University of Nebraska - Lincoln

DigitalCommons@University of Nebraska - Lincoln

Publications from USDA-ARS / UNL Faculty

U.S. Department of Agriculture: Agricultural
Research Service, Lincoln, Nebraska

2010

Proteomic analysis of the major cellular proteins of bovine trophoblast cell lines derived from IVP, parthenogenetic and nuclear transfer embryos: Reduced expression of annexins I and II in nuclear transfer-derived cell lines

Neil C. Talbot

United States Department of Agriculture, neil.talbot@ars.usda.gov

Anne M. Powell

United States Department of Agriculture, anne.powell@ars.usda.gov

Thomas J. Caperna

United States Department of Agriculture

Wesley M. Garrett

USDA-ARS, wesley.garrett@ars.usda.gov

Follow this and additional works at: <https://digitalcommons.unl.edu/usdaarsfacpub>

Part of the [Agricultural Science Commons](#)

Talbot, Neil C.; Powell, Anne M.; Caperna, Thomas J.; and Garrett, Wesley M., "Proteomic analysis of the major cellular proteins of bovine trophoblast cell lines derived from IVP, parthenogenetic and nuclear transfer embryos: Reduced expression of annexins I and II in nuclear transfer-derived cell lines" (2010).

Publications from USDA-ARS / UNL Faculty. 679.

<https://digitalcommons.unl.edu/usdaarsfacpub/679>

This Article is brought to you for free and open access by the U.S. Department of Agriculture: Agricultural Research Service, Lincoln, Nebraska at DigitalCommons@University of Nebraska - Lincoln. It has been accepted for inclusion in Publications from USDA-ARS / UNL Faculty by an authorized administrator of DigitalCommons@University of Nebraska - Lincoln.

Proteomic analysis of the major cellular proteins of bovine trophectoderm cell lines derived from IVP, parthenogenetic and nuclear transfer embryos: Reduced expression of annexins I and II in nuclear transfer-derived cell lines

Neil C. Talbot*, Anne M. Powell, Thomas J. Caperna, Wesley M. Garrett

U.S. Department of Agriculture, Agricultural Research Service USDA, ARS, Animal and Natural Resources Institute, Animal Biosciences and Biotechnology Laboratory, Beltsville Agricultural Research Center, Beltsville, MD 20705, United States

ARTICLE INFO

Article history:

Received 30 October 2009

Received in revised form 11 March 2010

Accepted 23 March 2010

Available online 29 March 2010

Keywords:

Bovine

Trophectoderm

Cell

Proteomics

ABSTRACT

Trophectoderm cell lines were established from 8-day *in vitro*-cultured embryos of cattle derived from fertilization (IVF), somatic cell nuclear transfer (NT), or parthenogenetic activation (P) of *in vitro*-matured oocytes and from five 8-day-old *in vivo* (V) embryos. The most abundant cellular proteins of 5 V-, 16 NT-, 12 P-, and 16 IVF-derived cell lines were compared by 2D-gel electrophoresis and mass spectrometry; that is, the unaltered thiourea/urea extract of each cell culture was analyzed. Common protein spots ($n = 118$) were examined, and 95% were identified with significant scores from protein and gene database searches. Of the proteins detected and identified, actin and cytokeratin-8 were found to be the most abundant. Other prominent cellular proteins were metabolic enzymes such as aldose reductase, phosphoglycerate mutase, enolase, triosephosphate isomerase, cytoskeletal interacting proteins transgelin and stratifin, anti-oxidant proteins peroxiredoxin 1 and anti-oxidant protein 2, and the calcium-dependent lipid-binding proteins annexins I and II. In comparative analysis of the 2D-gels, the NT-derived trophectoderm had less annexins I and II in comparison to the IVF- and P-derived trophectoderm. Because annexins I and II are abundant in the placenta and have functions important to the maintenance of placentation, the down-regulation of the annexin genes in the cultured NT trophectoderm may be related to the frequent failures of NT pregnancies.

Published by Elsevier B.V.

1. Introduction

Animal cloning by somatic cell nuclear transfer (NT) was first demonstrated in ungulates (Wilmot et al., 1997; Cibelli et al., 1998) and is a promising technology with the potential for increasing the efficiency of making precise modifications to the mammalian genome. However, as currently practiced 90–98% of NT embryos are lost *in utero*, and

at least 20% of offspring resulting from NT experience lethal developmental abnormalities (De Sousa et al., 2001; Hill et al., 1999, 2000, 2002). Thus, the incidence of pregnancy failure and developmental abnormalities in NT cattle reduces the effectiveness of NT as a means of genetic improvement.

The gene expression of embryos and fetuses produced by normal fertilization with sperm in comparison with embryos produced by NT shows numerous differences (Wrenzycki et al., 2001; Nowak-Imialek et al., 2008; Perecin et al., 2009). Conservation of appropriate DNA methylation patterns appears to be a problem in NT embryos (Bourc'his et al., 2001; Kang et al., 2002; Cezar et al., 2003; Santos et al., 2003). Histones and other DNA accessory proteins which control the form and function of the chro-

* Corresponding author at: USDA, ARS, ANRI, ABBL, Bldg. 200, Rm. 13, BARC-East, Beltsville, MD 20705-2350, United States.
Tel.: +1 301 504 8216; fax: +1 301 504 8414.

E-mail address: neil.talbot@ars.usda.gov (N.C. Talbot).

matin are also changed as a result of NT (Tamada et al., 2006; Laurincik and Maddox-Hyttel, 2007). This incomplete “nuclear reprogramming” that results in the loss of normal epigenetic modifications to the DNA may be the major cause of the developmental failures of NT embryos (Surani, 2001; Humpherys et al., 2001).

Among the most common of the developmental abnormalities that arise from NT reprogramming errors are deficiencies in placentation or placental hyperplasia (Hill et al., 2000; Tanaka et al., 2001). The failure to form a normal placenta may be responsible for many NT pregnancy losses in cattle (Hill et al., 2000; De Sousa et al., 2001; Hashizume et al., 2002; Lee et al., 2004; Patel et al., 2004). The trophoctoderm is the embryonic tissue that mediates placental connections with the uterine epithelium, and it was recently reported that DNA methylation anomalies were specifically localized to this tissue in NT cattle embryos (Kang et al., 2002). Also, changes in the expression of key secretory proteins of trophoctoderm have been found in NT embryos of cattle (Wrenzycki et al., 2001; Talbot et al., 2008). The analysis of trophoctoderm cells might therefore be useful in determining epigenetic deficiencies that lead to placental dysfunction in NT cattle.

Cellular proteins of IVF-, parthenogenote-, NT-, and *in vivo*-derived cattle embryos were compared using trophoctoderm cell lines derived from each type of embryo. The use of cell lines enabled the production of relatively large amounts of embryo-specific and tissue-specific sample protein that would otherwise have been very limited due to the small size of 8-day *in vitro*-cultured cattle embryos. Two-dimensional gel (2D-gel) analysis and mass spectroscopy were applied to determine the profiles of the most abundant proteins in the cultured trophoctoderm cells. Among these, at least two proteins, annexin I and annexin II, were found to be down-regulated in the NT-derived trophoctoderm cell lines. The potential involvement of annexin expression and function in NT pregnancy failure of cattle is discussed.

2. Materials and methods

2.1. Cell culture and reagents

All cells were grown on 25 cm² tissue culture flasks (T25; Greiner, Frickenhausen, Germany). Fetal bovine serum (FBS) was purchased from Hyclone, Logan UT. Cell culture reagents were purchased from InVitrogen, Gaithersburg, MD. Trophoctoderm cells were grown on irradiated STO mouse fibroblast (CRL 1503, American Type Culture Collection, Rockville, MD) feeder cell layers. Feeder-layers were prepared by exposing a suspension of STO cells to 8 Krad of gamma radiation and plating the cells at 6×10^4 cells/cm². STO feeder-layers were maintained by refeeding every 6–7 days with DMEM supplemented with 4 mM L-glutamine and 10% FBS (10% DMEM). Cultures were routinely maintained at 37 °C and in a 5% CO₂ atmosphere.

2.2. Trophoctoderm cell line establishment

In vitro-produced (IVP) blastocysts of cattle derived from either *in vitro* maturation, fertilization, and culture

(IVF), or somatic cell nuclear transfer (NT), or parthenogenetic activation (P) were produced from oocytes obtained from slaughter house ovaries as previously described (Powell et al., 2004; Talbot et al., 2007a, 2008). Blastocysts of good morphological quality were used to establish trophoctoderm cell lines. Briefly, *in vivo* blastocyst-derived cell lines were established from 8-day “hatched” blastocysts with clearly visible ICMs, obtained after artificial insemination (AI) as previously described in Talbot et al., 2007a. For the AI, sperm collected from a F1 transgenic Jersey bull was used (Powell et al., 2004; Talbot et al., 2007a). For NT, IVF, and P cell lines, the blastocysts used were from 8 to 11 days of culture, with the majority being from 8 to 9 days of culture. IVF blastocysts had usually “hatched” from their zona pellucida. The zona pellucida of parthenogenetic embryos was rarely ruptured by 8 or 9 days of culture so the trophoctoderm cell lines were usually started from expanded blastocysts with a very thin zona pellucida. Most of the NT blastocysts had protruded through the slit in the zona pellucida made by the enucleation micropipette, and they therefore formed a typical dumbbell-shaped “hatching” blastocyst. NT blastocysts were produced from two bovine fetal fibroblast cell lines as the nuclear donor source, and two of the three GFP expressing NT trophoctoderm cell lines were colony-cloned 2 times in order to produce a population of cells that were 100% GFP expressing (see Table 1). To establish blastocyst-derived bovine trophoctoderm cell lines, whole blastocysts were culture on STO feeder cells for 2–3 weeks and primary trophoctoderm outgrowths were identified and isolated as previously described (Talbot et al., 2000, 2007a, 2008). Primary trophoctoderm colonies were passaged to T25 flasks of STO feeder cells by physical disaggregation and were similarly passaged for 3–12 more times until established as cell lines, i.e., showed uniform growth (Talbot et al., 2000). The bovine trophoctoderm cell lines were designated according to the source blastocysts, i.e., IVF blastocyst-derived cell lines were designated, FT-, NT-derived cell lines, NT-, P-derived cell lines, PT-, and *in vivo* blastocyst-derived cell lines, VIVOT.

2.3. Two-dimensional gel electrophoresis of cellular proteins

Cellular proteins were extracted from a single T25 tissue culture flask where the cells had reached 75–100% confluency. Generally the period of time to reach this level of confluency was 1–2 weeks post-passage, but some cultures, particularly at earlier passage levels, were grown for up to 3 weeks before harvesting for protein. The culture medium was removed by aspiration and flasks were washed 4 times with ice cold 0.25 M sorbitol solution (4–5 ml). The final sorbitol wash was drained well and thoroughly aspirated, and the flasks were immediately frozen and stored at –70 °C. Flasks were thawed and 1 ml of lysis buffer (7 M urea, 2 M thiourea, 50 mM DTT, 4% CHAPS, 0.5% Bio-Lytes) was added to each flask which was incubated on ice for 20 min. Cells were scraped and disrupted by sonication. Insoluble material was removed by centrifugation at $\sim 12,000 \times g$ for 20 min at 22 °C and the cell lysates were frozen at –80 °C. The first dimension

Table 1

Biological origins of nuclear transfer-derived trophectoderm cell lines.

Cell line name	Nuclear donor cell line	^a Oocyte source	Passage tested	Comments
NT2-5	BFF-10	BOMED	P2	2005; ^b 0.5% FBS donor cell; ^c G1/G2 egg culture/low O ₂
NT2-11	BFF-10	BOMED	P2	2005; ^b 0.5% FBS donor cell; ^c G1/G2 egg culture/low O ₂
NT2-16	BFF-10	BOMED	P3	2005; ^b 0.5% FBS donor cell; ^c G1/G2 egg culture/low O ₂
NT2-20	BFF-10	BOMED	P2	2005; ^b 0.5% FBS donor cell; ^c G1/G2 egg culture/low O ₂
NT6-169	BFF-10	BOMED	P2	2005; ^b 0.5% FBS donor cell; ^c G1/G2 egg culture/low O ₂
NT6-170	BFF-10	BOMED	P2	2005; ^b 0.5% FBS donor cell; ^c G1/G2 egg culture/low O ₂
NT6-173	BFF-10	BOMED	P7	2005; ^b 0.5% FBS donor cell; ^c G1/G2 egg culture/low O ₂
NT6-175	BFF-10	BOMED	P8	2005; ^b 0.5% FBS donor cell; ^c G1/G2 egg culture/low O ₂
NT6-176	BFF-10	BOMED	P2	2005; ^b 0.5% FBS donor cell; ^c G1/G2 egg culture/low O ₂
NT6-181	BFF-10	BOMED	P6	2005; ^b 0.5% FBS donor cell; ^c G1/G2 egg culture/low O ₂
NT30-6	BFF-10	MOPAC	P20	2003; ^b 0.5% FBS donor cell; ^c G1/G2 egg culture/low O ₂
NT30-8	BFF-10	MOPAC	P5	2003; ^b 0.5% FBS donor cell; ^c G1/G2 egg culture/low O ₂
NT30-11	BFF-10	MOPAC	P5	2003; ^d 10% FBS donor cell; ^c G1/G2 egg culture/low O ₂
GFP-CT-2	BFF-8	MOPAC	P14	1998; ^c CR1aa medium egg culture in ambient O ₂ ; cell line not colony-cloned
GFP-CT-3A1	BFF-8	MOPAC	P8	1998; ^c CR1aa medium egg culture in ambient O ₂ ; cell line colony-cloned 2X
GFP-CT4-A1	BFF-8	MOPAC	P25	1998; ^c CR1aa medium egg culture in ambient O ₂ ; cell line colony-cloned 2X

^a Slaughterhouse ovaries from Bomed Inc., Madison, WI (oocytes matured in transit) or Moyer Packing Co., Philadelphia, PA (oocytes matured in-house).^b Nuclear donor cells cultured in medium with 0.5% FBS ("serum-starved") prior to NT.^c See Powell et al. (2004) and Talbot et al. (2007a).^d Nuclear donor cells cultured in medium with 10% FBS prior to NT.

isoelectric focusing was performed using 7 cm IPG strips (pH 3–10, Bio-Rad) in the IPGphor II system (GE Healthcare, Piscataway, NJ). All IPG strips were rehydrated in the presence of approximately ~225 µg sample protein in a total volume 125 µl rehydration buffer; additional rehydration buffer contained the following: 8 M urea, 4% CHAPS, 50 mM DTT, 0.5% Bio-Lytes, 0.001% bromophenol blue. The strips were passively rehydrated for ~14 h at rt and voltage settings for isoelectric focusing were 500 V for 30 min, 1000 V for 30 min, and 8000 V maximum to a total of approximately 7000 kVh. Second dimension separations were done as previously described (Talbot et al., 2007b) on 10% polyacrylamide gels (8 × 10 cm). The proteins in the gel were visualized by staining with Colloidal Coomassie Blue G-250 (Gradipure®; Life Therapeutics, Frenchs Forest, Australia) and the gel was scanned using laser densitometry (PDSI, GE Healthcare, Piscataway, NJ). Gels were stored in a 20% ammonium sulfate solution in seal-o-matic plastic bags (KAPAK, Minneapolis, MN). Gel images were processed using 2D Elite analytical software (GE Healthcare) to enumerate and define individual spots of interest. Spot volumes were determined using local background for subtraction. All spot volume and relative spot volume values (relative to aldose reductase or relative to phosphoglycerate mutase) were analyzed by GLM ANOVA (NCSS 2000, Kaysville, UT) and significant differences were separated by Fisher's LSD multiple-comparison test. In each graph (Fig. 6), mean values with different letters are different ($P < 0.05$). Error bars are the standard error of the means.

2.4. MALDI-TOF-MS analysis of tryptic peptides

A Voyager DE-STR MALDI-TOF mass spectrometer (Applied Biosystems, Framingham, MA) operated in positive ion reflector mode was used to analyze tryptic peptides. Samples were co-crystallized with α -cyanohydroxycinnamic acid (CHCA) matrix, and spectra were acquired with 50 shots of a 337 nm Nitrogen Laser

operating at 20 Hz. Spectra were calibrated using the trypsin autolysis peaks at m/z 842.51 and 2211.10 as internal standards.

2.5. MS/MS analysis of tryptic peptides

A Thermo Finnigan LCQ Deca XP plus Ion Trap mass spectrometer was used to analyze proteins that were not positively identified by MALDI-TOF mass spectrometry. Peptides were separated on a reverse phase column using a 30 min gradient of 5–40% acetonitrile:water containing 0.1% formic acid. Following a survey scan from 300 to 2000 Da, MS/MS spectra were acquired on the three most abundant ions identified in the survey scan. Dynamic exclusion was employed to prevent the continuous analysis of the same ions. The raw data was processed by Sequest to generate DTA files for database searching. The merge.pl script from Matrix science was used to convert multiple Sequest DTA files in to a single mascot generic file suitable for searching in Mascot.

Protein identification was performed by searching the NCBI non-redundant database using the Mascot search engine (<http://www.matrixscience.com>), which uses a probability based scoring algorithm. The following variables were used for database searches with MALDI-TOF peptide mass fingerprinting data: monoisotopic mass, 25 ppm mass accuracy, trypsin as digesting enzyme with 1 missed cleavage allowed, oxidation of methionine, N-terminal pyroglutamic acid from glutamic acid or glutamine as allowable variable modifications. For database searches with MS/MS spectra the following parameters were used, average mass, 1.5 Da peptide and MS/MS mass tolerance, peptide charge of +1, +2, or +3, trypsin as digesting enzyme with 1 missed cleavage allowed, oxidation of methionine, N-terminal pyroglutamic acid from glutamic acid or glutamine as allowable variable modifications. Taxonomy was limited to mammals for both MALDI and MS/MS ion searches. For MALDI-TOF-MS data to qualify as

Fig. 1. Examples of trophoblast of cattle in primary culture and established cell lines. (A) IVF bovine blastocyst's primary trophoblast outgrowth after 10 days of culture on STO feeder cells. Magnification $\approx 35\times$. Arrows indicate borders of the primary colony. (B) Bovine trophoblast cell line (VIVOT-199) growing in a T25 flask on STO feeder cells at passage 15. Arrow indicates individual colony among many that form after each physical disaggregation passage. Magnification $\approx 20\times$. (C) Phase-contrast micrograph of bovine trophoblast cells of the *in vivo*-derived VIVOT-199 cell line at $300\times$ magnification. Note dome formation lower right (arrows).

a positive identification, a protein's score had to equal or exceed the minimum significant score. Positive identification of proteins by MS/MS analysis required a minimum of two unique peptides, with at least one peptide having a significant ion score.

3. Results

Fig. 1 shows the typical appearance of primary bovine trophoblast cultures and cell lines at low magnification, and of the trophoblast cells at high magnification. As previously shown, IVF- and NT-derived primary outgrowths occur at a similar success rate, P-derived primary outgrowths occur at a lower rate (Talbot et al., 2008).

Fig. 2. 2D-gels of the PT28-2 parthenogenetic trophoblast cell line cellular proteins and of STO feeder cell cellular proteins. Annexin I, annexin II, aldose reductase, actin, and cytokeratin 8 are indicated in the PT28-2 gels. Loading was more than 6-fold that present in trophoblast cell line samples for the STO feeder cell sample.

Table 2

Trophoblast abundant cellular proteins of cattle identified by MALDI-TOF and LC-MS/MS.

Spot ID #	MW	PI	Protein ID	^a Peptides matched	^b Sequence coverage	^c Mowse score	^d Expected value	NCBI	Method
31	41,664	5.15	Chain A, beta-actin–profilin complex [<i>Bos taurus</i>]	9	29%	89	0.00068	gi 2624850	MALDI
33	47,266	8.6	Creatine kinase, mitochondrial 1 (ubiquitous) [<i>Bos taurus</i>]	15	29%	119	6.10E–07	gi 73587293	MALDI
34	35,791	8.67	Heterogeneous nuclear ribonucleoproteins A2/B1 (hnRNP A2/hnRNP B1) isoform 7 [<i>Bos taurus</i>]	13	46%	111	3.90E–06	gi 76662749	MALDI
35	27,686	8.87	Galectin-3 (galactose-specific lectin 3) [<i>Bos taurus</i>]	8	28%	111	3.90E–06	gi 76668658	MALDI
36	18,730	8.22	Cofilin 1 (non-muscle) [<i>Homo sapiens</i>]	3	19%	203		gi 15147369	MS/MS
37	53,552	5.7	Keratin 8 [<i>Bos taurus</i>]	29	50%	307	1.00E–25	gi 75812916	MALDI
38	47,639	6.37	Enolase 1 [<i>Bos taurus</i>]	11	27%	132	3.30E–08	gi 87196501	MALDI
39	27,946	4.65	Stratifyn [<i>Ovis aries</i>]	11	37%	78	0.0051	gi 3249019	MALDI
40	36,319	5.76	Aldose reductase (EC 1.1.1.21) [<i>Bos taurus</i>]	8	21%	312		gi 113594	MS/MS
41	38,873	6.92	Annexin A2 [<i>Bos taurus</i>]	23	58%	233	1.40E–18	gi 27807289	MALDI
44	39,411	8.45	(Muscle-type aldolase) isoform 2 [<i>Bos taurus</i>]	11	35%	107	1.00E–05	gi 76653611	MALDI
45	35,714	8.52	Glyceraldehyde-3-phosphate dehydrogenase [<i>Bos taurus</i>]	6	20%	62	0.03	G3P.BOVIN	MALDI
47	38,927	6.37	Annexin I [<i>Bos taurus</i>]	12	35%	144	2.10E–09	gi 73853762	MALDI
48	29,096	6.41	Carbonic anhydrase II [<i>Bos taurus</i>]	9	37%	116	1.30E–06	gi 30466252	MALDI
52	22,412	8.39	Transgelin 2 [<i>Bos taurus</i>]	11	49%	133	2.50E–08	gi 61888874	MALDI
53	58,792	5.09	Keratin 10 [<i>Homo sapiens</i>]	15	29%	81	0.0039	gi 40354192	MALDI
54	17,727	8.37	Peptidyl-prolyl cis-trans isomerase A (EC 5.2.1.8) (PPIase A) (rotamase A) (cyclophilin A) (cyclosporin A-binding protein) [<i>Bos taurus</i>]	7	44%	108	7.50E–07	PPIA.BOVIN	MALDI
56	28,802	6.67	Phosphoglycerate mutase 1 (brain) [<i>Homo sapiens</i>]	12	50%	79	0.0062	gi 38566176	MALDI
57	26,673	6.45	Triosephosphate isomerase [<i>Bos taurus</i>]	11	44%	80	0.0048	gi 61888856	MALDI
58	25,051	6	Anti-oxidant protein 2 (non-selenium glutathione peroxidase, acidic calcium-independent phospholipase A2) [<i>Bos taurus</i>]	10	45%	99	6.10E–05	gi 27807167	MALDI
59	22,665	5.77	Heat shock 27 kDa protein 1 [<i>Bos taurus</i>]	14	54%	199	6.40E–15	gi 71037405	MALDI
60	22,195	8.59	Peroxisomal protein 1 [<i>Bos taurus</i>]	13	40%	188	8.00E–14	gi 66773956	MALDI
75	67,863	7.56	Transketolase [<i>Bos taurus</i>]	12	20%	127	1.10E–07	gi 51491841	MALDI
76	54,939	6.51	Protein disulfide-isomerase (EC 5.3.4.1) ER60 precursor [<i>Bos taurus</i>]	26	50%	276	1.40E–22	gi 1083063	MALDI
77	42,369	5.13	Cytokeratin 8 (370 AA) [<i>Bos taurus</i>]	28	70%	318	8.80E–27	gi 481	MALDI

Table 2 (Continued)

Spot ID #	MW	PI	Protein ID	^a Peptides matched	^b Sequence coverage	^c Mowse score	^d Expected value	NCBI	Method
78	47,736	4.7	Tubulin, beta polypeptide [<i>Homo sapiens</i>]	17	35%	129	7.00E–08	gi 57209813	MALDI
78	51,397	4.97	Chain E, bovine F1-ATPase [<i>Bos taurus</i>]	15	44%	108	8.80E–06	gi 3660252	MALDI
79	38,852	6.17	Gelsolin-like capping protein [<i>Bos taurus</i>]	9	25%	90	0.00062	gi 30466254	MALDI
80	37,591	5.89	Galactose mutarotase (aldose 1-epimerase) [<i>Bos taurus</i>]	10	34%	80	0.0062	gi 77736588	MALDI
81	43,858	4.92	Cytokeratin 19 [<i>Bos taurus</i>]	24	67%	306	1.40E–25	gi 62751472	MALDI
82	32,846	4.69	Tropomyosin 1-alpha chain isoform 5 [<i>Homo sapiens</i>]	22	47%	175	1.80E–12	gi 27597085	MALDI
83	28,505	4.65	Tropomyosin alpha-4 chain (tropomyosin-4) (platelet beta tropomyosin)	18	43%	168	8.80E–12	gi 20178270	MALDI
84	28,965	6.4	Chain A, carbonic anhydrase II [<i>Bos taurus</i>]	7	32%	104	2.20E–05	gi 46016008	MALDI
85	23,598	6.89	Glutathione-S-transferase pi [<i>Bos taurus</i>]	5	39%	70	0.051	gi 29135329	MALDI
86	20,714	6.62	Phosphatidylethanolamine binding protein [<i>Bos taurus</i>]	8	58%	87	0.0011	gi 4389366	MALDI
87	55,199	8.27	Chain A, mitochondrial F1-atpase [<i>Bos taurus</i>]	15	41%	89	0.00065	gi 1943080	MALDI
88	54,751	6.55	Fascin homolog 1, actin-bundling protein [<i>Bos taurus</i>]	10	27%	111	4.40E–06	gi 78045491	MALDI
89	62,442	6.08	Hypothetical protein LOC617109 [<i>Bos taurus</i>]	28	44%	170	5.60E–12	gi 78369310	MALDI
90	69,278	5.82	Albumin [<i>Bos taurus</i>]	14	22%	111	4.30E–06	gi 30794280	MALDI
91	73,696	5.97	Heat shock 70 kDa protein 9B (mortalin-2) [<i>Bos taurus</i>]	26	47%	206	1.40E–15	gi 77735995	MALDI
93	89,274	5.13	Hypothetical protein LOC507345 [<i>Bos taurus</i>]	35	41%	178	8.60E–13	gi 77735541	MALDI
94	92,369	4.76	Tumor rejection antigen (gp96) 1 [<i>Bos taurus</i>]	34	38%	218	8.60E–17	gi 27807263	MALDI
95	36,764	5.3	Annexin A8 [<i>Bos taurus</i>]	18	52%	214	2.20E–16	gi 27806317	MALDI
96	32,006	5.92	Purine nucleoside phosphorylase [<i>Bos taurus</i>]	11	46%	84	0.0019	gi 61553356	MALDI
97	62,027	5.14	Keratin 9 [<i>Homo sapiens</i>]	16	26%	100	6.10E–05	gi 55956899	MALDI
99	12,3787	5.58	Predicted: similar to vinculin isoform meta-VCL isoform 1 [<i>Bos taurus</i>]	15	17%	72	0.036	gi 119918578	MALDI
100	57,230	4.8	Procollagen-proline, 2-oxoglutarate 4-dioxygenase [<i>Bos taurus</i>]	19	37%	239	6.80E–19	gi 27806501	MALDI
102	37,687	5.43	Cathepsin D {EC 3.4.23.5} [<i>Bos taurus</i>]	11	30%	101	4.40E–05	gi 299522	MALDI
103	35,971	8.67	Heterogenous nuclear ribonucleoprotein A2/B1 [<i>Mus musculus</i>]	17	52%	170	5.50E–12	gi 3329498	MALDI
105	30,821	8.82	Voltage-dependent anion channel 1 [<i>Bos taurus</i>]	14	53%	121	4.40E–07	gi 73853754	MALDI
106	38,327	8.09	GNB2L1 protein [<i>Bos taurus</i>]	5	10%	242		gi 74354951	MS/MS

Table 2 (Continued)

Spot ID #	MW	PI	Protein ID	^a Peptides matched	^b Sequence coverage	^c Mowse score	^d Expected value	NCBI	Method
107	30,329	8	Voltage-dependent anion channel 2 [<i>Homo sapiens</i>]	5	19%	68	0.052	gi 57209199	MALDI
108	35,935	4.86	Annexin V = CaBP37 isoform [<i>Bos taurus</i>]	21	64%	207	1.10E–15	gi 260138	MALDI
109	28,193	5.2	Predicted: similar to cytoplasmic beta-actin isoform 2 [<i>Macaca mulatta</i>]	8	31%	94	0.00023	gi 109119055	MALDI
110	27,696	4.73	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide [<i>Homo sapiens</i>]	14	55%	114	2.20E–06	gi 68085578	MALDI
111	33,716	5.26	Beta-actin [Lama glama]	7	29%	64	0.2	gi 29603621	MALDI
112	31,774	4.84	Laminin-binding protein [<i>Homo sapiens</i>]	4	16%	290		gi 34234	MS/MS
112	30,919	4.71	B23 nucleophosmin (280 AA) [<i>Homo sapiens</i>]	7	15%	259		gi 825671	MS/MS
113	32,356	5.28	Acidic ribosomal phosphoprotein PO [<i>Bos taurus</i>]	10	37%	83	0.003	gi 2293577	MALDI
114	33,720	6.02	Capping protein (actin filament) muscle Z-line, beta [<i>Bos taurus</i>]	6	13%	68	0.087	gi 28603770	MALDI
115	88,14	6.67	Chain B, E-trypsin (E.C.3.4.21.4) [<i>Sus scrofa</i>]	3	46%	44	20	gi 999627	MALDI
116	22,118	6.58	Biliverdin reductase B (flavin reductase (NADPH)) [<i>Bos taurus</i>]	7	43%	78	0.0089	gi 27806297	MALDI
117	69,190	5.88	ALB protein [<i>Bos taurus</i>]	9	14%	61	0.47	gi 74267962	MALDI
118	19,525	4.9	Translationally controlled tumor protein (TCTP)	7	46%	71	0.04	gi 6175056	MALDI
119	19,635	4.71	Myosin: regulatory light chain	8	51%	117	1.10E–06	gi 228542	MALDI
121	23,196	8.62	Peroxisomal protein 5 precursor [<i>Bos taurus</i>]	7	34%	72	0.036	gi 27807445	MALDI
122	17,127	6.13	Ubiquitin-conjugating enzyme E2N	2	11%	98		gi 4507793	MS/MS
125	68,717	6.06	Villin 2 [<i>Bos taurus</i>]	28	41%	217	1.10E–16	gi 27806351	MALDI
126	86,014	5.76	Gelsolin	5	5%	161		gi 51854227	MS/MS
127	60,957	5.78	Similar to 60 kDa heat shock protein, mitochondrial precursor (Hsp60) (60 kDa chaperonin) (CPN60) (heat shock protein 60) (HSP-60)	15	31%	101	4.40E–05	gi 74005078	MALDI
127	58,423	7.96	Similar to LOC512571 protein [<i>Bos taurus</i>]	12	29%	75	0.019	gi 119902010	MALDI
128	49,978	7.01	Fumarate hydratase, mitochondrial (fumarase)	2	4%	111		gi 120604	MS/MS
129	32,030	7	Asparaginase-like 1 protein [<i>Bos taurus</i>]	5	14%	298		gi 116004289	MS/MS
131	41,144	6.46	Acetyl-coenzyme A acetyltransferase 2 [<i>Bos taurus</i>]	5	12%	206		gi 109659291	MS/MS
132	36,574	8.12	Lactate dehydrogenase A [<i>Bos taurus</i>]	13	36%	138	9.10E–09	gi 27806559	MALDI
133	23,407	5.12	Rho GDP dissociation inhibitor (GDI) alpha [<i>Bos taurus</i>]	9	43%	106	1.40E–05	gi 28603774	MALDI

Table 2 (Continued)

Spot ID #	MW	PI	Protein ID	^a Peptides matched	^b Sequence coverage	^c Mowse score	^d Expected value	NCBI	Method
135	95,307	6.41	Eukaryotic translation elongation factor 2 [<i>Bos taurus</i>]	16	18%	129	7.20E–08	gi 115497900	MALDI
136	60,301	5.71	T-complex protein 1 subunit alpha (TCP-1-alpha) (CCT-alpha) (65 kDa antigen) Chinese hamster	12	22%	94	0.00024	gi 135536	MALDI
137	61,389	8.62	Pyruvate kinase [<i>Bos taurus</i>]	10	21%	85	0.0016	gi 73587283	MALDI
139	60,863	6.05	Chain A, crystal structure of bovine Hsc70(Aa1-554)e213aD214A mutant	14	27%	111	4.50E–06	gi 78101017	MALDI
140	43,702	6.09	Chain A, mitochondrial Ef-Tu	14	38%	156	1.40E–10	gi 6137414	MALDI
142	50,456	5.94	GDP dissociation inhibitor 2 [<i>Bos taurus</i>]	16	35%	155	1.80E–10	gi 76253900	MALDI
143	48,544	5.59	Proteasome (prosome, macropain) 26S subunit, ATPase 2 [<i>Rattus norvegicus</i>]	13	28%	80	0.0059	gi 15100181	MALDI
144	47,283	5.71	ARP3 actin-related protein 3 homolog (yeast) [<i>Bos taurus</i>]	10	21%	109	7.20E–06	gi 115304856	MALDI
146	46,353	4.31	Calreticulin, brain isoform 1 [<i>Bos taurus</i>]	11	15%	103	4.31	gi 631545	MALDI
148	57,422	6.01	Chaperonin containing TCP1, subunit 2 (beta) [<i>Rattus norvegicus</i>]	14	35%	114	2.30E–06	gi 54400730	MALDI
149	50,122	6.02	Predicted: similar to TIP49 [<i>Bos taurus</i>]	10	30%	79	0.0076	gi 119914925	MALDI
153	53,655	5.06	Vimentin [<i>Mus musculus</i>]	31	64%	333	2.90E–28	gi 31982755	MALDI
155	36,594	6.8	Aldo-keto reductase family 1, member A1 (aldehyde reductase) [<i>Bos taurus</i>]	9	27%	108	9.10E–06	gi 115495641	MALDI
159	43,261	7.23	Psmc6 protein [<i>Rattus norvegicus</i>]	12	37%	120	5.70E–07	gi 81294202	MALDI
160	38,075	7.14	Sorbitol dehydrogenase [<i>Bos taurus</i>]	6	24%	70	0.059	gi 82617550	MALDI
162	46,729	6.13	Isocitrate dehydrogenase 1 (NADP+), soluble [<i>Bos taurus</i>]	18	49%	148	9.10E–10	gi 75832090	MALDI
163	41,801	5.21	Chain A, beta-actin–profilin complex	10	32%	71	0.034	gi 576368	MALDI
166	29,802	5.57	Prohibitin [<i>Mus musculus</i>]	8	36%	82	0.0038	gi 6679299	MALDI
171	59,707	8.22	Chain A, structure of human muscle pyruvate kinase (Pkm2)	11	20%	77	0.012	gi 67464392	MALDI
172	58,423	7.96	Predicted: similar to LOC512571 protein [<i>Bos taurus</i>]	15	34%	98	8.70E–05	gi 119902010	MALDI
173	55,728	6.87	IMP (inosine monophosphate) dehydrogenase 2 [<i>Bos taurus</i>]	15	31%	120	5.70E–07	gi 77735779	MALDI
175	35,509	9.77	TSH receptor suppressor element-binding protein-1; TSEP-1 [<i>Rattus sp.</i>]	11	31%	71	0.031	gi 9653686	MALDI

Table 2 (Continued)

Spot ID #	MW	PI	Protein ID	^a Peptides matched	^b Sequence coverage	^c Mowse score	^d Expected value	NCBI	Method
178	38,214	6.13	Predicted: similar to Glycerol-3-phosphate dehydrogenase 1-like [<i>Bos taurus</i>]	10	27%	92	0.00034	gi 119914253	MALDI
180	35,749	5.41	Chain A, crystal structure of phosphorylation-mimicking mutant T6d Of annexin IV	14	42%	128	9.10E–08	gi 14278334	MALDI
181	17,582	4.74	Predicted: similar to tropomyosin 3 isoform 2 isoform 3 [<i>Canis familiaris</i>]	14	52%	156	1.40E–10	gi 73961067	MALDI
216	17,351	5.92	PRDX5 protein [<i>Bos taurus</i>]	9	50%	132	3.60E–08	gi 74354725	MALDI
218	16,220	5.87	Actin-related protein 2/3 complex, subunit 5, 16 kDa [<i>Bos taurus</i>]	7	51%	88	0.00093	gi 78369476	MALDI

^a Peptides matched refers to the number of peptides identified from the primary sequence.

^b Sequence coverage refers to the percentage of the protein sequence represented by the matched peptides.

^c MOWSE score is the absolute probability that the observed match is a random event expressed as $-10 \times \log_{10}(P)$, where P is the absolute probability (Pappin et al., 1993).

^d The number of times one would expect to obtain an equal or higher score, purely by chance; analogous to P -value.

Fig. 3. Representative 2D-gel of trophectoderm cellular proteins from cell line NT30-11 with enumeration of the protein spots listed in Table 2.

Fig. 4. Replicate 2D-gels runs ($n=4$) of the cellular proteins from cell line NT2-16 at two passage levels and from frozen/thawed samples or unfrozen samples.

There was no difference noted in the success rate of cell line establishment from any of the sources of blastocysts, except that the *in vivo*-derived blastocysts produced cell lines with 100% efficiency, well above that of the IVP blastocysts (Talbot et al., 2007a). However, the number of *in vivo* blastocysts obtained was small in comparison to the number of IVP blastocysts (Talbot et al., 2007a). In total, 16 NT-, 12 P-, 16 IVF- and 5 *in vivo*-derived cell lines were compared by 2D-gel electrophoresis in the study. Details of the origins of the NT cell lines are briefly summarized in Table 1.

The proteins from the untreated, non-fractionated samples of each trophoblast cell line, harvested from 1 T25 flask each, were separated by 2D-gel electrophoresis, e.g., Fig. 2, and the protein spots were analyzed and identified by mass spectroscopy (Table 2; Fig. 3). Fig. 2 shows the 2D-gel protein profile of PT28-2, a parthenogenetic blastocyst-derived trophoblast cell line, and it is typical of all the cell lines. Because no more than 225 μ g of total protein could be loaded on a 7-cm IPG strip without hampering the electrophoretic separation of the proteins (Table 2), is a compellation of the most abundant trophoblast proteins that have been identified to date. Replication of 2D-gel separations was observed in four independent gel runs from two independent sample preparations of the NT2-16 cell line (Fig. 4). Fig. 4 shows a close-up view of the same area of the gels containing three principle spots; annexin I (spot # 47), annexin II (spot # 41), and aldose reductase (spot # 40). STO feeder cell protein was a minor component of the total protein harvested from each T25 flask of the trophoblast cell lines. That STO feeder cell proteins are a minor proportion of the visible protein spots is apparent from Fig. 2 which shows a 2D-gel where more

than 6 times as much sample from an STO feeder cell-only T25 flask was electrophoretically separated (based on equivalent trophoblast cell line sample volume). Also, data base species identification was often specific for cattle but not cells of mice (STO cells are mouse fibroblasts, Table 2). Extensive visual inspections of the gels were conducted to see if any of the cellular proteins varied according to the type of blastocyst the cell lines originated from and this was confirmed by densitometry and 2D-gel-spot software analysis. The comparison of the gels indicated two proteins, identified as annexin I (spot # 47) and annexin II (spot # 41), were different when comparing FT and P cell lines to NT cell lines. A side-by-side comparison of the portion of the gel containing the annexin I, annexin II, and aldose reductase proteins from the 16 NT-, 16 IVF-, 12 P-, and 5 *in vivo*-derived cell lines (VIVOT-) is shown in Fig. 5. Annexin I content is clearly reduced in, or absent from, the NT-derived cell lines in comparison to the IVF-, parthenogenetic-, and *in vivo*-derived cell lines (designated FT, PT, and VIVOT, respectively). Annexin II protein spots were also reduced in the NT-derived cell lines although this was not as apparent from visual inspection. Densitometry measurements of the three proteins from all the gels verified that both annexins I and II were reduced ($P \leq 0.05$) in the NT trophoblast cell lines when compared to the internal less variant control proteins, aldose reductase and phosphoglycerate mutase (Fig. 6). The absolute values of annexins I and II were not different ($P \leq 0.05$) between NT- and *in vivo*-derived cell lines probably because of the small sample size of VIVOT cell lines ($n=5$). However, by visual inspection, 4 out of the 5 VIVOT cell lines had annexin I, and 5 out of 5 had annexin II (Fig. 5). A difference ($P \leq 0.05$) was found for annexin I when normalized to aldose reductase or phosphoglycerate mutase, two less variant proteins (Fig. 6).

4. Discussion

Modulation of annexins, and also of S100 protein family members that interact with some annexins, has been observed in proteomic surveys of cow and pig NT placentas and human placenta. Kim et al., 2005, found down-regulation of annexin I in term NT placentas of cattle when compared to normal control placentas. In 26-day pig NT extraembryonic tissue, annexins I, II, and IV were up-regulated in comparison to the extraembryonic tissue from fetuses of normally bred sows (Chae et al., 2006). In term pig NT placenta, proteomic analysis again showed up-regulation of annexins I and IV (V and VIII, also) compared to normal controls, but annexin II was down-regulated (Lee et al., 2007). A few proteomic studies have investigated human placental tissues, either from *in vivo* samples, or *in vitro* samples from primary tissue cultures of cytotrophoblast cells as a model of trophoblast differentiation and function. In an *in vitro* differential oxygen model of cytotrophoblast differentiation and invasion, Hoang et al. (2001), found that the amount of annexin II in the cells increased under hypoxic conditions. In another *in vitro* study of human cytotrophoblast cells involving the modeling of the role of neurokinin-B (NKB) in preeclampsia, annexin II and the annexin I partner protein, S100A11

Fig. 5. Side-by-side comparison of that portion of all the gels containing annexins I and II, and aldose reductase. NT cell lines designated GFP-CT were from blastocysts expressing the GFP gene.

Fig. 6. Comparison of the means of densitometry measurements of the proteins spots of annexins I and II, or when normalized to the densitometry of aldose reductase and phosphoglycerate mutase, in 2D-gels of the proteins of NT-, FT-, PT-, and *in vivo*-derived (INV) bovine trophoblast cell lines.

(calgizzarin; Streicher et al., 2009), were both down-regulated in response to NKB treatment (Sawicki et al., 2003). Annexin I binding partner, S100A11 protein, was also found to be down-regulated in the placental villous tissue of women suffering from early loss of pregnancy (Liu et al., 2006). Together, these previous proteomic studies and results from the present research, associate annexins I and

II, and their S100 partner proteins, with pathophysiological conditions or responses in the placenta and with NT placentas.

Although the specific roles of annexins in the physiology of placentation and pregnancy are not well understood to date, various studies draw attention to their involvement in placental function and parturition (Myatt et al., 1992;

Bogic et al., 1999). The location and function of annexins have been studied mainly in human and rodent placenta or in *in vitro* models thereof. The placenta is a rich source of annexins, one study estimating that the annexins make up 2% of the total placental membrane proteins (Buhl et al., 1991), and annexins I and II can be readily purified from placental tissue (Moore et al., 1992). Why placental tissue contains so much and so many annexins is also not understood. At least one annexin family member, annexin V, is important to placental function and integrity, and this appears to be due to its anti-coagulation activity on the surface of the syncytiotrophoblast cells (Wang et al., 1999). Annexins I and II are in greater amounts in the amnion, chorion, and trophoblast cells (Myatt et al., 1992; Kristoffersen and Matre, 1996; Sun et al., 1996; Bogic et al., 1999). Annexin I, a/k/a lipocortin I, is a calcium-dependent phospholipid-binding protein that may stimulate or inhibit phospholipase A2 (PLA₂) and thus influence the production of arachidonic acid and subsequently prostaglandin production in the trophoblast and, possibly, the endometrium through a potential paracrine mode of action (Bennett et al., 1994; Aarli et al., 1997). Prostaglandin synthesis, that can participate in an inflammatory response or in a maternal “recognition of pregnancy” response might, therefore, be influenced by annexin I in the peri-implantation embryo’s trophoblast tissue (Lawrence et al., 2002; Gavins et al., 2005; Meier et al., 2008). Also, annexin II, a/k/a lipocortin II or calpactin I, is involved with PLA₂ activity and prostaglandin synthesis through its interaction with protein S100A10 (Wu et al., 1997; Akiba et al., 2000). Potentially as important, however, is that annexin II in conjunction with the S100A10 protein, has been identified as the receptor for tissue plasminogen activator (tPA), and as such, is involved in extracellular matrix (ECM) remodeling via activation of cell surface bound plasminogen to plasmin, and subsequently activation of matrix metalloproteinases (Hajjar and Hamel, 1990; Bogic et al., 1999). Because ECM remodeling and adjustments of cellular architecture are important in establishing the intimate fetomaternal connection in the placentomes of ruminants, down-regulation of annexin II might lead to placental failure in NT fetuses (Hashizume et al., 2002; Hirata et al., 2003; Hoffert et al., 2005; Pfarrer, 2006). Further, both annexins I and II directly affect the migratory behavior of epithelial cells in several cancer cell models, and down-regulation of these annexins causes a loss of cell migration and “invasiveness”, as measured by *in vitro* assay (Ling et al., 2004; Liu et al., 2003; Babbin et al., 2006). So, again, it is possible that down-regulation of annexins I and II in the trophoblast of NT embryos hampers cotyledonary interaction with the maternal caruncles due to inhibition of trophoblast cell-mediated ECM remodeling and cell migratory processes (Ling et al., 2004; Liu et al., 2003; Babbin et al., 2006).

Further investigations are necessary to confirm the observation of the lesser amounts of annexins I and II in the cultured trophoblast cells of NT embryos. First, because all of the NT trophoblast cell lines in the study were derived from NT embryos created with Jersey cattle fetal fibroblasts (BFF-8 and BFF-10 nuclear donor cells; see Powell et al., 2004 and Talbot et al., 2007a) further comparative examinations are warranted. For example, NT

trophoblast cell lines could be created with nuclear donor cells from other cattle breeds and their protein profiles similarly analyzed by 2D-gel electrophoresis. This might identify if Jersey cattle trophoblast cells are naturally low in annexins I and II—although in the present study the AI-derived trophoblast cell lines were from Holstein cows inseminated with Jersey semen and all five had high levels of annexin II and four of the five had high levels of annexin I. Secondly, the analysis of NT trophoblast cell lines created from NT embryos made with donor nuclei from several more independent fetal fibroblast cell lines needs to be performed. This would clarify the possibility that the down-regulation of annexin I and II genes might only be associated with the use of certain nuclear donor cell lines, or, by extension, certain cell types, e.g., fibroblast compared to epithelial nuclear donor cells. Third, a comparison of annexins I and II in intact IVP embryos, ideally NT and IVF embryos developed *in vivo* to the filamentous stage and in comparison to matched AI embryos, needs to be performed to ascertain that the down-regulated expression of annexins I and II observed in the *in vitro*-cultured trophoblast is also present in the *in vivo* trophoblast of the early peri-implantation embryo.

If the same aberration in annexins I and II levels is observed with *in vivo*-developed trophoblast tissue, regardless of donor cell breed or embryo source, the annexins might serve as markers for monitoring embryo fitness or for monitoring modifications to NT methodology to make NT embryos more fit, i.e., to see if aberrant annexin levels could be changed to the “normal” levels of IVF embryos. Because NT methodology will always be *in vitro*, the protein profile of *in vitro*-produced embryos that establish pregnancies at “state of the art” efficiency, i.e., 40–50%, is the appropriate comparative positive control. However, differences in protein profile that can be elucidated in comparison to *in vivo*-derived embryos or embryonic tissue will always be of value in understanding basic developmental biology and fetomaternal interaction. In any case, embryo transfers of putatively “improved NT embryos” could then test the assumption that NT embryos with IVF-like protein profiles will in fact have greater fitness for survival and development to term. Also tested would be the major corollary to this assumption that the protein profile at the early blastocyst stage of development, prior to embryo transfer, will be a good indicator of successful reprogramming for later stages of development, i.e., will normal early gene expression translate to normal gene expression during all stages of development? The available literature does not answer this question (Hashizume et al., 2002). Abnormal proteins levels in NT embryos may also provide “markers” for the assessment of nuclear reprogramming that can be used for the creation of rapid, non-destructive assays of embryo fitness. Currently there are no real-time procedures for assessing whether a nuclear cloned embryo is properly “reprogrammed” for normal developmental gene expression prior to its transfer into the surrogate mother. Immunocytochemical, anti-viral assay, or ELISA based comparisons of individual NT, IVF, and P embryos might be devised and tested for this purpose from genomic and proteomic data, e.g., Kato et al. (2007) and Talbot et al. (2008). Finally, the proteomic analysis of the trophoblast

toderm cell lines of cattle, as it continues, will develop a database from which comparative analyses can be performed from other related proteomic and genomic studies to broaden the fundamental knowledge of developmental biology and reproductive physiology.

The 2D-gel electrophoresis of the total thiourea/urea extracted cellular proteins of the trophectoderm cell lines resolved protein spots representing the most abundant proteins in the crude cell lysate. To identify the many thousands of proteins that must actually comprise the cell lysates, fractionation of the total cell lysate (or the use of subcellular organelle preparations) must be done to drastically reduce the percentage content of proteins such as actin and cytokeratin-8, which, because of their abundance, preclude loading enough protein on the IPG strips to see the less abundant proteins. From 149 spots selected and analyzed by mass spectroscopy, 104 unique proteins were identified. Some of the protein spots that were analyzed contained mixtures of proteins and will require greater electrophoretic resolution in one or both dimensions to resolve. Also, some were not identifiable in the protein data bases, but the data bases are always improving over time. In any case, many of the proteins were in common with those reported in proteomic analyses of the *in vivo* tissues of ungulate NT placentas and of human placentas or cytotrophoblast cells (Hoang et al., 2001; Sawicki et al., 2003; Kim et al., 2005; Lee et al., 2007; Zhang et al., 2008). As discussed above, annexins I and II were found in the samples of trophectoderm cell lines, but also, annexins V and VIII were found. Proteins found the most frequently represented metabolic/cytosolic enzymes such as lactate dehydrogenase, creatine kinase, aldose reductase, phosphoglycerate mutase, enolase, triosephosphate isomerase, carbonic anhydrase, glyceraldehyde-3-phosphate dehydrogenase, aldehyde reductase, triosephosphate isomerase, and transketolase. The most abundant proteins were cytoskeletal proteins such as actin, tubulin, myosin, cytokeratin-8, -9, -18, and -19, and cytoskeletal interacting proteins such as tropomyosin, profilin, villin, cofilin, gelsolin, transgelin and stratifin. The intermediate filament protein, vimentin, was also found but was species identified as *Mus musculus*, and, therefore, was probably from the STO feeder cell protein component of the cell lysate. Chaperone or stress proteins such as heat shock proteins and TCP1 subunit 2 were found, and anti-oxidant proteins such as peroxiredoxin 1, peroxiredoxin 5, anti-oxidant protein 2, and glutathione-S-transferase were present. Finally, protein-degradation proteins such as proteasome 26S subunit and ubiquitin-conjugating enzyme E2 were represented, a calcium-binding protein, calreticulin, was found, and voltage-dependent anion channel 1 and 2 proteins were found.

In conclusion, the majority of NT pregnancies in cattle are lost around the time of placentation and placental abnormalities are frequently associated with NT pregnancies (De Sousa et al., 2001; Hill et al., 2002; Hashizume et al., 2002; Powell et al., 2004; Chae et al., 2006; Arnold et al., 2006). An 18-day NT embryo–maternal interaction, i.e., during the early implantation stage, had abnormalities in the endometrial tissue that were already measurable in terms of endometrial mRNA responses (Bauersachs et al.,

2009)—so what is the NT conceptus doing or not doing to cause this? Aberrant expression of annexin proteins are probably pertinent to this question since they are abundant in the placenta, specifically at the fetomaternal interface (Myatt et al., 1992; Kristoffersen and Matre, 1996; Bogic et al., 1999), and because annexins, or their biologically active externalized soluble peptide fragments, have effects on so many diverse biological processes, including tissue immunomodulation, apoptosis (annexin V), inhibition of placental blood coagulation (annexin V), mediation of intracellular membrane fusion (annexins I and II and synexin or annexin VII), signal transduction for cell differentiation, extracellular matrix remodeling via activation of plasminogen and, indirectly, matrix metalloproteinases (MMPs), and prostaglandin synthesis regulation via high affinity, Ca^{2+} -dependent, binding of phospholipids (Creutz, 2003; Buckland and Wilton, 2003).

Acknowledgements

We thank Dr. Julie Long for her helpful editorial and scientific comments on the manuscript. We also thank Mr. Paul Graninger for technical assistance in tissue culture, 2D-gel electrophoresis, and preparation of protein samples for MALDI-TOF analysis, Ms. Anne Powell for the production of cattle embryos, and Ms. Amy Shannon for technical assistance in the preparation of protein samples for MALDI-TOF analysis.

Mention of trade name, proprietary product or vendor does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture or imply its approval to the exclusion of other products or vendors that also may be suitable.

References

- Aarli, A., Kristoffersen, E.K., Jensen, T.S., Ulvestad, E., Matre, R., 1997. Suppressive effect on lymphoproliferation *in vitro* by soluble annexin II released from isolated placental membranes. *Am. J. Reprod. Immunol.* 38, 313–319.
- Akiba, S., Hatazawa, R., Ono, K., Hayama, M., Matsui, H., Sato, T., 2000. Transforming growth factor- α stimulates prostaglandin generation through cytosolic phospholipase A(2) under the control of p11 in rat gastric epithelial cells. *Br. J. Pharmacol.* 131, 1004–1010.
- Arnold, D.R., Bordignon, V., Lefebvre, R., Murphy, B.D., Smith, L.C., 2006. Somatic cell nuclear transfer alters peri-implantation trophoblast differentiation in bovine embryos. *Reproduction* 132, 279–290.
- Babbin, B.A., Lee, W.Y., Parkos, C.A., Winfree, L.M., Akyildiz, A., Perretti, M., Nusrat, A., 2006. Annexin I regulates SKCO-15 cell invasion by signaling through formyl peptidoreceptors. *J. Biol. Chem.* 281, 19588–19599.
- Bauersachs, S., Ulbrich, S.E., Zakhartchenko, V., Minten, M., Reichenbach, M., Reichenbach, H.D., Blum, H., Spencer, T.E., Wolf, E., 2009. The endometrium responds differently to cloned versus fertilized embryos. *Proc. Natl. Acad. Sci. U.S.A.* 106, 5681–5686.
- Bennett, P., Slater, D., Berger, L., Moor, G., 1994. The expression of phospholipase A2 and lipocortins (annexins) I, II and V in human fetal membranes and placenta in association with labour. *Prostaglandins* 48, 81–90.
- Bogic, L.V., Ohira, R.H., Yamamoto, S.Y., Okazaki, K.J., Millar, K., Bryant-Greenwood, G.D., 1999. Tissue plasminogen activator and its receptor in the human amnion, chorion, and decidua at preterm and term. *Biol. Reprod.* 60, 1006–1012.
- Bourc'his, D., Le Bourhis, D., Patin, D., Niveleau, A., Comizzoli, P., Renard, J.P., Viegas-Péquignot, 2001. Delayed and incomplete reprogramming of chromosome methylation patterns in bovine cloned embryos. *Curr. Biol.* 11, 1542–1546.
- Buckland, A.G., Wilton, D.C., 2003. Annexins and phospholipases. In: Bandorowicz-Pikula, J. (Ed.), *Annexins*. Kluwer Academic/Plenum, London, pp. 207–217.

- Buhl, W.J., García, M.T., Zipfel, M., Schiebler, W., Gehring, U., 1991. A series of annexins from human placenta and their characterization by use of an endogenous phospholipase A2. *Eur. J. Cell Biol.* 56, 381–390.
- Cezar, G.G., Bartolomei, M.S., Forsberg, E.J., First, N.L., Bishop, M.D., Eilertsen, K.J., 2003. Genome-wide epigenetic alterations in cloned bovine fetuses. *Biol. Reprod.* 68, 1009–1014.
- Chae, J.I., Cho, S.K., Seo, J.W., Yoon, T.S., Lee, K.S., Kim, J.H., Lee, K.K., Han, Y.M., Yu, K., 2006. Proteomic analysis of the extraembryonic tissue from cloned porcine embryos. *Mol. Cell. Proteomics* 5, 1559–1566.
- Cibelli, J.B., Stice, S.L., Golueke, P.J., Kane, J.J., Jerry, J., Blackwell, C., Ponce de León, F.A., Robl, J.M., 1998. Cloned transgenic calves produced from non-quiescent fetal fibroblasts. *Science* 280, 1256–1258.
- Creutz, C.E., 2003. Reflections on twenty-five years of annexin research. In: Bendorowicz-Pikula, J. (Ed.), *Annexins*. Kluwer Academic/Plenum, London, pp. 1–20.
- De Sousa, P.A., King, T., Harkness, L., Young, L.E., Walker, S.K., Wilmut, I., 2001. Evaluation of gestational deficiencies in cloned sheep fetuses and placentae. *Biol. Reprod.* 65, 23–30.
- Gavins, F.N., Sawmynaden, P., Chatterjee, B.E., Perretti, M., 2005. A twist in anti-inflammation: annexin I acts via the lipoxin A4 receptor. *Prostaglandins Leukot. Essent. Fatty Acids* 73, 211–219.
- Hajjar, K.A., Hamel, N.M., 1990. Identification and characterization of human endothelial cell membrane binding sites for tissue plasminogen activator and urokinase. *J. Biol. Chem.* 265, 2908–2916.
- Hashizume, K., Ishiwata, H., Kizaki, K., Yamada, O., Takahashi, T., Imai, K., Patel, O.V., Akagi, S., Shimizu, M., Takahashi, S., Katsuma, S., Shiojima, S., Hirasawa, A., Tsujimoto, G., Todoroki, J., Izaïke, Y., 2002. Implantation and placental development in somatic cell clone recipient cows. *Cloning Stem Cells* 4, 197–209.
- Hill, J.R., Roussel, A.J., Cibelli, J.B., Edwards, J.F., Hooper, N.L., Miller, M.W., Thompson, J.A., Looney, C.R., Westhusin, M.E., Robl, J.M., Stice, S.L., 1999. Clinical and pathologic features of cloned transgenic calves and fetuses (13 case studies). *Theriogenology* 51, 1451–1465.
- Hill, J.R., Burghardt, R.C., Jones, K., Long, C.R., Looney, C.R., Shin, T., Spencer, T.E., Thompson, J.A., Winger, Q.A., Westhusin, M.E., 2000. Evidence for placental abnormality as the major cause of mortality in first-trimester somatic cell cloned bovine fetuses. *Biol. Reprod.* 63, 1787–1794.
- Hill, J.R., Schlafer, D.H., Fisher, P.J., Davies, C.J., 2002. Abnormal expression of trophoblast major histocompatibility complex class I antigens in cloned bovine pregnancies is associated with a pronounced endometrial lymphocytic response. *Biol. Reprod.* 67, 55–63.
- Hirata, M., Sato, T., Tsumagari, M., Shimada, A., Nakano, H., Hashizume, K., Ito, A., 2003. Differential regulation of the expression of matrix metalloproteinases and tissue inhibitors of metalloproteinases by cytokines and growth factors in bovine endometrial stromal cells and trophoblast cell line BT-1 in vitro. *Biol. Reprod.* 68, 1276–1281.
- Hoang, V.M., Foulk, R., Clauser, K., Burlingame, A., Gibson, B.W., Fisher, S.J., 2001. Functional proteomics: examining the effects of hypoxia on the cytotrophoblast protein repertoire. *Biochemistry* 40, 4077–4086.
- Hoffert, K.A., Batchelder, C.A., Bertolini, M., Moyer, A.L., Famula, T.R., Anderson, D.L., Anderson, G.B., 2005. Measures of maternal-fetal interaction in day-30 bovine pregnancies derived from nuclear transfer. *Cloning Stem Cells* 7, 289–305.
- Humpherys, D., Eggan, K., Akutsu, H., Hochedlinger, K., Rideout III, W.M., Binarskiewicz, D., Yanagimachi, R., Janisch, R., 2001. Epigenetic instability in ES cells and cloned mice. *Science* 293, 95–97.
- Kang, Y.K., Park, J.S., Koo, D.B., Choi, Y.H., Kim, S.U., Lee, K.K., Han, Y.M., 2002. Limited demethylation leaves mosaic-type methylation states in cloned bovine pre-implantation embryos. *EMBO J.* 21, 1092–1100.
- Kato, Y., Li, X., Amarnath, D., Ushizawa, K., Hashizume, K., Tokunaga, T., Taniguchi, M., Tsunoda, Y., 2007. Comparative gene expression analysis of bovine nuclear-transferred embryos with different developmental potential by cDNA microarray and real-time PCR to determine genes that might reflect calf normality. *Cloning Stem Cells* 9, 495–511.
- Kim, H.R., Kang, J.K., Yoon, J.T., Seong, H.H., Jung, J.K., Lee, H.M., Sik Park, C., Jin, D.I., 2005. Protein profiles of bovine placenta derived from somatic cell nuclear transfer. *Proteomics* 5, 4264–4273.
- Kristoffersen, E.K., Matre, R., 1996. Surface annexin II on placental membranes of the fetomaternal interface. *Am. J. Reprod. Immunol.* 36, 141–149.
- Laurincik, J., Maddox-Hyttel, P., 2007. Nucleolar remodeling in nuclear transfer embryos. *Adv. Exp. Med. Biol.* 591, 84–92.
- Lawrence, T., Willoughby, D.A., Gilroy, D.W., 2002. Anti-inflammatory lipid mediators and insights into the resolution of inflammation. *Nat. Rev. Immunol.* 2, 787–795.
- Lee, R.S.F., Peterson, A.J., Donnison, M.J., Ravelich, S., Legard, A.M., Ning, L., Oliver, J.E., Miller, A.L., Tucker, F.C., Breier, B., Wells, D.N., 2004. Cloned cattle fetuses with the same nuclear genetics are more variable than contemporary half-siblings resulting from artificial insemination and exhibit fetal and placental growth deregulation even in the first trimester. *Biol. Reprod.* 70, 1–11.
- Lee, S.Y., Park, J.Y., Choi, Y.J., Cho, S.K., Ahn, J.D., Kwon, D.N., Hwang, K.C., Kang, S.J., Paik, S.S., Seo, H.G., Lee, H.T., Kim, J.H., 2007. Comparative proteomic analysis associated with term placental insufficiency in cloned pig. *Proteomics* 7, 1303–1315.
- Ling, Q., Jacovina, A.T., Deora, A., Febbraio, M., Simantov, R., Silverstein, R.L., Hempstead, B., Mark, W.H., Hajjar, K.A., 2004. Annexin II regulates fibrin homeostasis and neoangiogenesis in vivo. *J. Clin. Invest.* 113, 38–48.
- Liu, J.W., Shen, J.J., Tanzillo-Swartz, A., Bhatia, B., Maldonado, C.M., Person, M.D., Lau, S.S., Tang, D.G., 2003. Annexin II expression is reduced or lost in prostate cancer cells and its re-expression inhibits prostate cancer cell migration. *Oncogene* 22, 1475–1485.
- Liu, A.X., Jin, F., Zhang, W.W., Zhou, T.H., Zhou, C.Y., Yao, W.M., Qian, Y.L., Huang, H.F., 2006. Proteomic analysis on the alteration of protein expression in the placental villous tissue of early pregnancy loss. *Biol. Reprod.* 75, 414–420.
- Meier, S., Ledgard, A.M., Sato, T.A., Peterson, A.J., Mitchell, M.D., 2008. Polyunsaturated fatty acids differentially alter PGF(2alpha) and PGE(2) release from bovine trophoblast and endometrial tissues during short-term culture. *Anim. Reprod. Sci.* 111, 353–360.
- Moore, K.G., Goulet, F., Sartorelli, A.C., 1992. Purification of annexin I and annexin II from human placental membranes by high-performance liquid chromatography. *Protein Expr. Purif.* 3, 1–7.
- Myatt, L., Hirth, J., Everson, W.V., 1992. Changes in annexin (lipocortin) content in human amnion and chorion at parturition. *J. Cell. Biochem.* 50, 363–373.
- Nowak-Imialek, M., Wrenzycki, C., Herrmann, D., Lucas-Hahn, A., Lagutina, I., Lemme, E., Lazzari, G., Galli, C., Niemann, H., 2008. Messenger RNA expression patterns of histone-associated genes in bovine preimplantation embryos derived from different origins. *Mol. Reprod. Dev.* 75, 731–743.
- Pappin, D.J., Hojrup, P., Bleasby, A.J., 1993. Rapid identification of proteins by peptide-mass fingerprinting. *Curr. Biol.* 3, 327–332.
- Patel, O.V., Yamada, O., Kizaki, K., Takahashi, T., Imai, K., Takahashi, S., Izaïke, Y., Schuler, L.A., Takezawa, T., Hashizume, K., 2004. Expression of trophoblast cell-specific pregnancy-related genes in somatic cell-cloned bovine pregnancies. *Biol. Reprod.* 70, 1114–1120.
- Perecin, F., Méo, S.C., Yamazaki, W., Ferreira, C.R., Merighe, G.K., Meirelles, F.V., Garcia, J.M., 2009. Imprinted gene expression in *in vivo*- and *in vitro*-produced bovine embryos and chorio-allantoic membranes. *Genet. Mol. Res.* 8, 76–85.
- Pfarrer, C.D., 2006. Characterization of the bovine placenta by cytoskeleton, integrin receptors, and extracellular matrix. *Methods Mol. Med.* 121, 323–335.
- Powell, A.M., Talbot, N.C., Wells, K.D., Kerr, D.E., Pursell, V.G., Wall, R.J., 2004. Cell donor influences success of producing cattle by somatic cell nuclear transfer. *Biol. Reprod.* 71, 210–216.
- Santos, F., Zakhartchenko, V., Stojkovic, M., Peters, A., Jenuwein, T., Wolf, E., Reik, W., Dean, W., 2003. Epigenetic marking correlates with developmental potential in cloned bovine preimplantation embryos. *Curr. Biol.* 13, 1116–1121.
- Sawicki, G., Dakour, J., Morrish, D.W., 2003. Functional proteomics of neurokinin B in the placenta indicates a novel role in regulating cytotrophoblast antioxidant defenses. *Proteomics* 3, 2044–2051.
- Streicher, W.W., Lopez, M.M., Makhatazde, G.I., 2009. Annexin I and annexin II N-terminal peptides binding to S100 protein family members: specificity and thermodynamic characterization. *Biochemistry* 48, 2788–2798.
- Sun, M., Liu, Y., Gibb, W., 1996. Distribution of annexin I and II in term human fetal membranes, decidua and placenta. *Placenta* 17, 181–184.
- Surani, M.A., 2001. Reprogramming of genome functions through epigenetic inheritance. *Nature* 414, 122–128.
- Talbot, N.C., Caperna, T.J., Edwards, J.L., Garrett, W., Wells, K.D., Ealy, A.D., 2000. Establishment of bovine blastocyst-derived trophoblast and endoderm cell lines: use of interferon-tau and serum protein expression as respective *in vitro* markers. *Biol. Reprod.* 62, 235–247.
- Talbot, N.C., Powell, A.M., Camp, M., Ealy, A.D., 2007a. Establishment of a bovine blastocyst-derived cell line collection for the comparative analysis of embryos created *in vivo* and by *in vitro* fertilization, somatic cell nuclear transfer, or parthenogenetic activation. *In Vitro Cell. Dev. Biol. Anim.* 43, 59–71.
- Talbot, N.C., Blomberg, Ie, A., Mahmood, A., Caperna, T.J., Garrett, W.M., 2007b. Isolation and characterization of porcine visceral endoderm cell lines derived from *in vivo* 11-day blastocysts. *In Vitro Cell. Dev. Biol. Anim.* 43, 72–86.

- Talbot, N.C., Powell, A.M., Ocón, O.M., Caperna, T.J., Camp, M., Garrett, W.M., Ealy, A.D., 2008. Comparison of the interferon-tau expression from primary trophectoderm outgrowths derived from IVP, NT, and parthenogenote bovine blastocysts. *Mol. Reprod. Dev.* 75, 299–308.
- Tamada, H., Van Thuan, N., Reed, P., Nelson, D., Katoku-Kikyo, N., Wudel, J., Wakayama, T., Kikyo, N., 2006. Chromatin decondensation and nuclear reprogramming by nucleoplasmin. *Mol. Cell. Biol.* 26, 1259–1271.
- Tanaka, S., Oda, M., Toyoshima, Y., Wakayama, T., Tanaka, M., Yoshida, N., Hattori, N., Ohgane, J., Yanagimachi, R., Shiota, K., 2001. Placentomegaly in cloned mouse concept caused by expansion of the spongiotrophoblast layer. *Biol. Reprod.* 65, 1813–1821.
- Wang, X., Campos, B., Kaetzel, M.A., Dedman, J.R., 1999. Annexin V is critical in the maintenance of murine placental integrity. *Am. J. Obstet. Gynecol.* 180, 1008–1016.
- Wilmut, I., Schnieke, A.E., McWhir, J., Kind, A.J., Campbell, K.H.S., 1997. Viable offspring derived from fetal and adult mammalian cells. *Nature* 385, 810–813.
- Wrenzycki, C., Wells, D., Herrman, D., Miller, A., Oliver, J., Tervit, R., Niemann, H., 2001. Nuclear transfer protocol affects messenger RNA expression patterns in cloned bovine blastocysts. *Biol. Reprod.* 65, 309–317.
- Wu, T., Angus, C.W., Yao, X.L., Logun, C., Shelhamer, J.H., 1997. P11, a unique member of the S100 family of calcium-binding proteins, interacts with and inhibits the activity of the 85-kDa cytosolic phospholipase A2. *J. Biol. Chem.* 272, 17145–17153.
- Zhang, Y., Zhang, Y.L., Feng, C., Wu, Y.T., Liu, A.X., Sheng, J.Z., Cai, J., Huang, H.F., 2008. Comparative proteomic analysis of human placenta derived from assisted reproductive technology. *Proteomics* 8, 4344–4356.