Discovery of porcine maternal factors related to nuclear reprogramming and early embryo development by proteomic analysis

Qi Zhao, Zheng Guo, Shanhua Piao, Chunsheng Wang* and Tiezhu An*

Abstract

Background: Differentiated cell nuclei can be reprogrammed to a pluripotent state in several ways, including incubation with oocyte extracts, transfer into enucleated oocytes, and induced pluripotent stem cell technology. Nuclear transfer-mediated reprogramming has been proven to be the most efficient method. Maternal factors stored in oocytes have critical roles on nuclear reprogramming and early embryo development, but remain elusive.

Results: In this study, we showed most of porcine oocytes became nuclear matured at 33 h of IVM and the rate had no significant difference with oocytes at 42 h of IVM (p > 0.05). Moreover, the cleavage and blastocyst rates of SCNT and PA embryos derived from 42O were significantly higher than that of 33O (p < 0.05). But 33O could sustain IVF embryo development with higher cleavage and blastocyst rates comparing to 42O (p < 0.05). To clarify the development potential difference between 33O and 42O, 18 differentially expressed proteins were identified by proteomic analysis, and randomly selected proteins were confirmed by Western blot. Bioinformatic analysis of these proteins revealed that 33O highly synthesized proteins related to fertilization, and 42O was rich in nuclear reprogramming factors.

Conclusions: These results present a unique insight into maternal factors related to nuclear reprogramming and early embryo development.

Keywords: Proteomics, IVF, Nuclear transfer, Maternal factors

Introduction

Reprogramming of sperm or somatic cells into pluripotency has great attraction and value in both research and therapy. Recent advances in iPS have clearly indicated that differentiated cells can acquire pluripotency by overexpressing a small number of transcript factors, such as Oct4, Sox2, Klf4, and c-myc [1]. However, the reprogramming process is slow (at least 1 to 2wk), and the efficiency is low (typically about 1 %) [2]. In contrast, reprogramming by oocytes occurs within one or two cell cycles, and often in a majority of embryos, suggesting oocyte-mediated reprogramming resets differentiated cell states more efficiently than the iPS method and more factors must participate in the process [3–5]. So, understanding of these oocyte factors will provide us important information on nuclear reprogramming and embryo development.

Given how little is known about the factors in mammalian oocyte, there are several recent reports utilizing proteomics approaches to explore the oocyte proteomes in cattle, pig and mouse [6–15]. For example, Calvert et al. identified 8 highly abundant HSPs and related chaperones in the mature mouse egg by two-dimensional electrophoresis [16]. Vitale et al. used 2DE and MS to identify 12 proteins that appeared to be differentially expressed between GV and MII murine oocytes [12]. However, it has some limitations in identifying proteins that are related to nuclear reprogramming and early embryo development. So, a comparison of the proteomes of oocytes with different abilities of nuclear reprogramming and development may aid in the identification of the maternal factors.

In the study, we checked the effect of oocyte maturation time on early development of IVF, PA and SCNT embryos. Moreover, mass spectrometry was applied to
identify the maternal factors related to nuclear reprogramming and early embryo development, and we successfully discovered 18 differentially expressed proteins between 33O and 42O in pig.

**Materials and methods**

**IVM of porcine oocytes**

Porcine ovaries were collected from a local slaughter house and kept in saline at 32–37 °C. Antral follicles (3–5 mm indiameter) were aspirated with an 18-gauge needle. Aspirated oocytes with an evenly granulated cytoplasm and at least three uniform layers of compact cumulus cells were selected and cultured in four-well plates (Nunc, Naperville, IL, USA) containing 500 μL of maturation medium that was TCM199 (Gibco) based medium plus 0.05 μg/ml EGF, 0.5 μg/ml LH and FSH, at 39 °C in 5 % CO₂ in air [17]. The rates of polarbody extrusion were calculated from 16 h–42 h of IVM. Matured porcine oocyte was obtained at 33 h and 42 h for further experiments.

**IVF and cloned embryos production and in vitro culture**

For IVF, freshly ejaculated sperm-rich fractions were collected from fertile boars, and following a short incubation at 38.5 °C, the semen was resuspended and washed three times in Dulbecco’s Phosphate Buffered Saline (DPBS) supplemented with 0.1 % (w/v) bovine serum albumin (BSA) by centrifugation at 1500 × g for 4 min. The spermatozoa concentration was measured using a hemocytometer, and the proportion of motile sperm was determined. The spermatozoa were diluted with modified Tris-buffered medium (mTBM) to an optimal concentration. Cumulus-free oocytes at 33 h or 42 h of IVM were washed three times in mTBM. Approximately 30 oocytes were inseminated in 50-ml drops of mTBM at a final sperm concentration of 3 × 10⁶/ml for 6 h.

After 12 h co-incubation with spermatozoa, oocytes were fixed for 30 min in 4 % paraformaldehyde, and permeabilized for 20 min in 0.1 % triton X 100 following wash in PBS. Then oocytes were stained with 10 μg/ml Hoechst 33342 to visualize the nuclei. Samples were observed and photographed by using a Nikon Eclipse 80i epifluorescence microscope (Nikon, Tokyo, Japan). Oocytes with one or more swollen sperm head(s) and/or male pronuclei were considered as fertilized and fertilization rate was evaluated.

The procedure for porcine SCNT has been described previously [18]. After 33 h or 42 h of IVM, the oocytes were treated with 1 mg/ml hyaluronidase (H3506, Sigma-Aldrich, Missouri, USA) to remove the surrounding cumulus cells. Oocytes with a clearly extruded first polar body were selected as recipient cytoplasts. Cumulus-free oocytes were enucleated by aspirating the first polar body with a glass pipette 25 mm in diameter in TCM199-Hepes plus 0.3 % BSA and 7.5 mg/ml Cytochalasin B. Porcine ear fetal fibroblasts at passage 5 served as donor cells and were injected into the perivitelline space of enucleated oocytes. Injected oocytes were placed in fusion/activation medium (0.3 M mannitol, 1.0 mM CaCl₂, 0.1 mM MgCl₂, and 0.5 mM HEPES). Fusion/activation was induced with 2 DC pulses of 1.2kv/cm for 30 msec on a BTX Elector-Cell Manipulator 2001 (BTX, San Diego, CA). Cumulus-free oocytes were directly activated by the same parameters as SCNT procedure for producing PA embryos.

The embryos were cultured in porcine zygote medium-3 (PZM-3) at 39 °C in 5 % CO₂ in air. The cleavage and blastocyst rates were assessed at 48 and 156 h after activation, and the number of blastocyst cells was examined by nuclear staining with 5 μg/ml Hoechst 33342.

**Oocyte collection and proteomic analysis**

Zona pelluccida of more than 10,000 33O and 42O were removed (Additional file 1: Figure S1) and total proteins were extracted using ultrasonic wave and lysis buffer. The lysis buffer consisted of 7 M urea, 2 M thiourea, 4 % (w/v) CHAPS, 65 mM DTT, 2 % (v/v), and 1 % (v/v) protease inhibitor cocktail. The protein concentration was determined by the Bradford method and pH was adjusted to 8.5 with 50 mM NaOH. In fluorescent 2D DIGE, protein from 33O and 42O were equally pooled together and labeled with Cy2 as internal standard, and the two samples were labeled with Cy3 or Cy5, separately. Labelled samples were mixed in the rehydration buffer before to be loaded onto the into 24-cm pH 3–10 IPG strips (Bio-Rad) and run in a single two-dimensional gel. Then, the gels were scanned using the Typhoon 9410 scanner with excitation/emission wavelengths of 488/520 nm for Cy2, 532/580 nm for Cy3 and 633/670 nm for Cy5. Image analysis were performed by Decyder software suite 5.02 (GE Healthcare) which allow the comparison of the different combination corresponding to the experimental conditions. An independent t-test was used to determine the significance between the experimental groups. P-values less than 0.05, and fold-changes greater than 1.5 were considered statistically significant.

For protein identification, the spots of interest were cut from the gels, and washed with 25 mM NH₄HCO₃ and 50 % ACN solution and dehydrated with 100 % ACN sequentially and dried by centrifugal lyophilization. The gels were digested with 15-20μl of 0.01μg/ul trypsin (Promega) in 25 mM ammonium bicarbonate for 15 h at 37 °C. The supernatants were collected, and the tryptic peptides were extracted from the gel sequentially with 5 % TFA at 40u for 1 h and with 2.5 % TFA, 50 % ACN at 30 °C for 1 h. The extracts were pooled and completely dried by centrifugal lyophilization. Digested peptides with matrix (50 % acetonitrile, 0.1 % TFA containing 3 mg/ml alpha-cyano-4-hydroxy cinnamic acid matrix) were then
spotted on the target plate. Samples were analyzed by MALDI-TOF-TOF MS (4800 Proteomics Analyzer, Applied Biosystems) in positive reflectron mode at fixed laser fluency with low mass gate and delayed extraction. Database searching was carried out using Mascot version 2.2 (MatrixScience, London, UK) via GPS explorer software (ABI) version 3.6 combining MS and MS/MS interrogations on NCBI pig proteins database. In the searching parameter, modification was set as carbamido-methylation, oxidation, and a maximum of one missed trypsin cleavage was permitted. Tolerance of precursor and fragment ions was both set to 0.2 Da. The reported proteins were always those with the highest number of peptide matches. All identified proteins were those with a statistical significance (p ≤ 0.05) and the best ion score.

**Quantitative real-time PCR**
Total RNA was extracted from at least 100 oocytes using the PureLink™ Micro-to-Midi system (Invitrogen) according to the manufacturer’s instructions, and reverse transcription was used to generate cDNAs using the PrimeScript™ RT Reagent Kit (TaKaRa). Real-time PCR was performed using SYBR Premix Ex Taq™ (TaKaRa) and the 7500 Real-Time PCR System (Applied Biosystems). The reaction parameters were 95 °C for 30 sec, followed by 40 two-step cycles of 95 °C for 5 sec and 60 °C for 34 sec. Primers for each gene were listed (Additional file 1: Table S1). 18S rRNA was used as a reference gene. C_\text{t} \text{ values were calculated using Sequence Detection System software} (Applied Biosystems), and the amount of target sequence normalized to the reference sequence was calculated as 2^{ΔΔCt}.

**Western blot**
Oocytes removed zona pellucida, stored at –80 °C, were transferred to 10 μl cold 40 mM phosphate, pH 7.6, containing 50 mM NaCl, 50 μM sodium orthovanadate, 10 mM sodium fluoride, 20 μM MG-132, 2 μM matrix metalloprotease inhibitor III (444264, Calbiochem, San Diego, USA), and 1 % protease inhibitor cocktail III (539134, Calbiochem, San Diego, USA). Homogenization was carried out at 4 °C with a Tekmar homogenizer by three 15 s bursts with a minute cooling in-between. Homogenates were centrifuged at 4 °C for 1 h at 100 000 x g. The supernatant solutions were referred to as “soluble” fractions. The pellets were suspended in the 0.2-0.25 ml complete buffer containing 1 % ASB-14 and were mixed every 15 min for 2 h with Radnoti glass pestles (Unitek, Monrovia, USA). After centrifugation at 4 °C for 1 h at 100 000 x g, the supernatants, referred to as “membrane extracts”, were removed and the pellets were discarded. About 50 oocytes of each soluble and membrane extract for each gene testing were separated by lithium dodecyl sulfate polyacrylamide gel electrophoresis on 4-12 % Bis-Tris NuPAGE gels and transferred to PVDF membranes (Invitrogen, Carlsbad, USA); nonspecific binding was blocked by overnight incubation in 1 % casein in PBS at room temperature. Blots were then probed for 2–4 h at room temperature with antibodies of PDIA3 (anti-PDIA3; ab13507, abcam, Cambridge, USA), SOD1 (anti-SOD1; ab13498, abcam, Cambridge, USA) and VIM (anti-VIM; V6630, Sigma, USA), Histone H2B (anti-H2B; ab40975, abcam, Cambridge, USA) served as loading control. After 2 h incubation at room temperature with secondary antibodies, protein bands were detected by enhanced chemiluminescence with the RPN2108 kit (Amersham, Piscataway, USA) and Kodak BioMax Light film.

**Statistical analysis**
Statistical analysis was performed using SPSS 13.0 for MicroSoft™ Windows. Data are shown as the mean ± SD. One-way ANOVA was used to assess any differences between groups. The Duncan method was employed for pairwise comparisons, followed by a Bonferroni correction. p < 0.05 (two-tailed) was considered statistically significant.

**Results**
**Maturation of porcine oocytes during IVM**
To get the time lapse characteristics of maturation of porcine oocytes during IVM, we detected the rate of 1st polarbody extrusion from 16 h to 42 h. The oocyte began to extrude polarbody mainly at 25 h (14.45 %) of IVM and reached a plateau at 33 h (Fig. 1), and there was no significant difference between the rates at 33 h (76.72 %) and 42 h (81.80 %) of IVM (Additional file 1: Table S2; \( p > 0.05 \)). We also found most of 33O (79.71 %) were arrested at the MII stage, and it had no remarkable difference with 42O (83.86 %; Table 1). Theoretically, the maturation quality of 42O must be better than 33O [17–21].

**Effects of 33O and 42O on porcine embryo development**
In order to test the effect of maturation time on nuclear reprogramming and embryo development, we employed 33O and 42O to produce IVF, SCNT and PA embryos (Table 2). The results showed the rates of cleavage and blastocyst of IVF embryos from 33O were significant higher than that from 42O (78.36 % vs 67.22 %, \( p < 0.05 \); 33.79 % vs 19.73 %, \( p < 0.05 \), respectively). Also, the rate of fertilization of 33O was significantly enhanced compared to 42O (85.19 % vs 78.09 %, \( p < 0.05 \); Additional file 1: Table S3). On the contrary, the rates of cleavage and blastocyst of SCNT embryos from 42O were significant higher than that from 33O (89.17 % vs 67.60 %, \( p < 0.05 \); 22.34 % vs 13.54 %, \( p < 0.05 \), respectively), and the rates of enucleation of oocyte in both 33O and 42O
reached up to 90% (Additional file 1: Table S4), excluding the influence of enucleation on cloned embryo development. Moreover, the rates of cleavage and blastocyst of PA embryos from 42O were also significantly higher than that from 33O (92.23% vs 43.74%, p < 0.05; 43.64% vs 17.84%, p < 0.05), and there was no significant difference of pronuclear rates at 6 h after artificial activation between 33O and 42O (Additional file 1: Table S5), suggesting lower development of 33O may be not simply due to lower activation compared to 42O. These results indicate that further maturation of oocyte after 1st polarbody extrusion can accumulate maternal factors for SCNT and PA embryos development, but it may be not necessary for IVF embryo.

Identification of maternal factors involved in nuclear reprogramming and embryo development
To uncover the basis for nuclear reprogramming and embryo development, global protein changes between 33O and 42O were examined by proteomic analysis. After oocytes collection and treatment, the total proteins were separated by 2D DIGE (Fig. 2). Analysis of the gel images showed 994 paired protein spots, and we used independent t-test to calculate differentially expressed proteins. We considered only spots with fold-changes greater than 1.5 to exclude the possibility of false positives.

Table 1 The rates of oocytes 1st polarbody extrusion at 33 h and 42 h of IVM

| Culture period | No. of oocytes (repeats) | No. of MII oocytes (% ± SEM) |
|---------------|--------------------------|-----------------------------|
| 33 h          | 119 (3)                  | 95 (79.71 ± 0.86)           |
| 42 h          | 125 (3)                  | 105 (83.86 ± 0.76)          |

Highlights:
1. The majority of porcine oocytes achieve nuclear maturation at 33 h of IVM and proceed with cytoplasmic maturation from 33 h to 42 h.
2. Porcine oocyte during 33 h of IVM is able to sustain IVF embryo development, and further cytoplasmic maturation is indispensable for development of SCNT and PA embryos.
3. 18 differentially expressed proteins between oocytes during 33 h and 42 h of IVM are identified by mass spectrometry.

Fig. 1 The porcine oocytes during IVM. a The polarbody extrusion during IVM of porcine oocytes. The polarbody extrusion mainly began at 25 h (14.45%) of IVM, and the rate at 33 h reached the plateau. The rates of porcine oocyte polarbody extrusion at 33 h (76.72%) and 42 h (81.80%) of IVM had no significant difference (p > 0.05). b Fluorescence micrographs of nuclei in porcine MII oocytes (200x). DNA was blue, and the MII nuclei was marked by *.
positives by multiple comparisons. In total, 25 protein spots were found to be differentially expressed with fold-changes greater than 1.5 ($p < 0.05$; Fig. 3a), and finally 18 proteins were identified by MALDI-TOF/MS. Based on 2D DIGE analysis, 7 proteins were down-regulated and 11 proteins were up-regulated in comparison of 42O to 33O (Fig. 3b and Table 3). The 7 over-expressed proteins in 33O were indentified as protein-arginine deiminase type-6 (PADI6), major vault protein isoform 1 (MVP), inositol polyphosphate-1-phosphatase (INPP1), glucose regulated protein 58 (PDIA3), glial fibrillary acidic protein (GFAP), 90-kDa heat shock protein (HSP90B1), and beta-actin (ACTG1). The 11 high expressed proteins in 42O were trypsinogen precursor (PRSS1), ADP-sugar pyrophosphatase-like isoform 1 (NUDT5), vimentin-like (VIM), heat shock 70 kDa protein 1 (HSP90AB1), eukaryotic translation elongation factor 1 alpha 1 (EEF1A1), similar to GLUD1 protein (GLUD1), glutathione S-transferase mu2 (GSTM2), superoxide dismutase [Cu-Zn] (SOD1), DJ-1 protein (PARK7), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). To confirm the results, 2D DIGE data were examined by Quantitative realtime PCR and Western blot (Fig. 4). Though PCR results of 13 genes were inconsistent with the 2D DIGE results (Fig. 4a), the gray values of 3 randomly selected spots (PDIA3, SOD1 and VIM) in the 2D DIGE (Fig. 4b) were in agreement with the protein expressions of these genes checked by Western blot (Fig. 4c).

### Functional categorization and pathway analysis of the identified factors

We categorized proteins identified in this study by searching Gene Ontology and performing a literature search. The differentially expressed proteins were classified into groups based on molecular function (structural constituent of cytoskeleton, unfolded protein binding, nucleotide binding, NAD or NADH binding, ribonucleotide binding, calcium ion binding and ATP binding), biological process (cellular homeostasis, response to inorganic substance, response to drug, intermediate, cell death, cellular ion homeostasis, maintenance of location, antioxidation and monosaccharide catabolic), and cellular component (cytoplasm, membrane, endoplasmic reticulum, cytoskeleton, cytosol, perinuclear region of cytoplasm and nucleus) with the UniProt database (Fig. 5). DAVID and Agilent Literature Search were combined by cytoscape to cluster most affected

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### Table 2 The effect of 33O and 42O on IVF, NT and PA embryo development

| Groups | Culture period | Repeats | No. oocytes | No. embryos fused (% ± SEM) | No. embryos cleaved (% ± SEM) | No. blastocysts (% ± SEM) | Total cell No. of blastocysts (mean ± SEM) |
|--------|----------------|---------|-------------|-----------------------------|-------------------------------|--------------------------|-------------------------------------------|
| IVF    | 33 h           | 5       | 314         | –                           | 247 (78.36 ± 1.19)a           | 106 (33.79 ± 1.89)a        | 39 ± 3 (n = 103)                           |
|        | 42 h           | 5       | 309         | –                           | 210 (67.22 ± 2.53)b           | 60 (19.73 ± 1.2)b          | 36 ± 5 (n = 50)                            |
| NT     | 33 h           | 4       | 411         | 280 (67.56 ± 4.71)          | 195 (67.60 ± 5.63)a           | 36 (13.54 ± 2.58)a         | 35 ± 2 (n = 33)                            |
|        | 42 h           | 4       | 750         | 467 (61.86 ± 4.68)          | 233 (89.17 ± 2.15)b           | 59 (22.34 ± 2.18)b         | 37 ± 2 (n = 57)                            |
| PA     | 33 h           | 7       | 537         | –                           | 232 (43.74 ± 2.62)a           | 88 (17.84 ± 2.06)a         | 36 ± 3 (n = 83)                            |
|        | 42 h           | 7       | 465         | –                           | 428 (92.23 ± 2.60)b           | 200 (43.64 ± 3.18)b        | 37 ± 4 (n = 96)                            |

Values with different superscripts in the same group differ significantly ($p < 0.05$)
molecular functions in oocyte maturation, namely embryonic development, embryogenesis, ovulation, epigenetic modification, fertilization, meiosis, ageing, chromatin remodeling and anti-ageing. Additionally, we employed a detailed analysis of embryonic development, using automated text-mining tool which enables the software to generate pathways from entries in the PubMed database and Gene Ontology. These analysis revealed that proteins related to fertilization were found to be highly synthesized in 33O, such as PDIA3 [16], and nuclear reprogramming was significantly affected by high expressed proteins in 42O, such as PARK7 that was proved to be required for successful development of porcine SCNT embryos [22].

Discussion
An increasing amount of data demonstrate that oocyte maturation involves major changes within the cytoplasmic compartment and these changes are essential for nuclear reprogramming and embryo development [14]. However, the molecular factors remain largely unknown. In the study, we found that 33O and 42O exhibited distinct developmental competences after IVF, SCNT and PA. Additionally, 18 differentially expressed proteins between 33O and 42O were identified by MALDI-TOF/MS in porcine oocytes.

Insufficient maturation of oocyte is not enough to support the subsequent embryo development, and oocyte will be senescent if overmatured [23]. It has been generally considered that porcine oocyte reaches full maturation at 42 h of IVM, and a number of research groups including us use oocytes matured at 42 h for IVF, PA and SCNT study [17–21]. In the study, we demonstrated the rate of 1st polarbody extrusion of porcine oocytes reached a plateau at 33 h of IVM and the most part of the oocytes arrested at the MII stage. The result was not consistent with a previous report that described porcine oocyte arrived at MII stage during 34.4-48 h of IVM [24], and this may result from the addition of EGF to the IVM medium in the experiments for this research, which may have accelerated the nuclear maturation as already observed in other study [25].

Oocyte maturation is among the most crucial factors affecting embryo development [26–29]. So, we described the effects of 33O and 42O on embryo development of IVF, SCNT and PA. In consistent with previous reports [26–28], the developmental competence of SCNT and PA embryos by 42O were markedly improved in comparison with 33O, indicating that full cytoplasmic maturation is indispensable for development of SCNT and PA embryos. However, surprisingly, 33O was able to sustain IVF embryo development with higher competence comparing to 42O. This is in line with previous results showing that a suitable culture period for IVM of porcine oocytes to IVF is 32 to 36 h [30]. These results could be explained by the following reasons: firstly, according to our results, the rate of fertilization of 33O was higher, so more zygotes may initiate subsequent embryo development; secondly, the nuclear reprogramming of sperm and somatic cell undergoes two distinct processes. The success of SCNT depends on several parameters that impact on the ability of oocyte to remodel the nuclear morphology of donor cell, to reverse the epigenetic modifications to totipotent status, and to activate the embryonic gene expression required for further
development [31]. So, the great requirement of reprogramming factors for SCNT must be different with IVF and 42O is rich in these factors [32, 33]; thirdly, the stress-coping mechanisms are probably relying on different molecules between SCNT and IVF embryos. This notion is supported by the fact that SCNT embryos exhibit higher rates of apoptosis, weaker stress-coping functions and are less tolerant to the in vitro culture environment than IVF embryos [34]. Lower level of gene expression related to cell protection from stress and apoptosis has been reported in oocyte with low cytoplasmic maturation quality [35], such as SOD1, one of the major antioxidant enzymes, which was proved to scavenge superoxide during early embryonic development [36, 37], which was found abundantly in 42O by our proteome analysis. Considering that, it is reasonable to deduce that transferred somatic nuclei undergo lower cellular stress than sperms in 42O, so that 42O can support better development of SCNT embryos.

During the last decade, several approaches analyzing the protein composition of oocyte have been conducted including the proteome signature of porcine oocyte [6–15], however, the disadvantage of these proteome analyses is that no factor related to nuclear reprogramming and embryo development has been described. In the study, efforts have been made to discover differentially expressed proteins between 33O and 42O by proteome analysis.

### Table 3 Protein identifications by MS/MS analysis of oocytes which were significantly increased or decreased upon 42O, as compared with 33O

| Spot no | Down-regulated | Accestion ID | Fold Diff. | Mw | Peptides matched | S cov % | T-test (p) | Classification |
|---------|----------------|-------------|------------|----|-----------------|---------|------------|----------------|
| 1-2     | PREDICTED: protein-arginine deiminase type-6 | 350585686 PADI6 | $-2.6/-1.73$ | 545 | 79159.2 | 18 | 41.723 | 4.90E-02 | enzyme |
| 3       | PREDICTED: major vault protein isomorph 1 | 335284397 MVP | $-1.85$ | 557 | 99592.5 | 36 | 66.638 | 1.40E-02 | enzyme |
| 4       | inositol polyphosphate-1-phosphatase | 350529419 INPP1 | $-1.61$ | 521 | 44727.2 | 6 | 19.694 | 2.10E-02 | enzyme |
| 5-6     | glucose regulated protein S8 | 301016769 PDIA3 | $-2.07/-1.91$ | 593 | 56822.9 | 21 | 55.766 | 4.10E-02 | oxidoreductase, isomerase |
| 7       | proteinlial fibrillary acidic | 223869 GFAP | $-1.86$ | 596 | 24946.7 | 7 | 3.791 | 5.70E-03 | Cytoskeleton |
| 8       | 90-kDa heat shock protein | 1945447 HSP90B1 | $-1.52$ | 493 | 84721.7 | 27 | 4.224 | 6.80E-03 | Chaperone |
| 9       | beta-actin | 112980807 ACTG1 | $-1.55$ | 55 | 29392.7 | 8 | 44.348 | 3.70E-02 | Cytoskeleton |
| 10      | glyceraldehyde-3-phosphate dehydrogenase | 229279 GAPDH | 1.96 | 6.9 | 35686.2 | 6 | 14.312 | 3.00E-02 | Metabolism |
| 11-12   | DJ-1 protein | 67038688 PARK7 | 1.94/1.52 | 6.33 | 19923.5 | 10 | 28.298 | 4.70E-02 | Chaperone, oncogene, antioxidation |
| 13      | superoxide dismutase [Cu-Zn] | 298677090 SOD1 | 1.74 | 6.03 | 15881.9 | 5 | 28.238 | 2.30E-02 | Antioxidation |
| 14      | glutathione S-transferase mu 2 | 116047847 GSTM2 | 1.69 | 6.9 | 25746.9 | 4 | 4.67 | 4.80E-02 | enzyme |
| 15      | PREDICTED: similar to GLUD1 protein | 194042282 GLUD1 | 1.6 | 10.28 | 38321.5 | 13 | 8.87 | 2.60E-02 | Metabolism |
| 16      | eukaryotic translation elongation factor 1 alpha 1 | 223019599 EEF1A1 | 2.08 | 9.1 | 50109.1 | 9 | 8.74 | 3.40E-02 | enzyme/signal transducer |
| 17      | PREDICTED: heat shock 90kD protein 1, beta | 194033931 HSP90AB1 | 1.86 | 4.96 | 83201.2 | 27 | 42.224 | 2.10E-02 | Chaperone |
| 18      | PREDICTED: heat shock 70 kDa protein 5 | 194033595 HSPA5 | 1.71 | 5.68 | 73767.5 | 24 | 40.616 | 1.40E-02 | Chaperone |
| 19      | PREDICTED: vimentin-like | 76097691 VM | 1.71 | 4.79 | 34288.2 | 6 | 15.727 | 1.20E-02 | Cytoskeleton |
| 20      | PREDICTED: ADP-sugar pyrophosphatase-like isomorph 1 | 350589625 NUDT5 | 1.53 | 5.14 | 24253.2 | 4 | 31.635 | 1.00E-02 | Metabolism |
| 21      | trypsinogen precursor | 238867676 PRSS1 | 1.96 | 6.85 | 25864.6 | 3 | 10.866 | 6.60E-03 | enzyme |

| Spot no | Up-regulated | Accestion ID | Fold Diff. | Mw | Peptides matched | S cov % | T-test (p) | Classification |
|---------|-------------|-------------|------------|----|-----------------|---------|------------|----------------|
| 10      | glyceraldehyde-3-phosphate dehydrogenase | 229279 GAPDH | 1.96 | 6.9 | 35686.2 | 6 | 14.312 | 3.00E-02 | Metabolism |
| 11-12   | DJ-1 protein | 67038688 PARK7 | 1.94/1.52 | 6.33 | 19923.5 | 10 | 28.298 | 4.70E-02 | Chaperone, oncogene, antioxidation |
| 13      | superoxide dismutase [Cu-Zn] | 298677090 SOD1 | 1.74 | 6.03 | 15881.9 | 5 | 28.238 | 2.30E-02 | Antioxidation |
| 14      | glutathione S-transferase mu 2 | 116047847 GSTM2 | 1.69 | 6.9 | 25746.9 | 4 | 4.67 | 4.80E-02 | enzyme |
| 15      | PREDICTED: similar to GLUD1 protein | 194042282 GLUD1 | 1.6 | 10.28 | 38321.5 | 13 | 8.87 | 2.60E-02 | Metabolism |
| 16      | eukaryotic translation elongation factor 1 alpha 1 | 223019599 EEF1A1 | 2.08 | 9.1 | 50109.1 | 9 | 8.74 | 3.40E-02 | enzyme/signal transducer |
| 17      | PREDICTED: heat shock 90kD protein 1, beta | 194033931 HSP90AB1 | 1.86 | 4.96 | 83201.2 | 27 | 42.224 | 2.10E-02 | Chaperone |
| 18      | PREDICTED: heat shock 70 kDa protein 5 | 194033595 HSPA5 | 1.71 | 5.68 | 73767.5 | 24 | 40.616 | 1.40E-02 | Chaperone |
| 19      | PREDICTED: vimentin-like | 76097691 VM | 1.71 | 4.79 | 34288.2 | 6 | 15.727 | 1.20E-02 | Cytoskeleton |
| 20      | PREDICTED: ADP-sugar pyrophosphatase-like isomorph 1 | 350589625 NUDT5 | 1.53 | 5.14 | 24253.2 | 4 | 31.635 | 1.00E-02 | Metabolism |
| 21      | trypsinogen precursor | 238867676 PRSS1 | 1.96 | 6.85 | 25864.6 | 3 | 10.866 | 6.60E-03 | enzyme |

*Fold change represents protein upregulate (+) or downregulate (−) in 42O compared with 33O
*Percentage sequence coverage (S cov %) if the mascot score was significant (P < 0.05)
*Theoretical isoelectric point (pI) and theoretical molecular weight (Mw)
*p-value from t-test of the protein level between 33O and 42O
unique protein spots were detected by 2D DIGE, and only 25 protein spots were found to be differentially expressed between 33O and 42O. That is to say, the difference was no more than 2 % (25/994), indicating the proteome signature of 33O and 42O were very similar. But the developmental competence of 33O and 42O were largely different, suggesting that the few differential proteins identified played important roles in embryo development. This is in line with previous results that only a few proteins which are indispensable for embryo development are largely synthesized from stored mRNAs in the final stage of oocyte maturation [32, 33].

To further clear the molecular basis for cytoplasmic maturation, we focused on function analysis of these identified proteins. Proteins, such as HSP90B1, HSPA5 and HSP90AB1, with chaperone function were most commonly found, reflecting a marked change of nuclear proteins during oocyte maturation [7, 13]. Cluster analysis of the 18 proteins with the UniProt database showed a clear trend towards molecular functions and biological processes related to protein synthesis, including ribonucleotide binding, nucleotide binding and cellular homeostasis, that confirm the points that accumulation of proteins happen constantly during cytoplasmic maturation [7, 13]. Moreover, the influence of the differential expression factors on development of IVF and SCNT embryos was unraveled by pathway analysis of these proteins using DAVID and Agilent Literature Search. INPP1 and PDIA3 are predominantly involved in fertilization and were found to be over-expressed in 33O. Also, a characterized nuclear reprogramming factor, PARK7, was more abundant in 42O [22]. This led us to suggest that the 18 identified proteins were mainly responsible for nuclear reprogramming and early embryo development.

**Conclusions**

In summary, the study identified the differentially expressed proteins between porcine oocytes during 33 h and 42 h of IVM. We showed that majority of porcine oocytes have achieved nuclear maturation at 33 h of IVM, and there are 18 protein factors related to nuclear reprogramming and embryo development discovered by proteome analysis of 33O and 44O. Analysis of these factors indicates several of them may be responsible for the development competence difference between 33O and 42O when used for IVF and SCNT. Our study
The classification of the identified proteins was performed according to the gene ontology term “molecular function”, "biological process" and "cellular component" provides valuable information to investigate the functions of maternal proteins. Such efforts will enable us to better understand the molecular mechanism of nuclear reprogramming and embryo development.

Additional file

Additional file 1: Figure S1. Representative pictures of 33O and 42O with and without zona pellucida. Table S1. Details of primers used for Realtime PCR analysis. Table S2. The rates of porcine oocyte polar body extrusion at 33h and 42h of IVM. Table S3. The effect of 33O and 42O on IVF. Table S4. The enucleation rates of 33O and 42O. Table S5. The pronuclear rates of 33O and 42O at 6h after artificial activation.

Abbreviations

SCNT: Somatic cell nuclear transfer; PA: Parthenogenetic activation; IVF: In vitro fertilization; IVM: In vitro maturation; 42O: Porcine oocytes with 1st polarbody during 42 h of IVM; 33O: Porcine oocytes with 1st polarbody during 33 h of IVM; GV: Germinal vesicle; GVBD: Germinal vesicle breakdown; MII: Metaphase II; IPS: Induced pluripotent stem cells; HSPs: Heat shock proteins; MS: Mass spectrometry; 2D DIGE: Two-dimensional difference gel electrophoresis.

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

ATZ and WCS designed and conceived the experiments; ZQ and GZ conducted the oocyte and embryo manipulations; ZQ and PSH conducted molecular experiments; ZQ contributed to bioinformation analysis; PSH conducted the cell manipulations. WCS and ZQ wrote and all authors reviewed the manuscript. All authors read and approved the final manuscript.

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