Proteomic analysis of porcine oocytes during in vitro maturation reveals essential role for the ubiquitin C-terminal hydrolase-L1

Andrej Susor, Zdenka Ellederova, Lucie Jelinkova, Petr Halada¹, Daniel Kavan¹, Michal Kubelka and Hana Kovarova

Institute of Animal Physiology and Genetics, Academy of Sciences of the Czech Republic, Rumburska str. 89, 277 21 Libechov, Czech Republic and ¹Institute of Microbiology, Academy of Sciences of the Czech Republic, 142 20 Prague, Czech Republic

Correspondence should be addressed to H Kovarova; Email: kovarova@iapg.cas.cz

Abstract

In this study, we performed proteomic analysis of porcine oocytes during in vitro maturation. Comparison of oocytes at the initial and final stages of meiotic division characterized candidate proteins that were differentially synthesized during in vitro maturation. While the biosynthesis of many of these proteins was significantly decreased, we found four proteins with increased biosynthetic rate, which are supposed to play an essential role in meiosis. Among them, the ubiquitin C-terminal hydrolase-L1 (UCH-L1) was identified by mass spectrometry. To study the regulatory role of UCH-L1 in the process of meiosis in pig model, we used a specific inhibitor of this enzyme, marked C30, belonging to the class of isatin O-acyl oximes. When germinal vesicle (GV) stage cumulus-enclosed oocytes were treated with C30, GV breakdown was inhibited after 28 h of culture, and most of the oocytes were arrested at the first meiosis after 44 h. The block of metaphase I–anaphase transition was not completely reversible. In addition, the inhibition of UCH-L1 resulted in elevated histone H1 kinase activity, corresponding to cyclin–dependent kinase (CDK1)–cyclin B1 complex, and a low level of monoubiquitin. These results supported the hypothesis that UCH-L1 might play a role in metaphase I–anaphase transition by regulating ubiquitin-dependent proteasome mechanisms. In summary, a proteomic approach coupled with protein verification study revealed an essential role of UCH-L1 in the completion of the first meiosis and its transition to anaphase.


Introduction

The mammalian oocyte is the cornerstone of reproductive biology. Recent advantages in technologies such as assisted reproduction, nuclear transfer, and embryonic or adult stem cell derivation have been significant, and development in this field is enormous. In spite of this progress, our knowledge of molecular networks underlying fine mechanisms of the reproductive processes remains elusive. Deeper understanding of these mechanisms should help to improve poor developmental potential of in vitro-produced embryos, their successful implantation, and maintenance of pregnancy.

Fully grown mammalian oocytes are arrested at the dictyate stage of meiosis I (M I) and can be induced to resume the meiotic cell cycle by a surge of luteinizing hormone or after the release from their follicles in vitro. Although several in vitro culture systems for mammalian oocyte maturation, fertilization, and embryo development have been established, the final outcome is still not satisfactory (Prather & Day 1998, Nagai 2001, Pavlok et al. 2005). Transcriptional activity of the mammalian oocyte rapidly decreases during maturation; therefore, it is expected that the important information necessary for full meiotic and developmental competence of oocytes could be retained at the protein level. Recent findings suggest that the coordination of a large number of events is controlled by a network of protein interactions and/or posttranslational modifications, such as phosphorylation (Dai et al. 2003, Kraft et al. 2003). The M-phase promoting factor (MPF) is one of the main regulators of meiosis. The CDK1 kinase, the catalytic subunit of MPF, is stored in oocytes as an inactive protein and its activation needs, at the first level, the association with cyclin B1 which is known as the regulatory subunit of MPF and whose synthesis and degradation oscillates during the cell cycle (Pines & Hunter 1990, Jackman et al. 2003). Other reports demonstrated, however, that mitogen-activated protein (MAP) kinases have an important role during M-phase. They can be involved not only in spindle assembly but also in regulation of cap-dependent translation initiation and its contribution to the changes in overall protein synthesis during in vitro meiotic maturation of mammalian oocytes (Sun & Nagai 2003, Ellederova et al. 2006, 2007). The precise timing of
protein synthesis and degradation plays an important role in controlling oocyte maturation (Moor & Dai 2001, Coenen et al. 2004). In this context, the involvement of the ubiquitin–proteasome complex, an essential component of the proteolytic pathway in eukaryotic cells, has been demonstrated in the regulation of pig oocyte meiotic maturation and fertilization; however, the major regulatory molecular events are unknown (Sun et al. 2004, Pines & Lindon 2005).

Until recently, there have been relatively few studies examining genomes and proteomes of germ cells as well as embryos at a global level (Colonna et al. 1989, Latham et al. 1991, Sasaki et al. 1999). Molecular investigations of gene expression or protein composition of germ cells or embryos have been sparse due to the paucity of sample cells and sufficiently sensitive procedures to analyze and identify them. With the progress of technologies, including linear amplification of cDNA populations, it has been possible to consider gene profiling in this biological model of extremely limited availability (Robert et al. 2001, Goto et al. 2002, Dalbies-Tran & Mermillod 2003, Zeng & Schultz 2003). In addition, the number of functional proteomic analyses identifying biologically relevant candidate proteins which may be involved in the regulation of oocyte maturation, embryo development, or oviductal proteome is gradually increasing in spite of the limited availability of human germ cells and lack of complete genome sequences of other mammalian species (Georgiou et al. 2005, Horiguchi et al. 2006, Massicotte et al. 2006, Vitale et al. 2007).

In this study, we used a proteomics approach to analyze changes in protein synthesis of porcine oocytes during in vitro maturation. Comparative analysis of the oocytes at the initial and final stages of meiotic division characterized candidate proteins that are differentially synthesized during in vitro maturation. While the biosynthesis of many of these proteins was significantly decreased, we found four proteins with increase in biosynthetic rate, which are supposed to play a critical role during oocyte meiotic maturation. Among them, the ubiquitin C-terminal hydrolase-L1 (UCH-L1) was identified by mass spectrometry. In addition, two-dimensional gel electrophoresis (2-DE) followed by immunoblot revealed the presence of three spots corresponding to UCH-L1 protein with the most acidic form increased at the final stage of oocyte maturation compared with the initial stage. An essential requirement of this oocyte protein for the process of meiosis in pig model was verified using a specific inhibitor of UCH-L1.

**Results**

**Protein patterns of biosynthesis during in vitro maturation of porcine oocytes and identification of UCH-L1**

To investigate overall changes in protein biosynthesis involved in the in vitro porcine oocyte maturation, 2-DE of biosynthetically labeled oocyte proteins was performed. Four gels for each type of cell population were used for comparative analysis of protein profiles of GV, M I, and M II stages of oocyte maturation. Using PDQuest analysis software version 7.1 (Bio-Rad), an average of 220 ± 44 (n = 4) and 201 ± 38 (n = 4) protein spots was mapped for GV and M I oocytes respectively, while 120 ± 23 (n = 4) were detected from M II oocytes (Fig. 1). A statistical comparison between the initial GV and final M II groups of gels using Student’s t-test implemented in PDQuest software identified 16 protein spots that were significantly (P < 0.05) attenuated or amplified during in vitro maturation. Most of the 16 significantly altered protein spots were down-regulated during oocyte meiotic division, but four were up-regulated. Among these four spots, the protein in one spot (SSP 1108) from the Coomassie-stained gel was identified by mass spectrometry. Attempts to identify other selected proteins failed due to their low abundance or the incomplete knowledge of the pig genome. Representative protein features are presented in Fig. 2.

Among the spots whose biosynthesis was apparently up-regulated in M II stage of oocyte matured in vitro, SSP 1108 corresponded to UCH-L1. The protein spot was unambiguously identified by peptide mass mapping approach (for details see Materials and Methods). Briefly, the protein was digested directly in the gel by trypsin and the generated peptide mixture was analyzed by MALDI mass spectrometry. Seven peptide fragments covering 35% of UCH-L1 sequence were found in the obtained MALDI spectrum (Fig. 3). In addition, steady-state level of UCH-L1 revealed by specific immunodetection was high and it increased slightly in the course of meiotic division. More detailed 2-DE followed by immunoblot revealed the presence of three spots corresponding to UCH-L1 protein with the most acidic form increased at the final stage of oocyte maturation when compared with the initial stage (Fig. 4).

**Inhibition of meiotic progression by UCH-L1 inhibition**

When COCs were cultured in maturation medium (MM) containing 10, 50, or 100 μM C30 for 28 h, meiosis resumption and GV breakdown (GVBD) were inhibited in oocytes treated with 50 and 100 μM C30, while 10 μM C30 did not affect these steps. In comparison, most oocytes underwent GVBD and progressed to M I in control group treated in inhibitor-free medium (Table 1).

The oocytes treated with 10, 50, or 100 μM C30 for 44 h were not capable to reach successfully M II stage in the presence of either 10 or 50 μM concentrations of C30 and approximately half of the oocytes remained in M I stage. For the highest used concentration of C30 (i.e., 100 μM), about 25% oocytes were still blocked in the GV stage, while 75% underwent GVBD and progressed...
to M I. On the contrary, the group of control oocytes cultured in the inhibitor-free medium comprised more than 80% of M II oocytes (Table 2).

When the oocytes that had been cultured with 100 μM C30 for 4, 8, 12, and 28 h were transferred to inhibitor-free medium and cultured until the total time period was 44 h, nearly half of the oocytes treated for 8 h in the presence of C30 were affected and remained in M I stage, while the second half was capable to reach M II. In the presence of inhibitor for the first 12 h of culture, about 20% of oocytes were still kept in GV stage and about 50% reached M I, while only about 30% of oocytes completed maturation to M II stage. The presence of C30 for 28 h followed by 16-h culture in MM results to the approximately same distribution of oocyte stages comparable to 12-h inhibition (Table 3).

**Regulation of CDK1 activity and monoubiquitin level in the course of UCH-L1 inhibition**

To assess whether UCH-L1 inhibition influences activity of CDK1 which is controlled by the availability of cyclin B1 and level of monoubiquitin, we examined the effect of 100 μM C30 using a kinase assay and Western blot respectively. The double kinase assay measured the ability of CDK1 kinase and myelin-binding protein (MBP) kinase (corresponding to ERK1/2 MAP kinase) to phosphorylate histone H1 and MBP respectively. Both histone H1 kinase and MBP kinase possess minimum activity levels at GV stage (time 0) but they are highly active after 20–24 h of culture, e.g., at the time when GVBD is observed. The activity of H1 kinase declines temporarily in untreated oocytes after 28 h of culture corresponding to metaphase I–anaphase transition. On
the contrary, H1 kinase activity in C30-treated oocytes increases continuously, peaking at 28 h of culture and then declining slowly until 44 h of culture. On the other hand, MBP kinase activity increases gradually and remains at a high level for up to 36 h of culture in both control and inhibitor-treated oocytes, although the onset of activity appears to be delayed in the presence of inhibitor. Furthermore, as revealed by specific immunodetection, a temporary decrease in the level of monoubiquitin occurs in C30-treated oocytes after 28 h of culture compared with untreated oocytes (Fig. 5).

Discussion

A major challenge for the improvement of poor developmental potential of in vitro-produced embryos, their successful implantation, and maintenance of pregnancy is to elucidate molecular networks underlying fine mechanisms of reproductive processes. Here, we utilized a proteomics approach and model of porcine oocytes maturing in vitro after their release from follicles. We considered this animal model suitable to initiate proteomic analyses with respect to relatively good availability of oocytes and its utilization in biomedical research (Vodicka et al. 2005). Pig oocytes at a GV stage can be obtained from ovaries of slaughtered noncycling gilts by the aspiration of antral follicles of about 3–5 mm. Their in vitro meiotic maturation resulted in the metaphase II stage in ~85% oocytes.

In a previous study, we profiled constituent proteins of pig oocytes using 2-DE and mass spectrometry. Remarkably, among identified proteins we found several proteins, including peroxiredoxins, ubiquitin carboxyl-terminal hydrolase isozyme L1, and spermine synthase, which are even more abundant than actin, usually the most abundant protein in somatic cells (Ellederova et al. 2004). Surprisingly, UCH-L1 alone constituted ~6% of total identified oocyte proteins.

In this study, we have focused on overall changes in protein biosynthesis involved in in vitro porcine oocyte maturation. We considered only biosynthetically labeled proteins that were significantly different at the 95% probability level in comparison between initial (GV) and final (M II) stages of oocyte maturation. Using these
criteria, we found 16 protein spots that displayed reproducible quantitative differences in relative intensities. Mass spectrometry identified satisfactorily only one up-regulated protein, UCH-L1. In addition to the results showing the increase in biosynthesis, further immunoblot analysis showed that overall level of UCH-L1 increases during maturation. These findings demonstrated a possibility that this protein plays a major role in pig oocyte function and its characterization indicated that ubiquitin-dependent processes would play an essential role during maturation of oocytes.

Ubiquitination and ubiquitin-dependent proteolysis are the major routes of intracellular protein degradation. Ubiquitin tagging of target proteins is a four-step process, including the activation of protein-ligating enzymes, and it operates in all eukaryotic cells (Doherty et al. 2002). The monoubiquitin-conjugated target protein can serve as an ubiquitylation substrate for repeated ubiquitination leading to polyubiquitination of the protein target. These polyubiquitylated proteins are then degraded by a multicomponent protease system, the 26S proteasome. The small peptides resulting from degradation are further processed for antigen presentation or hydrolyzed by other proteases. The role of deubiquitination in the regulation of this process is not clear, however, it may restrict the length of the ubiquitin chain, thus preventing selection for proteasomal degradation. Deubiquitinating enzymes are subdivided into ubiquitin-specific proteases and ubiquitin carboxy terminal hydrolases (UCHs). Mammalian UCHs, the isoenzymes UCH-L1 and UCH-L3, are small proteins consisting of about 220 amino acids with >40% sequence identity (Wilkinson et al. 1989). While UCH-L3 is ubiquitously distributed, UCH-L1 is normally selectively expressed in the neuronal cells and in the testis/ovary (Wilkinson et al. 1992, Kon et al. 1999). In addition to its hydrolase activity, it can act as an ubiquitin-protein ligase and catalyze ubiquitin transfer to a lysine residue of another ubiquitin molecule.

Figure 3 Tryptic peptide mass map of pig UCH-L1 protein. Peak labels correspond to \([M+H]^+\) ions of the individual peptides and amino acid positions of these fragments in UCH-L1 sequence. Underlined amino acid stretches of UCH-L1 sequence were verified by peptide mass mapping. Signals of trypsin autoproteolytic fragments are labeled with the letter T.

Figure 4 Immunoblot analysis of UCH-L1 during in vitro maturation of porcine oocytes. (A) Protein lysates prepared from oocytes in GV (initial), MI, and M II (final) stages of oocyte in vitro maturation were examined on immunoblot using a specific antibody-recognizing UCH-L1. (B) Additional 2-DE protein separation followed by immunoblot revealed the presence of three protein spots corresponding to UCH-L1.
Table 1 Morphology of pig oocytes treated with C30 inhibitor for 28 h.

<table>
<thead>
<tr>
<th>Treatment of oocytes</th>
<th>Total no. of oocytes (28h)</th>
<th>GV oocytes (%)</th>
<th>M I oocytes (%)</th>
<th>M II oocytes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 28h</td>
<td>129</td>
<td>11 (8.6) ab c</td>
<td>103 (79.8) ab c</td>
<td>15 (11.6) ab c</td>
</tr>
<tr>
<td>10 µM C30</td>
<td>57</td>
<td>7 (12.3)</td>
<td>44 (77.2)</td>
<td>6 (10.5)</td>
</tr>
<tr>
<td>50 µM C30</td>
<td>82</td>
<td>31 (37.8) a</td>
<td>44 (53.7) b</td>
<td>7 (8.5) a</td>
</tr>
<tr>
<td>100 µM C30</td>
<td>124</td>
<td>83 (66.9) b</td>
<td>38 (30.7) b</td>
<td>3 (2.4) b</td>
</tr>
</tbody>
</table>

Within groups (columns), values with the same letter are significantly different (P<0.001). GV, germinal vesicle stage of oocytes; M I, metaphase I stage of oocytes; M II, metaphase II stage of oocytes.

Table 2 Morphology of pig oocytes treated with C30 inhibitor for 44 h.

<table>
<thead>
<tr>
<th>Treatment of oocytes</th>
<th>Total no. of oocytes (44h)</th>
<th>GV oocytes (%)</th>
<th>M I oocytes (%)</th>
<th>M II oocytes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 44 h</td>
<td>122</td>
<td>5 (4.1) ab c</td>
<td>16 (13.1) ab c</td>
<td>101 (82.8) ab c</td>
</tr>
<tr>
<td>10 µM C30</td>
<td>148</td>
<td>7 (4.7) a</td>
<td>66 (44.6) a</td>
<td>75 (50.7) a</td>
</tr>
<tr>
<td>50 µM C30</td>
<td>175</td>
<td>11 (6.3) b</td>
<td>91 (52.0) b</td>
<td>73 (41.7) b</td>
</tr>
<tr>
<td>100 µM C30</td>
<td>216</td>
<td>54 (25.0) c</td>
<td>138 (63.9) c</td>
<td>24 (11.1) c</td>
</tr>
</tbody>
</table>

Within groups (columns), values with the same letter are significantly different (P<0.001). GV, germinal vesicle stage of oocytes; M I, metaphase I stage of oocytes; M II, metaphase II stage of oocytes.

However, this ligase activity requires dimerization of the enzyme (Liu et al. 2002). The enzyme has a variety of functions. UCH-L1, which constitutes about 5% of the brain-soluble proteins, appears to be associated with the stabilization of monoubiquitin in neural cells and it is a component of inclusions that are indicative of neurodegenerative diseases including Parkinson’s disease and Alzheimer’s disease (Lowe et al. 1990, Osaka et al. 2003). Furthermore, it has been suggested that UCH-L1 also functions as a regulator of apoptosis in spermatogonial cell and sperm maturation. The testes of the gracile axonal dystrophy mouse, which carries an intragenic deletion of the Uchl1 gene and thus lacks UCH-L1 expression, have a reduced ubiquitin level thus and are resistant to cryptorchid injury-mediated germ cell apoptosis (Kwon et al. 2005).

To study regulatory role of UCH-L1 during pig oocyte meiotic maturation, we used specific inhibition of this enzyme. By utilizing high-throughput screening to find inhibitors and traditional medical chemistry, Liu et al. (2003) identified a small-molecule inhibitor belonging to the class of isatin O-acyl oximes that selectively inhibit UCH-L1 when compared with its isoform UCH-L3. We used the representative of this group, marked C30, which has IC50 value of 0.88 µM for UCH-L1. Then GV stage cumulus-enclosed oocytes were treated with C30, GVBD was inhibited after 28 h of culture, and most of the oocytes were arrested at M I stage after 44 h. This is in contrast to the study of Sun & Nagai (2003) showing that specific inhibition of proteasome by MG-132 or lactacystin blocked the first meiosis resumption when observed at 44 h of culture (Sun et al. 2004). On the contrary, our results are consistent with the reports showing that specific proteasome inhibition by MG-132 blocked the metaphase I–anaphase transition of meiosis in mouse and rat oocytes (Josefsberg et al. 2000). While the maturation of pig and rodent oocytes is differentially regulated in many aspects, the present results supported the hypothesis that metaphase I–anaphase transition may be regulated by ubiquitin-dependent proteasome mechanisms in these species. The block of metaphase I–anaphase transition was not completely reversible. Only about 30% of oocytes arrested at GV stage by 100 µM C30 present for 12 or 28 h of the culture could resume meiosis to M II stage following culture in inhibitor-free medium until 44 h. Furthermore, inhibition of UCH-L1 resulted in elevated histone H1 kinase (MPF) activity after 28 h of culture. This is another possible explanation of meiotic arrest at M I stage since metaphase I–anaphase transition needs decrease in MPF activity. Since it is widely accepted that inactivation of MPF is associated with cyclin B1 degradation by ubiquitin–proteasome pathway (Tokumoto et al. 1997), we expect that high MPF activity observed in oocytes cultured in the presence UCH-L1 inhibitor reflects a restriction in cyclin B1 ubiquitination and as a consequence failure in its degradation by the proteasome system. This result is further corroborated by a low level of monoubiquitin in oocytes treated by C30 inhibitor for 28 h in comparison with those treated in inhibitor-free medium.

In summary, a proteomic approach followed by verification functional study revealed a major regulatory role for UCH-L1 in pig oocyte maturation and its essential requirement in completion of the first meiosis and its transition to anaphase. The mechanisms by which hydrolase as well as ligase activities of this enzyme affect the balance of ubiquitination and contribute to time and spatial control of ubiquitin-dependent processes during oocyte maturation require further study.

phosphine was purchased from Fluka (Buchs, Switzerland). Tetramethylenediamine were from Bio-Rad; and tributyl phosphate was purchased from Amersham Pharmacia Biotech; 3-((3-cholamidopropyl)dimethylammonio)-2-hydroxyl-1-propanesulfonate (CHAPS), urea, and dithiothreitol (DTT) were from Amersham Pharmacia Biotech; acrylamide/bis-acrylamide 30% solution, Tris–HCl, agarose, iodoacetamide, thiourea, glycine, silver nitrate, protease inhibitor complete tablets (Complete, Mini, Sigma, cat. No. 04693124001), and trifluoroacetic acid (TFA) were used for the proteomic study was based on the criteria recommendation), and 0.003% (v/v) bromophenol blue. The samples were loaded by gel rehydration on 7 cm immobilized, pH 3–10, nonlinear gradient strips for 2-DE. The samples were then placed in 40% acetic acid and observed with a phase contrast NU Zeiss microscope (Jena, Germany). The collection of the oocytes was used to verify GV (intact GV), M I (the first meiosis), and 44 h (M II; the second meiosis) during maturation. At the end of culture, the cumulus cells of the oocytes were removed by vortexing. Denuded oocytes were then washed thrice in PBS and were stored at −80 °C until use in the experiment. Morphological evaluation of oocytes was used to verify GV (intact GV), M I (the first meiotic spindle), or M II (extrusion of the first polar body) stage of in vitro maturation and quality of the oocytes collected for 2-DE. The oocytes were mounted on microscope slides with Vaseline, covered with a cover glass, and fixed in ethanol:acetic acid (3:1) for 24 h. Staining was performed with 2% orcein in 50% aqueous acetic acid in physiological saline at 20 °C to the laboratory. The ovaries were briefly washed for 20 s in 70% ethanol and then twice in physiological saline. The oocytes were obtained by aspiration of antral follicles about 5 mm in diameter. Only oocytes surrounded by compact cumuli (COC; cumulus–oocyte complex) were used for culture. COCs were cultured in droplets of maturation medium (MM) supplemented with 15% OCS and 20 μl Suigonan PG-600 Intervet (International BV, Boxmeer, Holland) at 38.5 °C in an atmosphere of 5% CO2 (Pavlok et al. 2005). The samples were collected at 0 (GV; germinal vesicle stage), 28 (M I; the first meiosis), and 44 h (M II; the second meiosis) during spontaneous in vitro maturation. At the end of culture, the cumulus cells of the oocytes were removed by vortexing. Denuded oocytes were then washed thrice in PBS and were stored at −80 °C until use in the experiment. Morphological evaluation of oocytes was used to verify GV (intact GV), M I (the first meiotic spindle), or M II (extrusion of the first polar body) stage of in vitro maturation and quality of the oocytes collected for 2-DE. The oocytes were mounted on microscope slides with Vaseline, covered with a cover glass, and fixed in ethanol:acetic acid (3:1) for 24 h. Staining was performed with 2% orcein in 50% aqueous acetic acid and 1% sodium citrate. The slides were then placed in 40% acetic acid and observed with a phase contrast NU Zeiss microscope (Jena, Germany). The collection of the oocytes used for the proteomic study was based on the criteria that at least 85% of oocytes reached an appropriate maturation stage.

**Materials and Methods**

**Materials**

Park’s medium H-199 was from Sevapharma (Prague, Czech Republic) and estrous cow serum (OCS), including inactivation for 30 min at 56 °C, was prepared in our laboratory and stored at −80 °C. Immobiline dry strips (immobilized pH gradient, pH 3–10 non-linear, 7 cm) and ampholytes (pH 3–10) were purchased from Amersham Pharmacia Biotech; 3-(3-cholamidopropyl)dimethylammonio)-2-hydroxyl-1-propanesulfonate (CHAPS), urea, and dithiothreitol (DTT) were from Amersham Pharmacia Biotech; acrylamide/bis-acrylamide 30% solution, Tris–HCl, agarose, iodoacetamide, thiourea, glycine, silver nitrate, protease inhibitor complete tablets (Complete, Mini, Sigma, cat. No. 04693124001), and trifluoroacetic acid (TFA) were from Sigma; ammonium persulfate, SDS, and N2,N2,N2’,N2’-tetramethylenediamine were from Bio-Rad; and tributyl phosphate was purchased from Fluka (Buchs, Switzerland).

**Table 3** Morphology of pig oocytes treated with 100 μM C30 inhibitor for different culture time; followed by culture in inhibitor-free medium to the 44 h of total time.

<table>
<thead>
<tr>
<th>Treatment of oocytes</th>
<th>Total no. of oocytes</th>
<th>GV oocytes (%)</th>
<th>M I oocytes (%)</th>
<th>M II oocytes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 44 h</td>
<td>46</td>
<td>2 (4.4)</td>
<td>6 (13.0)</td>
<td>38 (82.6)</td>
</tr>
<tr>
<td>4 h C30+40 h</td>
<td>48</td>
<td>1 (2.1)</td>
<td>7 (14.6)</td>
<td>40 (83.3)</td>
</tr>
<tr>
<td>8 h C30+36 h</td>
<td>68</td>
<td>0 (0.0)</td>
<td>31 (45.6)</td>
<td>37 (54.4)</td>
</tr>
<tr>
<td>12 h C30+32 h</td>
<td>77</td>
<td>15 (19.5)</td>
<td>37 (48.0)</td>
<td>25 (32.5)</td>
</tr>
<tr>
<td>28 h C30+16 h</td>
<td>69</td>
<td>10 (14.5)</td>
<td>38 (55.1)</td>
<td>21 (30.4)</td>
</tr>
</tbody>
</table>

Within groups (columns), values with the same letter are significantly different (P<0.001). GV, germinal vesicle stage of oocytes; M I, metaphase I stage of oocytes; M II, metaphase II stage of oocytes.

**Oocyte collection and culture**

Ovaries, collected from slaughtered pigs, were transported in physiological saline at 20 °C to the laboratory. The ovaries were briefly washed for 20 s in 70% ethanol and then twice in physiological saline. The oocytes were obtained by aspiration of antral follicles about 5 mm in diameter. Only oocytes surrounded by compact cumuli (COC; cumulus–oocyte complex) were used for culture. COCs were cultured in droplets of maturation medium (MM) supplemented with 15% OCS and 20 μl Suigonan PG-600 Intervet (International BV, Boxmeer, Holland) at 38.5 °C in an atmosphere of 5% CO2 (Pavlok et al. 2005). The samples were collected at 0 (GV; germinal vesicle stage), 28 (M I; the first meiosis), and 44 h (M II; the second meiosis) during spontaneous in vitro maturation. At the end of culture, the cumulus cells of the oocytes were removed by vortexing. Denuded oocytes were then washed thrice in PBS and were stored at −80 °C until use in the experiment. Morphological evaluation of oocytes was used to verify GV (intact GV), M I (the first meiotic spindle), or M II (extrusion of the first polar body) stage of in vitro maturation and quality of the oocytes collected for 2-DE. The oocytes were mounted on microscope slides with Vaseline, covered with a cover glass, and fixed in ethanol:acetic acid (3:1) for 24 h. Staining was performed with 2% orcein in 50% aqueous acetic acid and 1% sodium citrate. The slides were then placed in 40% acetic acid and observed with a phase contrast NU Zeiss microscope (Jena, Germany). The collection of the oocytes used for the proteomic study was based on the criteria that at least 85% of oocytes reached an appropriate maturation stage.

**Sample preparation and two-dimensional gel electrophoresis**

For analytical 2-DE, samples of 100 oocytes were labeled with 50 μCi [35S]-methionine for 4 h at time 0, 24, and 40 h during in vitro culture. Following labeling, oocytes were lysed in 30 μl of lysis buffer containing urea (9 M), CHAPS (4% w/v), Tris (40 mM), DTT (70 mM), 2% (v/v) ampholytes (pH 3–10), protease inhibitors (Complete, Mini, Sigma, cat. No. 04693124001, used according to the manufacturer’s recommendation), and 0.003% (v/v) bromophenol blue. The samples were loaded by gel rehydration on 7 cm immobilized, pH 3–10, nonlinear gradient strips for 2-DE. The separations were performed as described by Hochstrasser et al. (1992). The isoelectric focusing was carried out in a non-linear pH gradient

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Identification of UCH-L1 by mass spectrometry

A CBB-stained protein spot corresponding to the same spot on radiolabeled and silver stained gels was excised from the gel, cut into small pieces, and washed several times with 10 mM DDT, 0.1 M 4-ethylmorpholine acetate (pH 8.1) in 50% acetonitrile (MeCN). After complete destaining, the gel was washed with water, shrunk by dehydration in MeCN, and reswelled again in water. The supernatant was removed and the gel was partly dried in a SpeedVac concentrator. The gel pieces were then reconstituted in a cleavage buffer containing 0.01% 2-mercaptoethanol, 0.1 M 4-ethylmorpholine acetate, 10% MeCN, and sequencing grade trypsin (20 ng/µl; Promega). After overnight digestion, the resulting peptides were extracted to 40% MeCN/0.1% TFA.

A solution of p-cyano-4-hydroxycinnamic acid (Sigma) in aqueous 30% MeCN/30% MeOH/0.2% TFA (10 mg/ml) was used as a MALDI matrix. A 1 µl sample was deposited on the MALDI target and allowed to air-dry at room temperature. After complete evaporation, 1 µl of the matrix solution was added. Positive ion MALDI mass spectrum was measured on a Bruker BIFLEX II reflectron time-of-flight mass spectrometer (Bruker-Franzen, Bremen, Germany) equipped with SCOUT 26 sample cell (Bruker-Rad). For each gel, the spots were detected and quantified automatically using default spot detection. A manual spot editing was performed and the results were in agreement with those of the visual inspection. Quantification of spots was done in terms of parts per million. To compare and analyze the images of the gels in experiments, the MatchSet Tool was used and the Master, a synthetic image containing the data from all the gels in the MatchSet, was created. Four independently prepared samples for initial (GV) and final (M II) stages of oocyte maturation were evaluated. The Analysis Set Manager, including Student’s t-test, was used for determination of significant protein spot differences at the level of P<0.05.

Inhibition of meiotic maturation by inhibitor specific toward UCH-L1

To investigate the effect of UCH-L1 inhibition on meiosis resumption, specific inhibitor of UCH-L1 marked C30 (Liu et al. 2003) and kindly provided by Peter T Lansbury, Jr (Harvard Center for Neurodegeneration and Repair) was added to the MM at the beginning of culture of cumulus-enclosed GV oocytes. Meiosis progression was evaluated at 28 or 44 h after culture. To evaluate further impact and reversibility of the inhibitory effect, the GV oocytes were cultured in inhibitor-containing medium for the first 2, 8, 12, or 28 h, then washed thrice and placed into fresh inhibitor-free MM with supplements of OCS and Suigonan PG-600 until the total time of cultivation reached 44 h. In control groups, oocytes were cultured for either 28 or 44 h in the inhibitor-free medium supplemented by DMSO in the amount equal to working C30 solution. Stock solution of C30 was prepared in DMSO at the concentration of 5 mM and kept frozen at −20°C. Final working solution was diluted immediately before usage.

Histone H1 and myelin basic protein (MBP) double kinase double assay

CDK1 kinase and MAP kinase activities were measured in oocytes via their capacity to phosphorylate external substrates histone H1 and MBP respectively according to Motlik et al. (1996). At each time interval during the culture, ten oocytes per sample were lysed in 5 µl homogenization buffer containing 40 mM MOPS (pH 7.2), and inhibitors of phosphatases (20 mM NaF, 20 mM para-nitrophenyl phosphate, 40 mM β-glycerophosphate, 0.2 mM Na3VO4), and proteases (10 mM EGTA, 0.2 mM EDTA, 2 mM benzamidine, 20 µg/ml leupeptin and 20 µg/ml aprogin, 1 mM phenylmethylsulphonyl fluoride) by three cycles of freezing/thawing on dry ice. The kinase reaction was initiated by an addition of 5 µl kinase buffer containing 10 mg/ml histone H1, and 5 mg/ml MBP, together with 10 mCi/ml [γ-32P] ATP (Amersham Pharmacia Biotech). The reaction was stopped after 30 min by the addition of SDS-PAGE sample buffer and boiling for 3 min. After electrophoresis on 15% SDS-PAGE gel, the gels were stained with Coomassie Blue R250, destained overnight, dried, and autoradiographed.

Immunoblotting

Oocyte extracts used for 1-D immunoblotting were prepared by lysis of ten oocytes in reduced SDS buffer and separated by 15% SDS-PAGE. 2-D immunoblotting was performed using samples of 200 oocytes which were lysed and proteins were separated by 2-DE as described above. The proteins were then transferred to Immobilon P (Millipore, Bedford, MA, USA) membrane using a semidry blotting system (Biometra, Schoeller Instruments, Prague, Czech Republic). Blots were incubated with low-fat milk dissolved in 0.5% Tween-20 in Tris-buffered saline, pH 7.4. The antibody specific toward UCH-L1 (Chemicon, Temecula, CA, USA) and monoclonal Anti-Ubiquitin (Clone 6C1, Sigma) was diluted in the ratio of 1:1000 in 5% low-fat milk and
incubated with the membrane overnight at 4°C. Following washing and addition of secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA), immunodetected proteins were visualized by ECL-plus chemiluminiscent kit according to manufacturer’s instructions (Amersham Pharmacia Biotech).

Statistical analysis
SigmaStat software (SPSS Inc., Chicago, IL, USA) was used for statistical evaluation of the results. The effect of different C30 concentration on meiotic maturation, e.g., proportion of oocytes in GV, MI, and M II stages, was evaluated using χ² analysis. In addition, χ² analysis with Yates (continuity) correction was used for evaluation of the effect induced by 100 μM C30 followed by culture in inhibitor-free medium.

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References


Robert C, Gagne D, Bousquet D, Barnes FL & Sirard MA 2001
Differential display and suppressive subtractive hybridization used to
identify granulosa cell messenger RNA associated with bovine oocyte
Sasaki R, Nakayama T & Kato T 1999 Microelectrophoretic analysis of
changes in protein expression patterns in mouse oocytes and pre-
Sun QY & Nagai T 2003 Molecular mechanisms underlying pig oocyte
maturation and fertilization. *Journal of Reproduction and Development*
49 347–359.
Sun QY, Fuchimoto D & Nagai T 2004 Regulatory roles of ubiquitin-
proteasome pathway in pig oocyte meiotic maturation and fertilization.
*Theriogenology* 62 245–255.
Tokumoto T, Yamashita M, Tokumoto M, Katsu Y, Horiguchi R,
Kajiura H & Nagahama Y 1997 Initiation of cyclin B degradation
by the 26S proteasome upon egg activation. *Journal of Cell Biology*
138 1313–1322.
Vitale AM, Calvert ME, Mallavarapu M, Yurttas P, Perlin J, Herr J &
Coonrod S 2007 Proteomic profiling of murine oocyte maturation.
*Molecular Reproduction and Development* 74 608–616.
Vodicka P, Smetana K Jr, Dvorankova B, Emerick T, Xu YZ, Ourednik J,
Ourednik V & Motlik J 2005 The miniature pig as an animal model for
Biomedical research. *Annals of the New York Academy of Sciences* 1049
161–171.
1989 The neuron specific protein PGP 9.5 is a ubiquitin carboxyl-
Wilkinson KD, Deshpande S & Larssen CN 1992 Comparison of neuronal
(PGP9.5) and non-neuronal ubiquitin C-terminal hydrolases.
*Biochemical Society Transactions* 20 631–637.
Zeng F & Schultz RM 2003 Gene expression in mouse oocytes and
preimplantation embryos: use of suppression subtractive hybridization
to identify oocyte- and embryo-specific genes. *Biology of Reproduction*
68 31–39.

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