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RESEARCH ARTICLE

Comparative proteomic analysis of human placenta derived from assisted reproductive technology

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The aim of this study was to use proteomics-based approach to examine differences in protein expression in placenta derived from assisted reproductive technology (ART) and normal pregnancy. Using 2-DE we found that, compared with the control group, 12 spots in standard *in vitro* fertilization group and 18 spots in intracytoplasmic sperm injection group were identified as significantly differentially expressed proteins. Among them, six spots were differentially expressed in both standard IVF and ICSI groups with the same change tendency. Totally, 20 proteins were successfully identified by MALDI TOF/TOF MS, including proteins involved in the membrane traffic, metabolism, nucleic acid processing, stress response and cytoskeleton. Notably, five proteins detected to be differentially expressed in both ART groups were identified as annexin A3, hnRNP C1/C2, α-SNAP, FTL and ATP5A. Some of the proteins were confirmed by Western blot and immunohistochemistry analysis. Our study allowed for the initial identification of these proteins related to various functions in placentation with significantly altered abundance in ART groups. The present results reveal that abnormal protein profiles are involved in ART placenta and these differentially expressed proteins may be valuable for the evaluation of potential association between ART treatment and offspring outcome.

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1 Introduction

Assisted reproductive technology (ART), containing standard *in vitro* fertilization (IVF), intracytoplasmic sperm injection (ICSI) and their related procedures, have helped thousands of couples conceive children in the past 30 years. Early studies suggested ART was safe and children born from ART developed normally. However, in recent years, more and more well-designed studies have consistently documented associations between ART and an increased risk of birth defects [1, 2], low birth weight [3], chromosome abnormalities [4, 5] and possibly, childhood cancer. These results were also confirmed in a number of systematic reviews [6, 7]. In addition, a possible link has now been suggested between ART and epigenetic defects. Several clinical studies reported an increased frequency of imprinting defects among ART children [8, 9]. Although the precise significance and origin of these associations require confirmation and clarification, available evidence has suggested the concerns for the long-term safety of ART procedures.

As IVF was introduced into practice without formal evaluation of its effects on the health of the children conceived with this procedure [1], there is little information about the mechan-

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Abbreviations: **ART**, assisted reproductive technology; **ICSI**, intracytoplasmic sperm injection; **IHC**, immunohistochemistry; **IVF**, *in vitro* fertilization; **IVP**, *in vitro* embryo production; **RT**, room temperature

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isms underlying the increased health defects. It is also unclear at which stage (s) from the controlled ovarian hyperstimulation, gametes manipulation, *in vitro* culture, embryo transfer to the baby's birth, the risk may be raised. Since ICSI was introduced for male infertility, more concerns were also raised that ICSI would increase the risk of birth defects and genetic disorders as it bypasses almost all the selection mechanisms that operate in natural conception and the invasive procedure of ICSI may have a deleterious effect [10]. Moreover, possible causes leading to abnormalities of offspring derived from ART are much more complicated. Generally, it is difficult to determine whether birth defects after assisted reproduction result directly from the procedures used, or whether they reflect the suboptimal fertility of the parents. To determine whether ART is associated with health defects and what is the mechanism for this association, additional in-depth investigations are required.

The placenta is a temporary organ that consists of a fetal component and a maternal component and it is a structure unique to pregnancy functioning to sustain and protect the fetus until birth. The placenta also serves as an endocrine organ producing a wide range of important signals that ensure the maintenance of gestation and fetal well-being. Abnormal placentation has been observed following *in vitro* embryo production (IVP) in animals [11, 12]. It is also suggested that heavy fetal losses and abnormalities in animal somatic cell nuclear transfer might be associated with abnormal placenta development partially because of embryo manipulation and *in vitro* culture [13, 14]. Meanwhile, it has been reported that human placenta derived from ART are associated with more frequent pathological findings [15]. These results of abnormal placentation in assisted reproduction related procedures in animals and human being reveal partial evidence for the possibility of worse outcome of ART offspring. To overall evaluate the specific gene products in placenta following ART, global screening of protein expression in placenta is needed.

Proteomics, the large-scale study of proteins, is the currently most powerful technique for protein identification in complex cellular systems. High-resolution 2-DE in combination with MS is an excellent tool for proteomic analysis. It gives information in a high-throughput mode about protein expression profiles. Thus, proteomics may enable us to unravel particular molecular complexes or pathways in the pathogenesis of the human placentation following ART. In the present study, we analyzed differential protein patterns between placentas obtained from ART (standard IVF or ICSI) and normal placenta obtained from natural conception using 2-DE and MS to find potential effects of ART on the expression of proteins in placenta.

2 Materials and methods

2.1 Subjects and ethics

Placentas from ART (standard IVF and ICSI) and normal pregnancies were collected after caesarean delivery during the period of April 2006 to May 2007 in Women's Hospital, School of Medicine, Zhejiang University, China. We had access to complete clinical data for each individual. The inclusion criteria used for ART patients were as follows: maternal age between 25 to 35 years; full-term delivery, singleton pregnancy, child birth weight between 2500 to 4000 g, no indication of pregnancy complications and no birth defect. Couples referred for ART because of maternal oviduct obstruction for standard IVF group and male infertility (oligospermia and/or asthenozoospermia) with or without maternal oviduct problem for ICSI group. The control group was formed by age-matched women with natural, singleton pregnancy and underwent caesarean surgery without suffering any disease during pregnancy. The clinical application of ART was licensed by the Health Ministry of People's Republic of China. The study was approved by the Ethical Committee of Women's Hospital, School of Medicine, Zhejiang University and informed written consent was obtained from each woman for the use of placenta.

2.2 Placenta sample collection

The placenta was collected and dissected immediately following delivery. Fragments from the placental subchorial zone corresponding to umbilical cord insertion were dissected. Maternal membranes were eliminated, and floating villi were washed in ice-cold PBS. Placental samples were stored in liquid nitrogen just after collection for half an hour and finally stored at −80°C until further protein extraction.

2.3 Protein preparation

For experiments, a total of 300 mg of each placental villous sample was homogenized and solubilized in 1000 μL of lysis buffer containing 7 M urea, 2 M thiourea, 4% w/v CHAPS, 1% w/v DTT, 2% v/v IPG buffer (pH 4–-7), 1% v/v PMSF and 1% v/v protease inhibitor cocktail and kept on ice for 1 h. Sonication was carried out on ice at a power of 90 W for five bursts of 8 s, each interspersed with 16 s. Then, the lysed cells were centrifuged at 15 000 × *g* at 4°C for 60 min to remove debris. Protein concentrations were determined using the Bradford protein assay (Bio-Rad) [16]. The final protein samples were stored at −80°C prior to electrophoresis.

2.4 2-DE

The 2-DE was performed using the Protean IEF Cell and Protean Plus Dodeca Cell (Bio-Rad, USA) according to the manufacturer's instructions. For the first dimension, IEF was performed on Protean IEF Cell with ReadyStrip IPG strips (Linear, pH 4–7, 24 cm, Bio-Rad). 400 μg of total protein was mixed with a rehydration solution containing 7 M urea, 2 M thiourea, 2% w/v CHAPS, 0.4% w/v DTT, 0.5% IPG buffer (pH 4–7) and 0.001% bromophenol blue to a total volume of 450 μL. Following IEF separation, the strips were incubated for 15 min in an equilibration buffer (6 M urea,

50 mM Tris-HCl pH 8.8, 30% v/v glycerol, 2% SDS, and 0.001% bromophenol blue) containing 1% DTT and subsequently in the same equilibration buffer containing 2.5% iodoacetamide for 15 min. The second-dimensional separation was carried out on 12% SDS-PAGE gels using the Protean Plus Dodeca Cell, with the IPG strips sealed on the top of the gels with 0.5% agarose. SDS-PAGE was run at a constant power of 80 V for the first 45 min and 200 V for subsequent 7–8 h until the bromophenol blue line reached the bottom of the gels. For each sample, three separate experiments were run independently on three analytical 2-DE gels.

2.5 Gel staining, image analysis and statistical analysis

Modified silver staining method compatible with MS was performed according to the published procedures [17]. The stained gels were scanned by the high-resolution scanner GS-800 calibrated densitometer (Bio-Rad). The digitized images were analyzed with PDQuest software (v 7.1.1, Bio-Rad) for spot detection, spot matching and spot quantification. The same parameters were used to detect spots in all of the gels to guarantee comparability between the gels. To correct for variations due to silver staining and quantify protein spots, the individual spot quantity was normalized by the total quantity in valid spots on the gel. In this study, the gels were divided into three groups (standard IVF placenta, ICSI placenta and normal placenta) for comparative and statistical analysis. Expression intensity that showed at least 1.5-fold difference with $p < 0.05$ was set as a threshold indicating significant changes.

2.6 In-gel tryptic digestion

The protein spots of interest were manually excised from the gels. In-gel digestion was performed mainly as published procedures with some modifications [18]. The gel pieces were placed into V-bottom 96-well microplates loaded with 100 μL of 50% ACN and 50 mM ammonium bicarbonate solution per well. After being destained for 1 h, gel plugs were dehydrated with 100 μL 100% ACN for 20 min and then thoroughly dried. The samples were then rehydrated in a digestion buffer containing 20 mM ammonium bicarbonate and 12.5 ng/μl trypsin at 4°C. After 30-min incubation, the gels were digested for 12 h at 37°C. Peptides were then extracted twice using the solution containing 0.5% TFA and 50% ACN. The extracts were dried under the protection of N_2.

2.7 MALDI-TOF/TOF MS and MS/MS analysis and database search

The peptides were eluted onto the target plate with 0.7 μL matrix solution (CHCA in 0.1% TFA, 50% ACN) and allowed to air-dry. Mass analysis of peptide mixtures was performed on a 4700 MALDI-TOF/TOF Proteomics Analyzer (Applied Biosystems, USA). The UV laser was operated at a 200 Hz repetition rate with wavelength of 355 nm. The accelerating voltage was operated at 20 kV. Myoglobin digested by trypsin was used to calibrate the mass instrument with internal calibration mode. All acquired spectra of samples were processed using 4700 ExploreTM software (Applied Biosystems) in a default mode. Parent mass peaks with mass range 700–3200 Da and minimum S/N 20 were picked out for tandem TOF/TOF analysis. Combined MS and MS/MS spectra were submitted to MASCOT (V2.1, Matrix Science, UK) by GPS Explorer software (V3.6, Applied Biosystems) and searched with the following parameters: NCBInr database (release date: 2007.06.27), human species, trypsin digest with an allowance for up to one missed cleavage per peptide, none fixed modifications, MS tolerance of 0.2 Da, MS/MS tolerance of 0.6 Da, and possible oxidation of methionine. MASCOT protein scores (based on combined MS and MS/MS spectra) of greater than 64 were considered statistically significant ($p < 0.05$). The individual MS/MS spectrum with statistically significant (confidence interval >95%) best ion score (based on MS/MS spectra) were accepted.

2.8 Western blot analysis

Western blot on annexin A3, hnRNP C1/C2, α-SNAP and FTL was performed on 12 cases of placental tissues from the standard IVF, ICSI and the control groups respectively. An amount of 30 μg protein was subjected to 12% SDS-PAGE and then transferred to NC membranes. Membranes were incubated in blocking buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1% Tween 20 containing 5% non-fat milk) at room temperature (RT) for 1 h, and then incubated with rabbit anti-annexin A3 antibody (Abcam, ab33068, 1:1600 dilution), mouse anti-hnRNP C1/C2 antibody (Abcam, ab10294, 1:5000 dilution), mouse anti-α-SNAP antibody (Abcam, ab16391, 1:5000 dilution), goat anti-FTL antibody (Santa Cruz Biotechnology, sc14420, 1:200 dilution) and rabbit anti-Actin antibody (Santa Cruz Biotechnology, sc-1616-R, 1:5000 dilution) at 4°C overnight. Then the membranes were incubated with appropriate secondary antibody for 1 h at RT. The detection procedure was carried out in a dark room using ECL detection kits according to the manufacturer's instructions (Amersham Biosciences).

2.9 Immunohistochemistry

Immunohistochemistry (IHC) was performed on the analysis of annexin A3, hnRNP C1/C2 and α-SNAP. The detection of these proteins in the placental tissues was carried out by a two-step IHC procedure. Paraffin blocks were cut into 5-μm-thick sections. Then, the sections were deparaffinized in xylene and rehydrated in graded alcohol concentrations. Nonspecific binding was blocked by preincubation with blocking solution for 5 min, and then the sections were incubated for 2 h at RT with antibodies against annexin A3 (Abcam, 1:100 dilution), hnRNP C1/C2 (Abcam, 1:200 dilution) and α-SNAP (Abcam, 1:50 dilution). After incubation with primary antibodies, sections

were incubated with appropriate secondary antibody for 1 h. Substrate-chromogen DAB reagent was then added to each section following rinse, and finally hematoxylin solution was used to stain nuclei.

2.10 Statistics

The data were presented as mean ± SD. Student's *t*-test was used for the statistical analysis. Differences were considered significant at $p < 0.05$.

3 Results

3.1 Analysis of the ART and normal placenta proteomes by 2-DE

To investigate molecular-level difference in gene expression that might relate to the functional differences between ART and normal placenta, the protein patterns of six placentas from standard IVF pregnancy, six from ICSI pregnancy and six from natural pregnancy were compared using 2-DE (each group was composed of three boy and three girl pregnancies to equilibrate the difference between fetal gender). Each gel was detected approximately 1200 spots. The representative 2-DE images are shown in Fig. 1. Compared with the control group, 12 spots in the standard IVF group and 18 spots in the ICSI group were identified as significantly differentially expressed proteins. Among 12 spots in the IVF placentas, seven spots were up-regulated and five spots were down-regulated, while ten spots were up-regulated and eight spots were down-regulated among 18 spots in ICSI placentas. Furthermore, there were six differentially expressed spots presenting in IVF group as well as in ICSI group. Relative quantification of differentially expressed proteins is shown in Fig. 2. The representative protein spots in the ART and normal placenta are shown in Fig. 3.

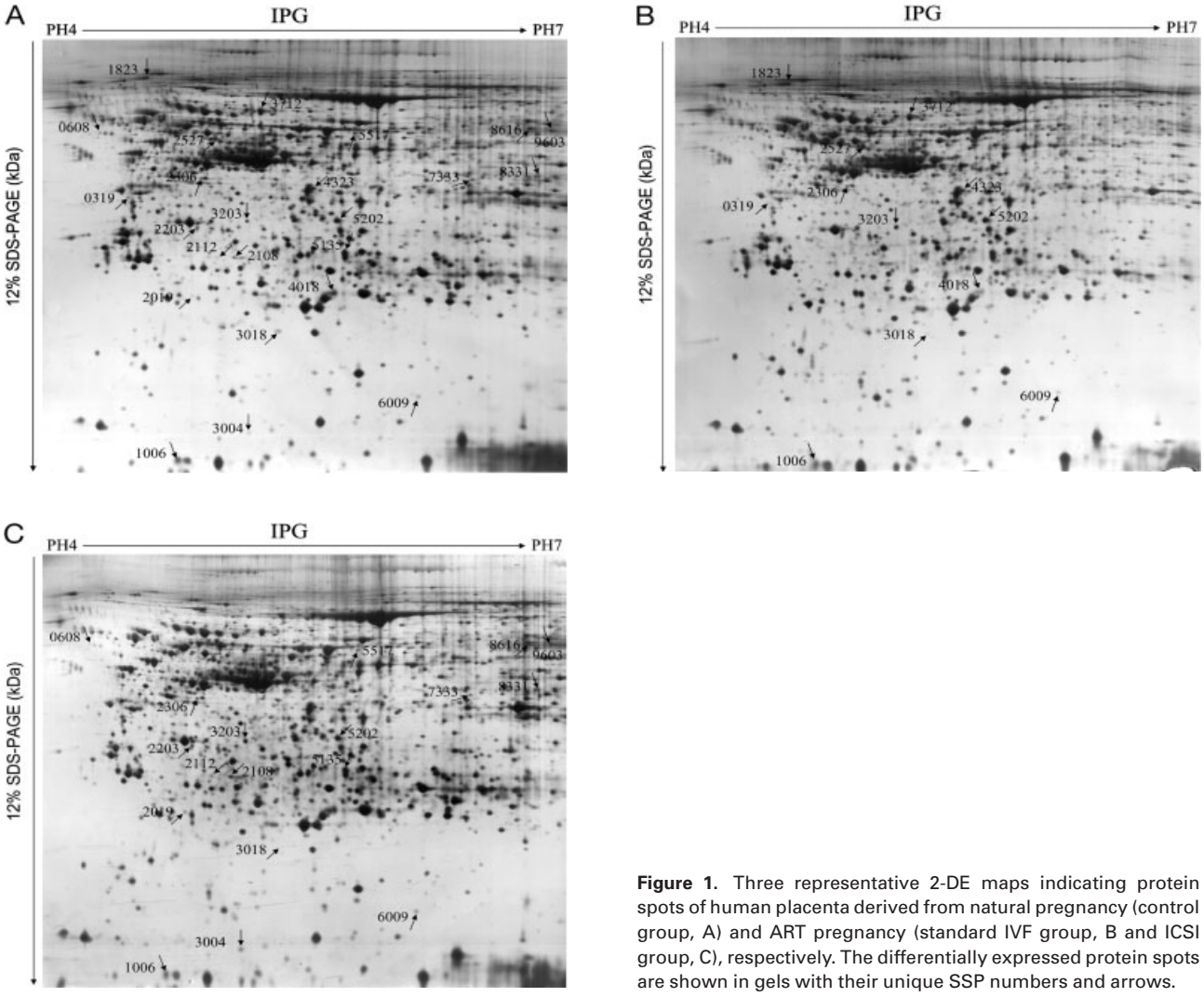


Figure 1. Three representative 2-DE maps indicating protein spots of human placenta derived from natural pregnancy (control group, A) and ART pregnancy (standard IVF group, B and ICSI group, C), respectively. The differentially expressed protein spots are shown in gels with their unique SSP numbers and arrows.

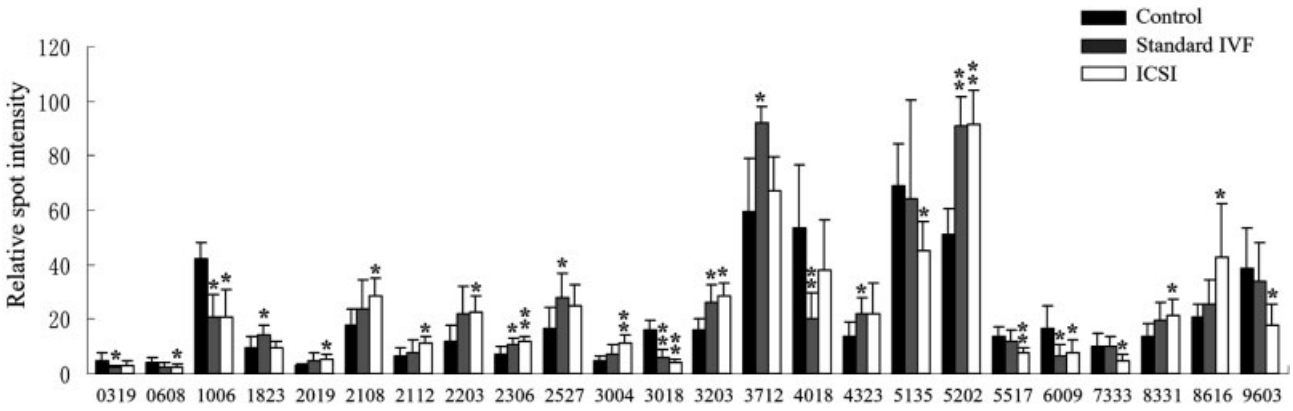


Figure 2. Relative quantification of differentially expressed proteins between normal and ART placenta detected by 2-DE. Each bar represents the intensity as means ± SD. Significantly different expression is labeled with asterisks (*p <0.05, **p <0.01, compared with the control group).

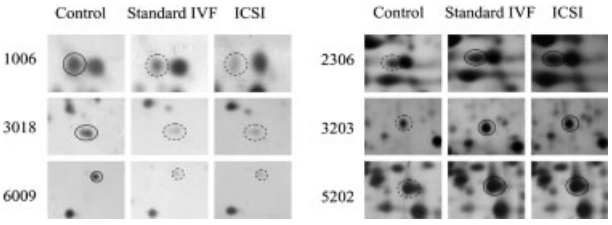


Figure 3. Magnified 2-DE maps of representative spots with differential expression between normal and ART placenta.

3.2 Identification of differentially expressed proteins

The differentially expressed spots were excised from gels and identified after tryptic digestion by MALDI-TOF/TOF MS. Twenty among the 24 spots were successfully identified. The search results were evaluated on the basis of accepted standards that take account of the number of peptides matched to the candidate protein, the difference in the number of matched peptides between the candidate protein and the next best fit, the coverage of the candidate proteins sequence by the matching peptides. Proteins identified and the supporting identification data are listed in Table 1 for standard IVF group and in Table 2 for ICSI group. The data of PMF and MS/MS identification of spots annexin A3 by MALDI-TOF-MS/MS are presented in Fig. 4 as a representative example.

3.3 Characteristics of the identified proteins

In the comparison of standard IVF and normal placenta, 12 protein spots were shown to be expressed differentially and nine were successfully identified, of which seven were up-regulated, including ORP150, hnRNP C1/C2, PDIR, α-SNAP, Hsp60, EIF3S2 and annexin A3, two were down-regulated, including ATP5A and FTL. In the comparison of ICSI and normal placenta, 18 protein spots were shown to be expressed differentially and 16 were successfully identified, of which nine were up-regulated, including CHP, PSMA3, COPE, hnRNP C1/C2, TBCA, α-SNAP, annexin A3, PTK9L and LAP3, seven were down-regulated, including RAD23A, ATP5A, FTL, CTSD, ALDH9A1, ENO1 and FSCN1. Among them, five proteins, ATP5A, hnRNP C1/C2, FTL, α-SNAP and annexin A3 were differentially expressed in both ART groups with the same change tendency. According to annotations from UniProt knowledgebase (Swiss-Prot/TrEMBL) and Gene Ontology Database, the identified proteins are mainly involved in the membrane traffic, metabolism, nucleic acid processing, stress response and cytoskeleton. These differentially expressed proteins mainly distributed within the cytoplasm, nucleus, membrane, mitochondrion, endoplasmic reticulum and lysosome (Tables 1 and 2 and Fig. 5).

3.4 Validation of differentially expressed proteins by Western blot

To further confirm the alterations of protein expression in placenta of ART group, four of the differentially expressed proteins in both standard IVF and ICSI groups were validated. As shown in Fig. 6, Annexin A3, hnRNP C1/C2 and α-SNAP all showed consistently increased expression in both ART groups. In contrast, FTL showed significantly decreased expression. The expression changes of these selected proteins were consistent with the 2-DE and silver-staining results.

3.5 Detection of differentially expressed proteins by IHC

To view the tissue localization of differentially expressed proteins in human placenta, we performed IHC on annexin A3, hnRNP C1/C2 and α-SNAP. In this analysis, we found that annexin A3 and α-SNAP were located in the cytoplasm or membrane of trophoblastic cells in placental villous tissues and hnRNP C1/C2 was located in the nucleus. The results are shown in Fig. 7.

Table 1. Identification of differentially expressed proteins in standard IVF placenta

Spot ID.[a]	Protein name	Abbreviation	Accession no.[b]	Theoretical *Mr*/p*I* (Da)	Match/ unmatch[c]	Protein score[d]	Cover-age(%)[e]	MS/MS sequence data[f]	Functional association	Subcellular location
Up-regulated proteins										
1823	Oxygen regulated protein precursor	ORP150	gi|5453832	111266.2/5.16	13/8	109	8	LQDLTLRDLEK LYQPEYQEVSTEEQR LYQPEYQEVSTEEQREEISGK	Stress response	Endoplasmic reticulum
2306	Heterogeneous nuclear ribonucleoprotein C (C1/C2)	hnRNP C1/C2	gi|13937888	33577.5/4.99	9/10	105	22	VPPPPPIAR GFAFVQYVNER MIAGQVLDINLAAEPK	mRNA splicing	Nucleus
2527	Protein disulfide isomerase-related protein 5	PDIR	gi|1710248	46170.2/4.95	15/17	208	33	GSFSEQGINEFLR GSTAPVGGGAFPTIVER	Stress response	Endoplasmic reticulum
3203	Alpha SNAP	α-SNAP	gi|3929617	33225.3/5.23	11/11	185	28	AIEIYTDMGR TIQGDEEDLR VAGYAALLEQYQK	Membrane fusion	Membrane
3712	Chaperonin	Hsp60	gi|31542947	61016.4/5.7	28/24	453	42	TVIIEQSWGSPK TLNDELEIIEGMKFDR LVQDVANNTNEE AGDGTTTATVLAR	Stress response	Mitochondrion
4323	Eukaryotic translation initiation factor 3, subunit 2 beta, 36kDa	EIF3S2	gi|4503513	36478.6/5.38	14/20	224	40	SYSSGGEDGYVR FFHLAFEEEFGR DPIVNVWYSVNGER	Protein synthesis	EIF3 complex
5202	Annexin A3	ANX3	gi|4826643	36352.7/5.63	22/23	301	47	LTFDEYR NTPAFLAER GIGTDEFTLNR	Signal transduction	Cytoplasm
Down-regulated proteins										
1006	ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit precursor	ATP5A	gi|4757810	59713.6/9.07	8/9	205	12	VLSIGDGIAR ILGADTSVDLEETGR TGAIVDVPVGEELLGR	ATP production	Mitochondrion
3018	Ferritin light chain	FTL	gi|48145547	20036.1/5.51	8/24	137	42	LNQALLDLHALGSAR LGGPEAGLGEYLFER	Cellular iron ion homeostasis	Ferritin complex

a) Spot ID is the unique SSP number, which refers to the labels in Fig. 1.
b) Accession no. is the MASCOT results of MALDI-TOF/TOF searched from the NCBInr database.
c) The number of peaks that match/unmatch to the trypsin peptides.
d) A score of more than 64 is significant ($p < 0.05$).
e) The number of amino acids spanned by the assigned peptides divided by the sequence length.
f) Peptides matched by using MASCOT MS/MS ion search.

4 Discussion

Although much epidemiological evidence suggests that there may be an increased risk of worse outcome in infants born following ART treatment, it is difficult to identify the causes, as assisted reproduction is a rather complicated procedure. In this study, to minimize the possibility of interference and to focus on the manipulations of ART itself, we strictly limited the selection criteria of subjects as described in Section 2.1. It has been shown that placenta abnormalities in human were usually associated with adverse effects on the growth and development of the fetus, and abnormal placentation was observed following ART treatment [19, 20]. Thus, identifying differentially expressed proteins involved in ART placenta is of great interest. In the current study, we have initiated the high-resolution proteomic analyses of human placenta derived from ART for further investigations of the underlying molecular pathophysiology. Twenty identified differentially expressed proteins fall into five functional classes: membrane traffic, metabolism, nucleic acid processing, stress response and cytoskeleton. Notably, five proteins, annexin A3, hnRNP C1/C2, α-SNAP, FTL and ATP5A, were detected to be differentially expressed in both standard IVF and ICSI groups. To the best of our knowledge,

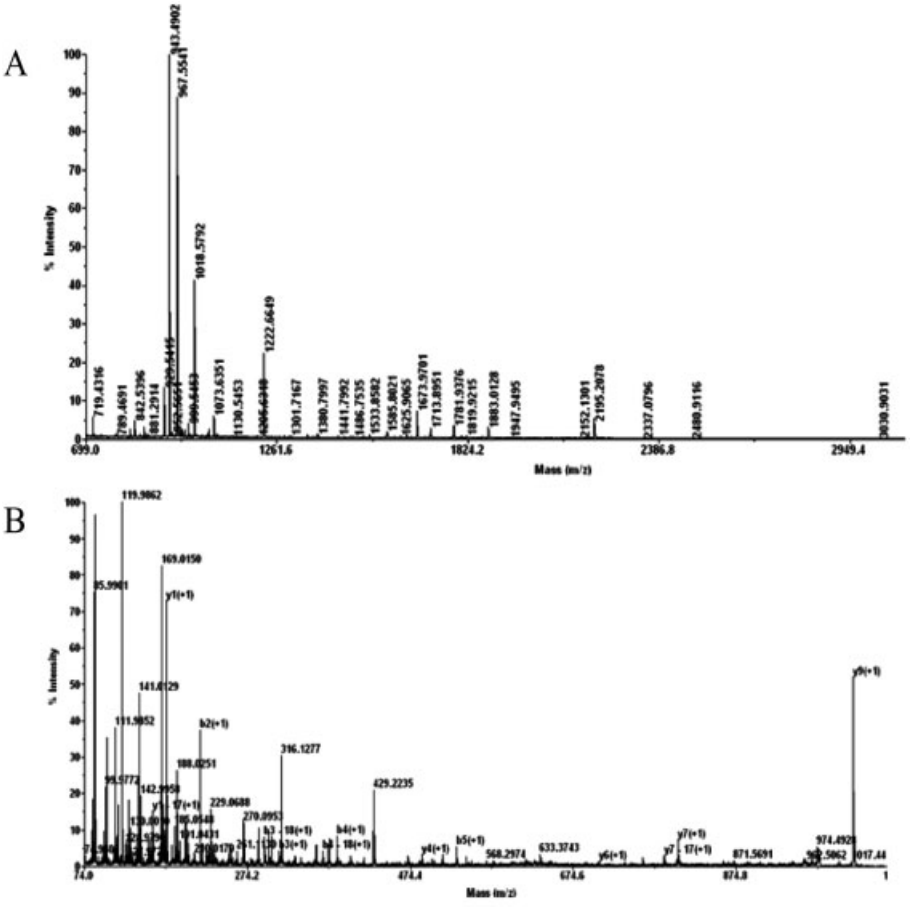
Table 2. Identification of differentially expressed proteins in ICSI placenta

Spot ID.[a)]	Protein name	Abbreviation	Accession no.[b)]	Theoretical *M*r/p*I* (Da)	Match/ unmatch[c)]	Protein score[d)]	Cover-age(%)[e)]	MS/MS sequence data[f)]	Functional association	Subcellular location
Up-regulated proteins										
2019	Calcium binding protein P22	CHP	gi|6005731	22442.3/4.98	18/29	258	74	LHFAFR IPELAINPLGDR TLAHFRPIEDNEK IINAFFPEGEDQVNFR	Membrane traffic	Cytoplasm
2108	Proteasome alpha 3 subunit isoform 2	PSMA3	gi|23110939	27629.7/5.19	12/7	172	28	LYEEGSNKR SNFGYNIPLK AVENSSTAIGIR	Protein catabolism	Cytoplasm Nucleus
2203	Epsilon subunit of coatomer protein complex isoform c	COPE	gi|40805827	28754.6/5.11	7/14	78	10	DVERDVFLYR	Membrane traffic	COPI vesicle coat
2306	Heterogeneous nuclear ribonucleoprotein C (C1/C2)	hnRNP C1/C2	gi|13937888	33577.5/4.99	9/10	105	22	VPPPPPIAR GFAFVQYVNER MIAGQVLDINLAAEPK	mRNA splicing	Nucleus
3004	Chain A, Human tubulin chaperone cofactor A	TBCA	gi|21730330	12802.5/5.11	6/7	123	18	QAEILQESR LEAAYLDLQR RLEAAYLDLQR	Cytoskeleton	Cytoplasm
3203	Alpha SNAP	α-SNAP	gi|3929617	33225.3/5.23	11/11	185	28	AIEIYTDMGR TIQGDEEDLR VAGYAALLEQYQK	Membrane fusion	Membrane
5202	Annexin A3	ANX3	gi|4826643	36352.7/5.63	22/23	301	47	LTFDEYR NTPAFLAER GIGTDEFTLNR	Signal transduction	Cytoplasm
8331	PTK9L	PTK9L	gi|48145581	39779.4/6.37	9/57	69	30	HQTLQGLAFPLQPEAQR	Cytoskeleton	Cytoplasm
8616	LAP3 protein	LAP3	gi|37588925	54354.7/6.8	25/21	372	47	TLIEFLLR GVLFASGQNLARLRETLNISGPPLK AAGIDEQENWHEGKENI	Proteolysis	Cytoplasm
Down-regulated proteins										
0608	RAD23 homolog A (S. cerevisiae)	RAD23A	gi|56789929	39540.6/4.56	6/16	82	9	ASYNNPHR DQPQFQNMR	Nucleotide excision repair	Nucleus
1006	ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit precursor	ATP5A	gi|4757810	59713.6/9.07	8/9	205	12	VLSIGDGIAR ILGADTSVDLEETGR TGAIVDVPVGEELLGR	ATP production	Mitochondrion
3018	Ferritin light chain	FTL	gi|48145547	20036.1/5.51	8/24	137	42	LNQALLDLHALGSAR LGGPEAGLGEYLFER	Cellular iron ion homeostasis	Ferritin complex
5135	Cathepsin D preproprotein	CTSD	gi|4503143	44523.6/6.1	14/16	142	19	YYTVFDR YYTVFDRDNNR LVDQNIFSFYLSR	Proteolysis	Lysosome
5517	Gamma-aminobutyraldehyde dehydrogenase	ALDH9A1	gi|1049219	53497.9/6.01	13/27	200	20	VTIEYYSQLK ANDTTFGLAAGVFTR MSTGTFVVSQPLNYR	Metabolism	Cytoplasm
7333	Enolase 1	ENO1	gi|4503571	47481.4/7.01	22/29	313	44	LAQANGWGVMVSHR VVIGMDVAASEFFR AAVPSGASTGIYEALELR	Glycolysis	Cytoplasm Nucleus

Table 2. Continued

Spot ID.[a]	Protein name	Abbreviation	Accession no.[b]	Theoretical *Mr*/p*I* (Da)	Match/ unmatch[c]	Protein score[d]	Cover-age(%)[e]	MS/MS sequence data[f]	Functional association	Subcellular location
9603	Fascin 1	FSCN1	gi|4507115	55123.3/6.84	23/53	355	53	GEHGFIGCR WSLQSEAHR FLIVAHDDGR LINRPIIVFR LVARPEPATGYTLEFR	Cytoskeleton	Actin cytoskele-ton

a) Spot ID is the unique SSP number, which refers to the labels in Fig. 1.
b) Accession no. is the MASCOT results of MALDI-TOF/TOF searched from the NCBInr database.
c) The number of peaks that match/unmatch to the trypsin peptides.
d) A score of more than 64 is significant ($p < 0.05$).
e) The number of amino acids spanned by the assigned peptides divided by the sequence length.
f) Peptides matched by using MASCOT MS/MS ion search.



C
1 MASIWVGHRGTVRDYPDFSPSVDAEAIQKAIRGIGTDEKMLISILTERSN
51 AQRQLIVKEYQAAYGKELKDDLKGDLSGHFEHLMVALVTPPAVFDAKQLK
101 KSMKGAGTNEDALIEILTTRTSRQMKDISQAYYTVYKKSLGDDISSETSG
151 DFRKALLTLADGRRDESLKVDEHLAKQDAQILYKAGENRWGTDEDKFTEI
201 LCLRSFPQLKLTFDEYRNISQKDIVDSIKGELSGHFEDLLLAIVNCVRNT
251 PAFLAERLHRALKGIGTDEFTLNRIMVSRSEIDLLDIRTEFKKHYGYSLY
301 SAIKSDTSGDYEITLLKICGGDD

Figure 4. Identification of annexin A3 by MALDI TOF-MS/MS. (A) The MS map of annexin A3, in which matched peptide peaks are labeled with mass value. (B) MS/MS spectra of peptide *m/z* 1018.5791, in which the tryptic peptide sequences were confirmed from the labeled b- and y-ions. The sequence of precursor at *m/z* 1018.5791 was analyzed by MS/MS to be NTPAFLAER. (C) The amino acid sequences of annexin A3 in which matched peptide sequences are underlined.

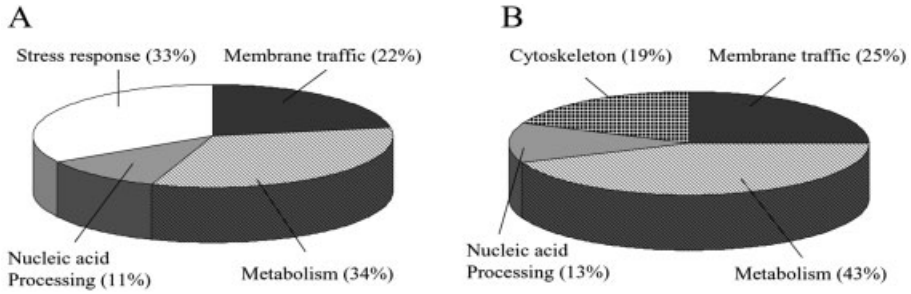


Figure 5. Functional classification of differentially expressed proteins in ART placenta identified by MALDI-TOF/TOF MS. (A) Functional classification of differentially expressed proteins in standard IVF group. (B) Functional classification of differentially expressed proteins in ICSI group.

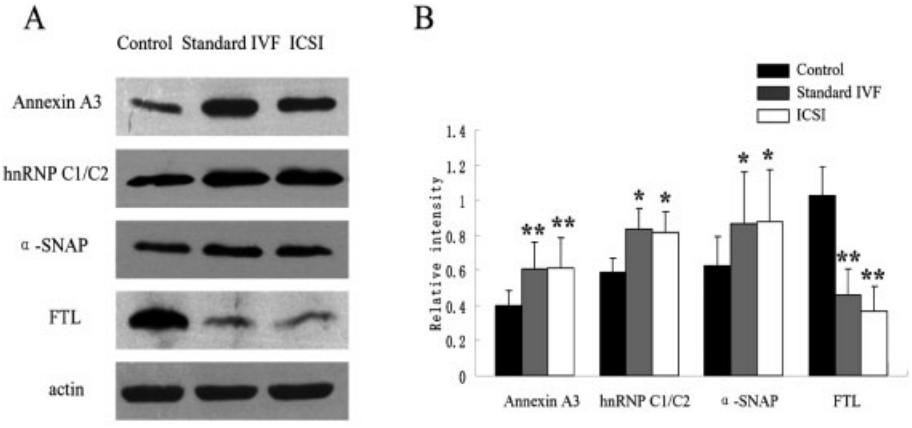


Figure 6. Western blot analysis of annexin A3, hnRNP C1/C2, α-SNAP and FTL for control, standard IVF and ICSI placenta samples. (A) The representative autoradiographs of annexin A3, hnRNP C1/C2, α-SNAP, FTL and Actin. Data were normalized by using the Actin signals. (B) Densitometric quantification of protein levels in control, standard IVF and ICSI placenta samples. Significantly different expression is labeled with asterisks (*p <0.05, **p <0.01, compared with the control group).

this is the first report of proteomic analysis performed to study the human placenta derived from ART.

4.1 Differentially expressed proteins in both ART groups

4.1.1 ART and membrane fusion interference

Membrane fusion, involving intracellular and intercellular membranes, is an event inevitably associated with basic cellular functions, such as secretion of hormones, uptake of vesicles, transport between organelles and multinucleated cellular organizations. In human, both types of fusion are required in embryo implantation and placentation [21]. Disturbances and imbalances of trophoblast fusion seem to be directly associated with pathological clinical conditions [22]. In this study, two proteins involved in membrane fusion, annexin A3 and α-SNAP, were identified as up-regulated proteins in both ART groups. Annexin A3 is a member of the annexin family, which is a Ca^{2+}-dependent phospholipid-binding protein group and proposed to exert various cellular functions [23]. Annexin A3 has been shown to mediate membrane-membrane contact during biological processes [24] and was reported as a potential angiogenic mediator that induced angiogenesis [25]. The α-SNAP is a well known protein essential for most fusion events through its interaction with NSF and the α-SNAP receptor (SNARE) complex and has been implicated in Ca^{2+}-dependent exocytosis in many cell types [26]. Accumulating evidence suggests that increase of the intracellular concentration of α-SNAP may lead to an enhancement of membrane fusion [27]. Thus, the increased expression of annexin A3 and α-SNAP in ART placenta indicated that manipulations of ART could affect placenta function through membrane fusion interference.

4.1.2 Identification of proteins involved in energy metabolism

Two proteins involved in energy metabolism, FTL and ATP5A, were identified to be significantly down-regulated in ART placenta. FTL is a light polypeptide of ferritin, which is the major iron-storage protein. Ferritin expression can enhance cell growth and protect trophoblast from free radical formation through sequestering free Fe^{2+} [28]. Artificial FTL down-regulated cells were inhibited of proliferation and displayed enhanced sensitivity to oxidative stress and to apoptosis [29]. ATP5A is part of the F1 enzymatic complex known to bind ADP, phosphate and ATP and acts as a central part in the mitochondrial energy-producing mechanism [30]. It is generally involved in cell energy metabolism. In animal research, it has been reported that mitochondrial ATP production and distribution were important to the placenta development, and placental metabolic insufficiency could result in intrauterine growth restriction [31]. Therefore, the significant down-regulation of FTL and ATP5A indicates the insufficiency of energy metabolism in ART placenta. This result provides possible reasons of higher incidence of low

birth weight after ART treatment although the mechanism is still unclear.

4.1.3 ART and increased expression of hnRNP C1/C2

HnRNP C1/C2 belongs to the hnRNP family and has been implicated in a variety of biological functions in mRNA metabolism, such as splicing, polyadenylation and turnover, and telomere/telomerase regulation [32, 33]. Subsets of hnRNP are strictly nuclear [33], and our result of IHC confirmed the nuclear location of hnRNP C1/C2 in human placenta. There is evidence that hnRNP C1/C2 could bind to chromatin in a DNA-damage-dependent manner, suggesting its unique role in DNA repair and/or damage response [34]. We infer that the significant up-regulation of hnRNP C1/C2 in ART placenta may be the result of DNA damage after ART treatments. Other hnRNP members also have been found with differential expression in pathological trophoblast [35]. Thus, the results from our present study and other laboratories suggest that the increased health risk of ART offspring might be related with certain nucleic gene metabolism dysfunction in placentation.

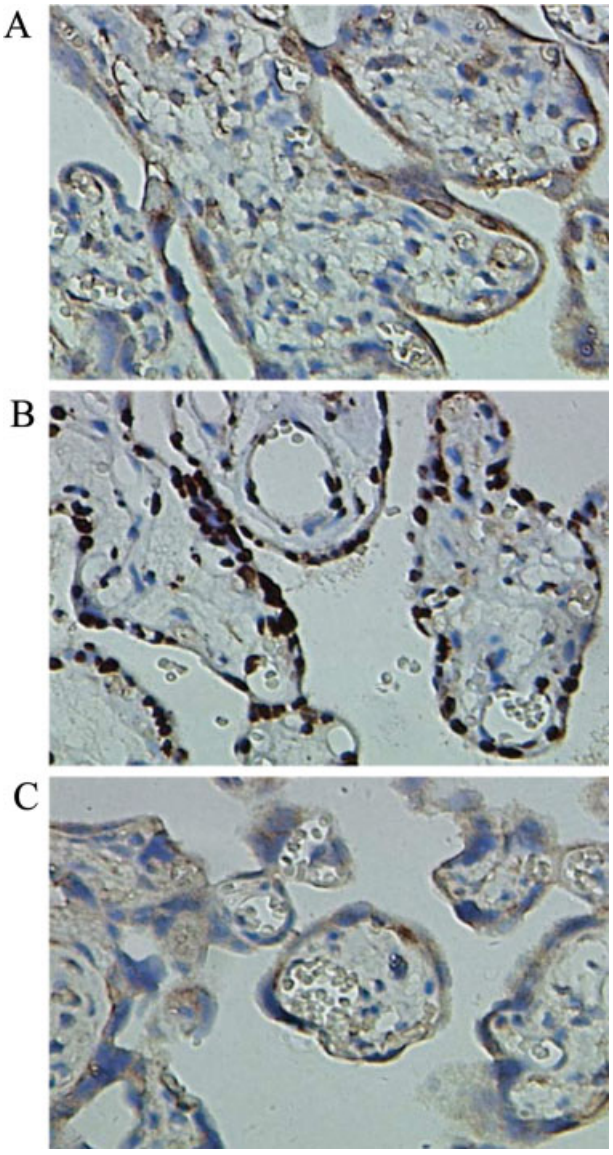


Figure 7. Immunohistochemistry on annexin A3, hnRNP C1/C2 and α-SNAP for the detection of cellular localization. (A) Annexin A3 (B) hnRNP C1/C2 and (C) α-SNAP. Annexin A3 and α-SNAP were located in the cytoplasm or membrane of trophoblastic cells in placental villous tissues and hnRNP C1/C2 was located in the nucleus.

4.2 Differentially expressed proteins in standard IVF group

4.2.1 IVF and stress response proteins

Interestingly, three proteins involved in stress response were identified of up-regulated expression in IVF group. They were ORP150, PDIR and Hsp60. ORP150 and Hsp60 are members of HSP family located in ER and mitochondrion, respectively, and known to be up-regulated by a variety of stresses [36]. PDIR is another protein involved in the ER stress-response pathway [37]. Since stress response appears to be a cellular protective mechanism, HSP and ER stress-response proteins can protect cells from structural and functional damage following exposure to adverse environmental conditions. The up-regulated expression of ORP150, PDIR and Hsp60 in IVF placenta suggests that environmental stress might be increased under the IVF treatment and the organism might have multiple defense systems against stress.

4.3 Differentially expressed proteins in ICSI group

4.3.1 Alteration of cytoskeleton proteins in ICSI group

Cytoskeleton, including microtubule and actin, participates in a wide variety of cellular functions, including endocytosis, motility, secretion, cell division and intracellular signal transduction [38]. The functions of tubulin and actin are regulated by a large number of their binding proteins. In this study, we found that two actin-binding proteins, PTK9L and FSCN1, were differentially expressed in ICSI placenta. Moreover, TBCA, a protein cofactor involved in the post-chaperonin tubulin folding pathway and required to maintain the supply of tubulin [39], was detected to be up-regulated. As the cytoskeletal arrangement has profound impact on cellular structure and function, and differential expression of cytoskeletal components has already been found to be associated with abnormal placentation in human [40], our results suggest that ICSI might have extra effect on placentation through the alteration of cytoskeleton. However, the functional significance of these modulations remains to be determined.

4.3.2 ICSI and membrane traffic interference

Other than annexin A3 and α-SNAP, two more proteins involved in membrane traffic were identified up-regulated in ICSI placenta. CHP is a member of EF hand Ca^{2+}-binding superfamily and has been shown to have function in vesicle formation and fusion and membrane traffic [41]. COPE, another up-regulated protein in ICSI group, is the subunit of coatomer protein complex, which is usually associated with trans-Golgi network membrane traffic [42]. The differential expression of these proteins in ICSI group indicated that, compared to standard IVF, male infertility and micromanipulation of ICSI may have additional effect on the placenta membrane traffic function.

4.3.3 ICSI and protein catabolism alteration

Three proteins involved in protein catabolism and proteolysis were identified as differential expression in ICSI placenta. Among them, CTSD was down-regulated, while PSMA3 and LAP3 were up-regulated. CTSD is the precursor of cathepsin D and its mostly described function is intracellular catabolism in lysosomal compartments. It has been shown that cathepsin D was associated with angiogenesis and vascular regression in placental trophoblasts [43]. PSMA3 is a subunit of proteasome, which is a proteolytic complex and plays a critical role in the regulation of many physiological processes [44]. LAP3, another proteolysis protein, is presumably involved in the processing and regular turnover of intracellular proteins. The differential expression of these proteins indicated the malfunction of protein metabolism in placenta following ICSI treatment.

4.4 Functional associations of differentially expressed proteins in ART placenta

Taking all together, in ART placenta, the significant down-regulation of proteins involved in energy metabolism and up-regulation of hnRNP C1/C2 involved in DNA damage indicated that ART treatment could interfere with the placenta energy production and cause gene metabolism dysfunction through certain mechanism and it might result in poor outcome of ART offspring. On the other hand, as increased placental angiogenesis in IVP animal placenta has been found and considered as a compensatory mechanism of the decreased fetal-maternal contact [11, 45], we speculate that it may also be a compensatory mechanism of increased expression of annexin A3 and α-SNAP in ART placentas, according to their angiogenic and membrane fusion mediator function. Moreover, in IVF placenta, simultaneously increased expression of three stress-response proteins also suggests a cellular protective mechanism of increased environmental stress. Surprisingly, none of these proteins was found to be changed in ICSI group. In contrast, more proteins involved in membrane traffic and protein metabolism were found to be differentially expressed in ICSI placenta. At the same time, additional alteration of cytoskeleton proteins also existed in ICSI group. The differences of protein profile in two ART groups suggest that male infertility and invasive micromanipulation of ICSI might have additional effect on the placenta development and subsequent influence on the offspring outcome.

4.5 Conclusions

In this study, we performed a proteomic approach on the human placenta derived from ART with a combination of 2-DE and MALDI-TOF/TOF MS. By the application of very strict selection criteria for the determination of potentially important proteins, a small number of molecules were defined and identified. Our study allowed for the initial identification of nine proteins in standard IVF group and 16 proteins in ICSI group with significantly altered abundance compared to the normal placenta. These proteins seem to be related to various functions in placentation. Our results reveal for the first time that abnormal protein profiles are involved in ART placenta and these proteins may be valuable for the evaluation of potential association between ART manipulation and offspring outcome. Clearly, more work is needed to understand the roles of the differentially expressed proteins in ART placenta with worse outcome. It is also of great interest to find out the differences in the expression of proteins between standard IVF and ICSI groups. Moreover, large-scale analyses will still certainly be required for the safety evaluation of ART offspring.

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