 23                   | 38,245 | 7.21                   | 38,020 | 6.31                  |
| 6        | Pyrroline-5-carboxylate reductase 1 isoform 1 | Q58DT4            | A                        |              | 80                            | 11               | 58              | 33                   | 35,274 | 6.75                   | 33,718 | 7.72                  |
| 7        | Hsp27       | Q5S1U1             | A, B                     |              | 83                            | 7                | 25              | 32                   | 24,871 | 6.04                   | 22,985 | 6.23                  |
| 8        | Ran, chain A | 1IBRA              | A                        |              | 135                           | 11               | 39              | 64                   | 20,251 | 8.28                   | 19,524 | 9.64                  |
| 9        | Voltage-dependent anion channel 1 | Q9MZ16            | A                        |              | 81                            | 8                | 38              | 34                   | 28,725 | 6.45                   | 30,822 | 8.62                  |
| 10       | Porcine hemoglobin (β subunit), chain B | 1QPWB             | A                        |              | 106                           | 8                | 23              | 79                   | 15,322 | 8.42                   | 16,082 | 6.76                  |
| 11       | Annexin A4  | P08132             | A                        |              | 90                            | 11               | 41              | 39                   | 36,024 | 6.72                   | 36,034 | 5.71                  |
| 12       | Lamin A#   | BAA02476           | P48678                   | A            | 73                            | 10               | 40              | 26                   | 47,742 | 6.48                   | 47,792 | 6.63                  |

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# Two-dimensional Gel Electrophoresis (2-DE)—2-DE was performed as described previously (25). IEF was carried out with the IPGphor unit (Amersham Biosciences) using precast 18-cm pH 3–11 nonlinear IPG gel strips (Amersham Biosciences). Total proteins from extraembryonic tissue were isolated with the protein extraction solution (1.0 mM PMSF, 1.0 mM EDTA, 1 μM pepstatin A, 1 μM leupeptin, and 0.1 μM aprotinin). 500 μg of total proteins were mixed with the rehydration solution (7 M urea, 30% (v/v) glycerol, and 2% (w/v) SDS). The second dimension separation was carried out using 10 and 12% SDS-PAGE gels. Then electrophoresis was performed using a Protean II xi 2-D cell (Bio-Rad) with 10 mA for first 20 min and 20 mA until the bromophenol blue reached the bottom of the gel. Electrophoresis was repeated three times in each sample to ensure reproducibility.

**Staining 2-DE Gels—** Silver staining kit (Amersham Biosciences) was used for 2-DE gels. The gels were fixed in 40% ethanol and 10% acetic acid for 30 min and sensitized in ethanol glutaraldehyde (25%, w/v), sodium thiosulfate (5%, w/v), and sodium acetate (17 g) for 30 min followed by three washes with water for 15 min each. Then the gels were immersed in silver nitrate (2.5%, w/v) and formaldehyde (37%, w/v) for 20 min, developed with sodium carbonate (6.25 g) and formaldehyde (37%, w/v) for 5 min, and stopped in EDTA-Na₂-2H₂O.

**Proteomic Analysis—** The silver-stained gels were scanned with the ImageScanner (Amersham Biosciences) and analyzed with the Phoretix Expression software 2005 version (Nonlinear Dynamics). Destaining and in-gel trypsin digestion of the protein spots were performed as described previously (26). Xcite (Shimadzu Biotech Co.), the automatic sample preparation system, was used for in-gel digestion, desalting, and plating. The desalting was performed with C₁₈ ZipTips (Millipore), and the samples were spotted with the 4-hydroxy-α-cyanocinnamic acid matrix solution onto a MALDI MS plate. In-gel digested peptides were analyzed with a MALDI MS/MS mass spectrometer, Ultraflex-TOF/TOF (Bruker Daltonics). Mass spectra were calibrated and processed using Flex Analysis and Biotool 2.2 software (Bruker Daltonics). Peptide mass fingerprinting (PMF) and MS/MS ion search were performed using MASCOT 2.0 software (Matrix Science) integrated with the Biotool 2.2 software. The Mass Spectrometry Protein Sequence Database (MSDB) (Version 09292005; 2,344,227 sequences) and The National Center for Biotechnology Information non-redundant (NCBI) (Version 04222006; 3,604,615 sequences) protein databases were searched with the following MASCOT settings: taxonomy as Mammalia (mammals); one incomplete tryptic cleavage allowed; peptide tolerance, 0.100 ppm; fragment tolerance, 1 ppm; monoisotopic mass; 1 + peptide charge state as 4-hydroxy-α-cyanocinnamic acid protonation; alkylation of cysteine by carbamidomethylation as a fixed modification; and oxidation of methionine as a variable modification. For PMF and MS/MS ion search, statistically significant (p < 0.05) matches by MASCOT 2.0 were regarded as correct hits. The threshold score were 67 in MSDB and 69–78 in NCBI database searches. For further analysis, two criteria were used: 1) porcine proteins matched were chosen although other species had higher ranked hits and 2) the protein matched to pI criteria were used: 1) porcine proteins matched were chosen although other proteins had higher ranked hits and 2) the protein matched to pI and molecular weight in the two-dimensional gel was chosen although other proteins had higher ranked hits.

**MALDI-TOF and MALDI-TOF/TOF Calibration—** The peptides for the calibration were: Bradykinin-[1-7] [M + H]⁺ mono (757.399), Angiotensin II-[M + H]⁺ mono (1046.541), Angiotensin I-[M + H]⁺ mono (1298.684), Substance P-[M + H]⁺ mono (1347.735), Bombesin-[M + H]⁺ mono (1619.822), Renin_Substrate-[M + H]⁺ mono (1758.933), ACTH_clip-[1-17]-[M + H]⁺ mono (2093.086),
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**TABLE II**

Down-regulated proteins in the SCNT extraembryonic tissue

| Spot no. | Protein name | NCBI accession no. | Swiss-Prot accession no. | Method of ID | Peptides matched | Peptides obtained | Sequence coverage | Experimental Molecular mass (Da) | Theoretical Molecular mass (Da) |
|----------|--------------|---------------------|--------------------------|-------------|-----------------|-----------------|-----------------|-------------------------------|-------------------------------|
| 13 | Transferrin | S01384 | A | 424 | 46 | 96 | 68 | 80,122 | 78,971 |
| 14 | AF542068 NID (serum albumin) | AAN17824 | P02769 | A, B | 93 | 13 | 40 | 18 | 72,011 | 71,274 |
| 15 | Glutamate dehydrogenase (NAD(P) (EC 1.4.1.3) precursor | P00367 | A | 76 | 12 | 56 | 29 | 62,087 | 61,701 |
| 16 | alpha enolase (EC 4.2.1.11) (2-phospho-o-glycerate hydrolyase) (Non-neural enolase) (NNE) (enolase 1) (phosphopyruvate hydratase) (c-myc promoter-binding protein) | P06733 | A | 121 | 14 | 57 | 33 | 47,112 | 47,350 |
| 17 | Mus musculus 0 day neonate thymus cDNA, RIKEN full-length enriched library, clone: A430106 I2, product: hypothetical protein, full insert sequence (fragment) | Q9CTR0 | A | 71 | 12 | 63 | 35 | 35,044 | 35,531 |
| 18 | Pigment epithelium-derived factor; PEDF (AF017058 NID) | AAC05733 | P97298 | A, B | 78 | 11 | 37 | 31 | 46,023 | 48,045 |
| 19 | Adenosylhomocysteinase (EC 3.3.1.1) (S-adenosyl-L-homocysteine hydratase) (AdoHcyase) | Q710C4 | A | 119 | 13 | 40 | 18 | 48,332 | 54,216 |
| 20 | 4-Hydroxyphenylpyruvate dioxygenase (EC 1.13.11.27) | S32821 | Q02110 | A | 125 | 15 | 62 | 51 | 45,887 | 45,216 |
| 21 | Phosphoglycerate kinase 1 (EC 2.7.2.3) | Q7SIB7 | A | 108 | 19 | 69 | 48 | 45,125 | 44,798 |
| 22 | Glyceraldehyde-3-phosphate dehydrogenase (phosphorylating) (EC 1.2.1.12) | DEPGG3 | A | 108 | 11 | 29 | 43 | 36,115 | 35,914 |
| 23 | Alcohol dehydrogenase (NADP+) (EC 1.1.1.12) (aldehyde reductase) (aldo-keto reductase family 1 member A1) | P50578 | A | 102 | 11 | 45 | 28 | 35,042 | 35,670 |
| 24 | Malate dehydrogenase (EC 1.1.1.37), chain A | 1MLDA | A | 188 | 19 | 50 | 59 | 35,003 | 33,390 |
| 25 | Apolipoprotein E | S33450 | P18650 | A | 208 | 29 | 81 | 62 | 36,002 | 36,634 |
| 26 | L-Lactate dehydrogenase (EC 1.1.1.27), chain M | DEPGLM | A | 116 | 14 | 42 | 37 | 36,117 | 36,749 |
| 27 | Pro-alpha (I) collagen (fragment) | CAC38832 | Q8ND8 | A, B | 95 | 8 | 22 | 30 | 32,987 | 32,134 |
| 28 | Phosphoglycerate mutase 1 (EC 5.4.2.1) (EC 5.4.2.4) (EC 3.1.3.13) (phosphoglycerate mutase isozyme B) (PGAM-B) (PGAM-dependent PGAM1) | CAC38832 | Q8ND8 | A, B | 95 | 8 | 22 | 30 | 32,987 | 32,134 |
| 29 | Triose-phosphate isomerase 1 | Q5E956 | A | 98 | 11 | 52 | 48 | 25,114 | 26,901 |
| 30 | Apolipoprotein A-I | A46018 | P18648 | A | 164 | 14 | 35 | 49 | 30,147 | 30,312 |
| 31 | Transthyretin precursor | S66595 | P50390 | A | 152 | 10 | 28 | 70 | 16,185 | 16,185 |
| 32 | Carboxypeptidase A1 precursor | NP_0.9409 | A | 78 | 10 | 46 | 26 | 47,024 | 47,320 |
| 33 | Thorinidoxin domain-containing 2* | AA9S05132 | Q86V03 | A | 80 | 10 | 40 | 25 | 51,721 | 53,637 |
| 34 | Peroxiredoxin 4 | AA9S05132 | Q9Z0V5 | A | 73 | 8 | 44 | 31 | 31,451 | 31,216 |
| 35 | Peroxiredoxin 5* | AAG13452 | Q9GLW8 | A | 134 | 11 | 43 | 53 | 18,034 | 17,484 |
| 36 | CRY | Q8SQ26 | Q8SQ26 | A | 110 | 12 | 51 | 39 | 34,377 | 35,504 |
| 37 | TTN protein (fragment) | Q6NSG0 | Q6NSG0 | A | 68.2 | 14 | 61 | 18 | 64,327 | 69,051 |
| 38 | alpha-1-Antichymotrypsin 3 | CAC07657 | Q8GMA8 | A | 86 | 9 | 47 | 35 | 23,421 | 22,883 |
| 39 | BC057898 NID | AAH57898 | Q8JM90 | A | 75 | 10 | 95 | 68 | 14,702 | 15,026 |

* Score thresholds were 69–78 for NCBI database.

ACTH_clip-(18–39) [M + H]" mono (2465.198), and Somatostatin [M + H]" mono (3147.471).

Western Blot Analysis—Western blot analyses were performed as described previously (27). Antibodies used in this study were purchased from Santa Cruz Biotechnology.

Caspase 3 Activity—Enzymatic activity of Caspase 3 was measured using the fluorescence assay kit (Peptron). The assay is based on the spectrophotometric detection of the fluorogenic substrate, which is cleaved by the activated Caspase 3. Fluorescent signal was measured using the excitation wavelength at 360 nm and the emission...
sion wavelength at 460 nm with a microplate reader (Victor3, PerkinElmer Life Sciences).

**TUNEL Assay**—The paraffin sections of the normal and SCNT placenta from the 26th day of pregnancy were prepared. TUNEL assay was carried out using the In Situ Cell Death Detection kit (Roche Applied Science). Tissue was dissected in Ringer’s solution, fixed in PBS containing 4% formaldehyde for 25 min, digested with 20 μg/ml proteinase K for 15 min, and incubated with the TUNEL reaction mixture.

**RESULTS AND DISCUSSION**

**Abnormally Small Extraembryonic Tissue from the SCNT Embryos**—Abnormal epigenetic reprogramming in the trophoblasts of the SCNT porcine blastocysts (13) suggests that extraembryonic tissue development will be abnormal because trophoblasts generate extraembryonic tissue. SCNT extraembryonic tissue from the 26th day of pregnancy showed abnormally small size and shape (Fig. 1B) compared with the wild type control (Fig. 1A). Interestingly the size and shape of the fetus and amnionic sac of both the control and experimental samples were similar. Extraembryonic tissue is a multifunctional organ developed from both fetal and maternal origins. During the pregnancy, it provides nutrition, gas exchange, waste removal, and endocrine and immune support for the developing fetus (6). This abnormally small extraembryonic tissue from SCNT embryos indicates that one of the main reasons for the low birth rate of cloned animals is due to the defective development of extraembryonic tissue.

**Proteomic Analysis of the SCNT Extraembryonic Tissue and Identifications of Up- and Down-regulated Proteins**—To investigate differences between the normal and SCNT extraembryonic tissues at the molecular level, we performed proteomic analysis (Fig. 2). Among ~2000 spots in the two-dimensional gel, 39 protein spots were changed at least 2-fold in their intensities between the control and SCNT extraembryonic tissues. After protein identifications with mass spectrometry and protein database search, 12 up-regulated and 27 down-regulated proteins were found in the SCNT extraembryonic tissue. They are listed in Tables I and II.

Lamins A and B1; Annexins A1, A2, and A4; voltage-dependent anion channel, and Hsp27 proteins were up-regulated in the SCNT extraembryonic tissue. Lamin is a nuclear
envelop protein and is cleaved by the interleukin-converting enzyme family during apoptosis (29). Annexins, structural proteins exhibiting Ca
$^{2+}$-dependent binding activity to phospholipids, are known biomarkers for apoptosis (30). Voltage-dependent anion channel is a major component of the permeability transition pore complex of the mitochondrial megachannel and is associated with members of the Bcl-2 family (31). Hsp27 is an apoptotic regulator that interacts with the key components of apoptotic signaling (32). These up-regulated proteins indicate that apoptosis occurred in the SCNT extraembryonic tissue as shown in Fig. 1.

Glyceraldehyde-3-phosphate dehydrogenase, enolase, and lactate dehydrogenase, enzymes functioning in anaerobic glucose metabolism, were down-regulated in the SCNT extraembryonic tissue (Table II). Placenta of rodents and large mammals including humans is a glucose-dependent tissue with limited mitochondrial respiration and mainly anaerobic conversion of glucose to lactate (33). Glyceraldehyde-3-phosphate dehydrogenase converts glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate by the reduction of NAD$^{+}$ to NADH. Enolase catalyzes 2-phosphoglycerate to phosphoenolpyruvate, and lactate dehydrogenase catalyzes the conversion of pyruvate to lactate. These down-regulations may be because of the size reduction of the SCNT extraembryonic tissue.

Other down-regulated proteins were transferrin and apolipoprotein E (Table II). Transferrin is a main component of iron transport and metabolism and has a cytoprotective role. In the mouse model, transferrin inhibits Fas-mediated hepatocyte death and liver failure (34). Apolipoprotein is a main protein moiety of circulating high density lipoproteins and protects vascular endothelial cells from apoptosis (35). These down-regulated proteins also indicate that apoptosis occurred in the SCNT extraembryonic tissue.

Classification of the Regulated Proteins and Down-regulated Antioxidant Enzymes—Thirty-nine regulated proteins were classified by their molecular functions (Fig. 3A). The major category is the oxidoreductase activity category in which one up-regulated and nine down-regulated proteins were classified. Peroxiredoxin 4 is one of the down-regulated proteins (Table II). Exogenous and endogenous oxidative stresses generate ROS that damage the macromolecules and cause apoptosis (36). ROS are degraded by superoxide dismutase, peroxiredoxin, glutathione peroxidase (GPx), and catalase, which convert H$_2$O$_2$ to O$_2$ and H$_2$O. In the Western blot analysis, catalase and GPx proteins were down-regulated in the SCNT extraembryonic tissue (Fig. 3B and C). These proteomic and Western blot data suggest that ROS accumulated in the SCNT extraembryonic tissue and may cause apoptosis.
Up-regulated Hsp27 and Apoptotic Marker Proteins in the SCNT Extraembryonic Tissue—In the 2-DE analysis, one of the up-regulated proteins in the SCNT extraembryonic tissue was Hsp27 protein. It was confirmed by the Western blot analysis in which Hsp27 protein from the SCNT extraembryonic tissue was up-regulated 2.5 times more than the controls (Fig. 4). This result also suggests that apoptosis occurred in the SCNT extraembryonic tissue because up-regulated Hsp27 induces apoptosis through the activating Caspase cascade (37). In Fig. 4, two sets of samples show the same Hsp27 up-regulations, indicating that these up-regulations are not sample-specific.

To study apoptosis in the SCNT extraembryonic tissue, expression of apoptotic marker proteins, including Bcl-2, Caspasas, and PARP, was examined by Western blot analysis (Fig. 5A). Caspase 3 enzymatic activity in the SCNT extraembryonic tissue was also examined (Fig. 5B). Antiapoptotic Bcl-2 protein expression was decreased in the SCNT extraembryonic tissue compared with the control. Pro- and active Caspase 8 and active Caspase 3 proteins were up-regulated in the SCNT extraembryonic tissue. In the Caspase 3 enzymatic assay, the SCNT extraembryonic tissue had higher Caspase-3 enzymatic activity than that of the normal control tissue (Fig. 5B). These results demonstrate that apoptosis occurred in the SCNT extraembryonic tissue. In Fig. 5, two sets of samples show the same results of protein regulations, indicating that these results are not sample-specific.

TUNEL Assay in the SCNT Extraembryonic Tissue—To investigate apoptosis in vivo, TUNEL assay was performed with the placenta from the normal and SCNT extraembryonic tissues. A very small portion of the cells was TUNEL-positive in the normal placenta (Fig. 5C), whereas a large portion of the cells was TUNEL-positive in the SCNT placenta (Fig. 5D) (yellow and green colored cells). The apoptotic cells in the SCNT placenta were cytrophoblasts, which play an important role during the pregnancy for the growth and development of placenta (28). This result also demonstrates that apoptosis occurred in the SCNT extraembryonic tissue.

Conclusion—In the proteomic analysis using the abnormally small extraembryonic tissue on the 26th day of pregnancy from the SCNT embryos, 39 proteins were identified as differentially regulated proteins. Among the up-regulated proteins, Annexins and Hsp27 were found. They are closely related to the processes of apoptosis. Among down-regulated proteins, anaerobic glucose metabolism enzymes were found. These findings may be due to the size reduction of the SCNT extraembryonic tissue that resulted from apoptosis. In the Western blot analysis, antioxidant enzymes were down-regulated, and caspases were up-regulated. This indicates that oxidative stress may be a main cause for inducing apoptosis in the SCNT extraembryonic tissue. Results of TUNEL analysis provide evidence that apoptosis occurred in the SCNT placenta. These results demonstrate that a major reason for the low birth rate in cloned animals is abnormal apoptosis in the extraembryonic tissue during early pregnancy.

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