Expression of adiponectin receptors and effects of adiponectin isoforms in mouse preimplantation embryos

Štefan Čikoš*, Ján Burkuš, Alexandra Bukovská, Dušan Fabian, Pavol Rehák, and Juraj Koppel

Institute of Animal Physiology, Slovak Academy of Sciences, Šoltésovej 4, 04001 Košice, Slovakia

*Correspondence address. Tel: +421-55-7287841; Fax: +421-55-7287842; E-mail: cikos@saske.sk

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BACKGROUND: Adiponectin, a pleiotropic hormone secreted from adipose tissue, can mediate some negative effects of obesity on female health, and can participate in the impaired reproductive performance of obese women. Using a mouse model, we investigated expression of adiponectin receptors in ovulated oocytes and in vivo derived preimplantation embryos, and tested effects of different adiponectin isoforms on development of preimplantation embryos in vitro.

METHODS AND RESULTS: Using RT–PCR and immunohistochemistry, we found expression of adiponectin receptors AdipoR1 and AdipoR2, at the mRNA and protein level, in mouse ovulated oocytes and preimplantation embryos. Quantitative real-time RT–PCR analysis showed a decrease in the amount of AdipoR1 and AdipoR2 mRNA after fertilization, which was followed by an increase in mRNA at the morula and blastocyst stage; mRNA for adiponectin was detected only at the blastocyst stage. Administration of full-length adiponectin significantly changed the distribution in numbers of cells of cultured preimplantation embryos, increasing the proportion of embryos with high cell numbers (>128 cells) and decreasing the proportion of embryos with lower cell numbers (<65 cells). Blastocysts possessed significantly higher cell numbers after full-length adiponectin treatment. Mutated trimeric adiponectin had the opposite effect, a significant decrease in the proportion of embryos with higher cell numbers (>96 cells) and increase in the proportion of embryos with lower cell numbers (<65 cells). Trimeric adiponectin also significantly decreased the cell number and increased cell death in blastocysts. Truncated globular adiponectin had no significant effect on development of mouse preimplantation embryos.

CONCLUSIONS: Our results indicate that adiponectin can directly influence the development of the preimplantation embryo, and the effects are isoform dependent.

Key words: adiponectin / adiponectin receptors / preimplantation embryo

Introduction

Adiponectin belongs to the group of proteins called ‘adipokines’ which are secreted from adipose tissue into the bloodstream and influence many physiological processes including glucose and lipid metabolism, inflammation and reproduction (Vettor et al., 2005; Garaulet et al., 2007; Zavala-Gómez et al., 2008). Serum levels of most adipokines (including the best-known one—leptin) are elevated in obesity, and they can mediate some negative consequences of obesity on health, including reproductive health (Norman et al., 2004; Mitchell et al., 2005; Campos et al., 2008; Van der Steeg et al., 2008). In contrast to other adipokines, the plasma level of adiponectin is inversely correlated with body fat amount in adults, i.e. it is decreased in obesity and in diseases associated with obesity, such as type 2 diabetes and cardiovascular diseases (Trujillo and Scherer, 2005; Oh et al., 2007).

Moreover, plasma concentrations of adiponectin are much higher than those of other adipokines (about 10 μg/ml, which is about a thousand times higher than the level of leptin) and show clear gender differences with females having higher levels (especially of high molecular adiponectin isoforms) than males (Nishizawa et al., 2002; Combs et al., 2003).

Two main domains can be distinguished in the adiponectin molecule—the collagen-like domain and the globular domain. The collagen-like domain contains amino acids cysteine and lysine, which enable the formation of hexamers and high-molecular-weight trimers. Full-length adiponectin can be found in circulation as a trimer, hexamer or large multimer. Moreover, full-length adiponectin can be cleaved and can exist in serum as the globular adiponectin, forming trimers (Wang et al., 2008). Two receptors binding adiponectin, AdipoR1 and AdipoR2, have been identified. The predicted structure
of the two receptors is similar to the topology of G-protein coupled receptors, but the intracellular/extracellular orientation of N-terminus and C-terminus is the opposite. There are differences in the binding preference of the receptors to the adiponectin isoforms as well as differences in the tissue distribution of the two receptors (Kadowaki and Yamauchi, 2005). Several signaling pathways mediating adiponectin effects in various cell types have been shown. The involvement of AMP-activated protein kinase (AMPK), peroxisome proliferators-activated receptor α (PPAR α), nuclear factor-κB (NF-κB), mitogen-activated protein kinases (MAPK) and other kinases has been demonstrated (Tsao et al., 2002; Yamauchi et al., 2007; Grossmann et al., 2008; Benaitreau et al., 2009).

Recent data suggest that, besides its influence on maternal health, adiponectin can also play a role in maintaining the normal reproductive function, and in the communication between mother and embryo. The influence of adiponectin on ovarian, endometrial and placental functions as well as on early embryo development has been suggested (Chen et al., 2006; Ledoux et al., 2006; Takemura et al., 2006; Archancé et al., 2007; Chappaz et al., 2008; Schmidt et al., 2008). To ascertain if adiponectin can directly interact with embryo cells at the earliest developmental stages, we investigated expression of adiponectin receptors in mouse ovulated oocytes and in in vivo derived preimplantation embryos. We also tested the effects of different adiponectin isoforms on development of mouse preimplantation embryos in vitro.

Materials and Methods

Embryo recovery

Female mice (ICR strain, Velaz, Prague, Czech Republic; 4–5 weeks old) underwent ovulation induction treatment by intraperitoneal injection of 5 IU of serum gonadotrophin (Folligon, Intervet International Bv, Boxmeer, Holland), followed 46 h later by administration of 5 IU of human chorionic gonadotrophin (hCG, Organon, Oss, Holland). The mice were killed by cervical dislocation and unfertilized oocytes (at metaphase II) were isolated by flushing the oviduct 24 h after hCG. To obtain preimplantation embryos, females were mated with males of the same strain overnight (mating was confirmed by identification of a vaginal plug), killed by cervical dislocation (57–72–86–98 h after hCG) and the embryos (4-cell embryos, 8–16-cell embryos, morulas and expanded blastocysts) were isolated by flushing the oviduct or uterus. Oocytes and embryos were washed in several drops of FHM medium (Lawits and Biggers, 1993) containing 1% bovine serum albumin (BSA), classified according to their morphology and pooled. Cumulus cells were removed with 0.1% hyaluronidase (Sevac, Prague, Czech Republic). All animal experiments were reviewed and approved by the Ethical Committees for animal experimentation of the Institute of Animal Physiology, approved by the State Veterinary and Food Administration of the Slovak Republic, and were performed in accordance with Slovakian legislation based on EC Directive 86/609/EEC on the protection of animals used for experimental and other scientific purposes.

RT–PCR and real-time PCR quantification of mRNA

Total RNA was extracted from batches of 90–100 mouse preimplantation embryos and unfertilized oocytes (the number of embryos/oocytes in each pool was exactly determined), and also from mouse adipose tissue and brain (positive controls). TRizol Reagent (Invitrogen Life technologies, Karlsruhe, Germany) was used for the extraction (according to the manufacturer’s instructions). Contaminating DNA in RNA preparations was digested with amplification-grade DNase I (Invitrogen Life Technologies). For the quantitative analysis, 0.2 pg of luciferase (Luc) mRNA (Promega, Madison, WI, USA) per embryo/oocyte was added to the TRizol lysis reagent before the RNA extraction to control for differences in RNA recovery and loading of RT–PCR reactions. Three independent experiments were performed, using separate batches of embryos/oocytes for the RNA isolation.

The RNA (from 90 to 100 embryos/oocytes or 0.5 μg RNA from brain or adipose tissue) was reverse transcribed with Superscript II RNase H– Reverse Transcriptase (Invitrogen Life Technologies) as described previously (Fabian et al., 2009). To check for the presence of genomic DNA contamination in the RNA preparations, reverse transcriptase negative controls (no reverse transcriptase in the reaction) were carried out in parallel, using a part of each RNA sample. The cDNA preparations were then cleaned by ethanol precipitation and the cDNA pellets from embryos/oocytes were diluted in an appropriate amount of 10 mM Tris (pH 8.3) so that 1 μl of the cDNA corresponded to 2.5 embryo/oocyte (and 1.25 pg Luc mRNA) equivalents.

PCR amplifications for the qualitative analysis were carried out in 25 μl volumes containing 1 μl of cDNA, 0.5 μl of each oligonucleotide primer, 50 μM KCl, 10 mM Tris–HCl pH 8.3, 2 mM MgCl₂, 0.2 mM dNTPs (dATP, dTTP, dCTP, dGTP) and 0.04 units/μl Platinum Taq DNA polymerase (Invitrogen Life Technologies). We designed primers for adiponectin (5′-CTCTGGAGAGAAGGAGAGAA-3′ and 5′-CGAATGGGTA CATTGGAAC-3′, 209 bp PCR product), and primers for adiponectin receptors were designed by Schmidt et al. (2008). For adiponectin, an initial denaturation step at 95°C for 2 min was followed by 30 (adipose tissue cDNA—the positive control) or 40 (oocytes/embryos) cycles of 94°C for 60 s, 63°C for 60 s and 72°C for 60 s. For AdipoR1 and AdipoR2 receptors, an initial denaturation step at 95°C for 2 min was followed by 30 (brain cDNA—the positive control) or 40 (oocytes/embryos cDNA) cycles of 94°C for 60 s, 60°C for 60 s and 72°C for 60 s. To check for the presence of cross contamination, a reaction with water instead of cDNA was performed concurrently (blank reaction). Detection of beta-actin transcript using beta-actin primers (Temeles et al., 1994) served as control for RNA integrity and the RT–PCR process. The PCR products were analyzed using electrophoresis on a 2% agarose gel stained with SYBR Green I. To confirm the amplification of the correct sequences, we digested the obtained PCR products with appropriate restriction enzymes (according to nucleotide sequences obtained from GenBank under accession numbers NM_009605, NM_028320, NM_197985).

PCR amplifications for the quantitative analysis were performed in the real-time PCR system Mx 3000P (Stratagene, La Jolla, CA, USA). The reactions were carried out in 20 μl volumes containing 0.8 μl of cDNA, SYBR Green/ROX PCR mix (PA-012, SuperArray Bioscience Corp., Frederick, MD, USA) and 0.5 μM primers (Schmidt et al., 2008). An initial step at 95°C for 15 min was followed by 40 cycles at 94°C for 30 s, 64°C (AdipoR1) or 62°C (AdipoR2) for 30 s, 72°C for 30 s and 76°C (AdipoR1) or 80°C (AdipoR2) for 30 s (acquiring of fluorescence). For amplification of luciferase, we used primers designed in our previous work (Fabian et al., 2009). An initial step at 95°C for 15 min was followed by 40 cycles at 95°C for 20 s, 60°C for 60 s and 82°C for 30 s (acquiring of fluorescence). Amplification specificity was checked by generation of a melting curve using 41 cycles with temperature increments of 1°C (starting with 55°C) and a fluorescence measurement in each cycle. The expression of AdipoR1 and AdipoR2 was normalized with the external control—luciferase mRNA in each sample (i.e. the mRNA relative quantity of AdipoR1 and AdipoR2 was divided by the Luc mRNA relative quantity). The fluorescence data obtained from amplifications were transformed to the values of relative mRNA quantity using three methods of analysis:
the relative standard curve method (using serial dilutions of mouse brain cDNA), the LinRegPCR-Ct method (fitting log-transformed fluorescence data from the exponential PCR phase with the line) and the SCF method (fitting fluorescence data with the sigmoid curve; Žižek and Koppel, 2009). All the methods showed similar results, indicating their reliability (quantification results obtained with the LinRegPCR-Ct method are presented).

**Immunostaining**

Preimplantation embryos and oocytes were isolated as described above and the zona pellucida was removed with 0.5% pronase in KSOM at 37°C. Zona-free oocytes and embryos were fixed in 4% paraformaldehyde. Free aldehyde groups were blocked with 0.1 M glycine (Merck, Darmstadt, Germany), and washed in PBS/BSA/SAP (PBS containing BSA and 0.05% saponin; Sigma-Aldrich, Munich, Germany). Non-specific immunoreactions were blocked with 10% normal goat serum (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 45 min at room temperature. Oocytes and preimplantation embryos at various developmental stages were incubated with primary antibodies raised against adiponectin receptor 1 or adiponectin receptor 2 (affinity-purified rabbit polyclonal antibodies, AdipoR12A, AdipoR22A, Alpha Diagnostic International, San Antonio, TX, USA) in PBS/BSA/SAP at 4°C overnight. Blastocysts were also incubated with primary antibody raised against the adiponectin (affinity-purified rabbit polyclonal antibody, LS-B440, LifeSpan Biosciences, Seattle, WA, USA). Specific secondary antibody coupled with Alexa Fluor 488 (Alexa Fluor 488 goat anti-rabbit IgG, Invitrogen Life Technologies, Karlsruhe, Germany) was used to visualize primary antibodies (30 min at room temperature). Cell nuclei were stained with Hoechst 33342 in PBS/BSA/SAP (20 μg/ml; Sigma-Aldrich, Germany). Afterwards, they were mounted in ProLong Gold antifade reagent (Molecular Probes, Invitrogen) on glass slides, sealed with coverslips and observed using an epifluorescence microscope (BX 51 Olympus, Japan). Negative control groups of oocytes and embryos were incubated without the primary antibody or without the primary and secondary antibody, or with the primary antibody pre-adsorbed with an excess (10 times by weight) of the corresponding immunizing peptide (Mouse AdipoR1 Blocking peptide—AdipoR12P or Mouse AdipoR2 Blocking peptide—AdipoR22P, Alpha Diagnostic International; Adiponectin synthetic peptide to LS-B440, LifeSpan Biosciences). Oocytes and embryos in each experimental group (incubated with primary antibody) were evaluated by comparison with negative control groups of oocytes and embryos.

**Embryo culture and morphological evaluation**

Four-cell stage embryos were flushed from the oviduct, then pooled, randomly divided into two groups, washed in KSOM culture medium (Specialty Media Group, Phillipsburg, NJ, USA), transferred to KSOM culture drops (1 embryo/1 μl KSOM, covered with mineral oil) containing adiponectin (experimental group) or equivalent amounts of solvent (control group), and cultured in standard conditions (5% CO2 and 37°C) for 65 h. Three isoforms of adiponectin were used in the experiments: full-length adiponectin (mouse Acrp30, recombinant protein, produced in HEK 293 cells, GenWay, San Diego, CA, USA), the truncated isoform—globular adiponectin (mouse Acrp30, globular recombinant protein, produced in Escherichia coli, GenWay), and mutated full-length adiponectin (mouse Acrp30, trimeric recombinant protein, produced in HEK 293 cells, GenWay) with cysteine 39 replaced with alanine (enabling trimer formation only). The final concentration of adiponectin in the culture medium was 10 μg/ml. To eliminate experimental bias, three independent experiments were performed for each adiponectin isoform and data were pooled.

After 65 h of incubation, cell nuclei were stained with cell-impermeant dye propidium iodide (PI, 10 μg/ml; Sigma-Aldrich; stains dead cells only), and fixed in 4% paraformaldehyde (Merck, Darmstadt, Germany) for 60 min. Fixed embryos were permeabilized with 1% Triton X-100 solution in PBS (v/w) at room temperature for 60 min. Embryos were then incubated in TUNEL assay reagents (TUNEL, In Situ Cell Death Detection Kit; Roche, Penzberg, Germany) for 60 min at 37°C in the dark. After TUNEL reaction, all embryos were counterstained with Hoechst 33342 (bisbenzimide 20 μg/ml in PBS; Sigma-Aldrich) for 5 min at room temperature. Finally, embryos were mounted on slides with Vectashield (Vector laboratories, Burlingame, CA, USA) and observed under a fluorescence microscope at ×400 magnification (BX 51 Olympus, Japan). The number of cell nuclei was determined in each embryo. For the analysis of embryo distribution, each embryo was assigned to one of five classes (cell numbers: <32, 32–64, 65–96, 97–128, >128) depending on its developmental stage. According to the nuclear morphology, the presence of specific DNA fragmentation in the nucleoplasm and PI positivity/negativity, embryonic cells were classified as: normal (without morphological changes in nuclei, without TUNEL labeling, and without PI inclusion into the nucleoplasm) or dead (showing at least one of following features: fragmented or condensed nucleus, positive TUNEL labeling or positive PI staining).

**Statistics**

Statistical analysis was performed using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA). The Kruskal–Wallis test was used for the comparison of differences in mRNA relative quantities between all preimplantation developmental stages, and the Mann–Whitney test was used for the comparison of differences in mRNA relative quantities between oocytes and other developmental stages. The unpaired Student’s t-test was used for estimating the embryo cell number. The standard χ2 test was used for analyzing embryo distribution and cell death incidence. Differences of P < 0.05 were considered significant.

**Results**

**Expression of adiponectin and adiponectin receptor mRNAs**

We detected PCR products corresponding to AdipoR1 (132 bp) and AdipoR2 (258 bp) receptors in oocytes as well as in embryos of all tested developmental stages; PCR product corresponding to adiponectin mRNA (209 bp) was detected only at the blastocyst stage (Fig. 1). The embryos produced a PCR fragment corresponding to beta-actin mRNA (539 bp) at all developmental stages, thus confirming the integrity of the RNA and the RT–PCR process (data not shown). No PCR products were detected in the reactions where reverse transcriptase or cDNA were omitted (data not shown). Digestion of the PCR products with appropriate restriction enzymes gave DNA fragments of the expected sizes: digestion of the 132 bp PCR product (amplified with AdipoR1 primers) with Hinf I gave 79 and 53 bp DNA fragments, digestion with Pst I gave 101 and 31 bp DNA fragments; digestion of the 258 bp PCR product (amplified with AdipoR2 primers) with Hpa II gave 174 and 84 bp DNA fragments; digestion of the 209 bp PCR product (amplified with adiponectin primers) with Alu I gave 130 and 79 bp DNA fragments, digestion with Hae III gave 122 and 87 bp DNA fragments (data not shown).
The AdipoR1 mRNA relative quantity was significantly lower in 4-cell embryos than in oocytes, and it increased to the oocyte level in 8- to 16-cell embryos; in morulas and blastocysts, the AdipoR1 mRNA reached significantly higher levels than in oocytes (Fig. 2). The AdipoR2 mRNA relative quantity was significantly lower in 4-cell embryos and 8- to 16-cell embryos than in oocytes, and it increased significantly above the oocyte level in morulas and blastocysts (Fig. 2).

**Immunohistochemical study**

The immunohistochemical study showed the presence of AdipoR1 and AdipoR2 receptor protein in all studied developmental stages (oocytes, 4-cell embryos, 8- to 16-cell embryos, blastocysts, Fig. 3), but we did not detect expression of adiponectin protein. The signal intensity was higher in AdipoR2 than in AdipoR1 at all tested stages. The specificity of the signal was confirmed using several negative controls: the intensity of immunostaining signal was significantly reduced in controls incubated with the primary antibody preadsorbed with the corresponding immunizing peptide (Fig. 3), and in controls incubated without the primary antibody or without the primary and the secondary antibody (data not shown).

**Effect of adiponectin isoforms on embryo development**

After 65 h in vitro culture of mouse 4-cell embryos in media supplemented with three different isoforms of adiponectin (using the same dose of the protein, 10 μg/ml), the majority of embryos in all groups reached the blastocyst stage. Table I shows the analysis of the distribution of such embryos. We found opposite effects of the full-length and the trimeric isoform of adiponectin on the embryo distribution. The proportion of embryos with lower cell numbers (32–64 cells) was decreased and the proportion of embryos with high cell numbers (>128 cells) was increased after the full-length adiponectin treatment. In contrast, the proportion of embryos with lower cell numbers (<65 cells) was increased and the proportion of embryos with higher cell numbers (>96 cells) was decreased after the trimeric adiponectin treatment. We found no significant effect of globular adiponectin on the embryo distribution (the increased proportion of embryos with high cell numbers (>128 cells) was not significant because of the low number of embryos at this developmental stage—one embryo in the control group and six embryos in the adiponectin-treated group).

The comparison of cell number in blastocysts confirmed the results in the embryo distribution. The cell number was significantly higher in the full-length adiponectin-treated blastocysts than in controls. On the contrary, the cell number was significantly lower in the trimeric adiponectin-treated blastocysts than in controls. The cell numbers in globular adiponectin-treated blastocysts and control blastocysts did not differ (Fig. 4). Analysis of cell death incidence in blastocysts showed a slightly lower proportion of dead cells in the full-length adiponectin-treated embryos than in controls (however, this difference did not reach statistical significance, \(P = 0.073\)). In contrast, the proportion of dead cells was significantly higher in the trimeric adiponectin-treated blastocysts when compared with controls (\(P = 0.0103\), Table II).
Most information about the roles of adipokines in reproduction refers to leptin, and much less is known about the role of adiponectin and other adipokines. In this study, we showed the expression of adiponectin receptors in mouse ovulated oocytes and preimplantation embryos. We found different effects of full-length adiponectin, globular adiponectin and mutated (trimeric) full-length adiponectin on the development of mouse preimplantation embryos in vitro.

Adiponectin receptors AdipoR1 and AdipoR2 have been shown to be expressed in organs of the female reproductive system, which indicates a potential role of these receptors in the female reproductive system.
with the finding of these receptors in rat and bovine oocytes (Chabrolle et al., 2007; Maillard et al., 2010; Tabandeh et al., 2010), mouse, rabbit and bovine blastocysts (Schmidt et al., 2008; Maillard et al., 2010), in vitro matured pig oocytes, and parthenogenetically activated pig 2-cell embryos and blastocysts (Chappaz et al., 2008). Our quantitative real-time RT–PCR analysis showed that mRNAs for AdipoR1 and AdipoR2 were synthesized in the mouse oocyte, and then (after fertilization) the amounts of both mRNAs dropped, but they increased above the oocyte level from the morula stage. In contrast, Whitworth et al. (2005) found no difference between the pig pre-ovulatory oocyte and 4-cell embryo AdipoR1 and Adipo R2 mRNA amounts, and no change between the oocyte and blastocyst AdipoR2 mRNA, but they found about 1.7× lower levels of AdipoR1 mRNA in blastocysts than in pre-ovulatory oocytes. The discrepancy between these results could be caused by technical reasons, i.e. the use of a less sensitive quantification technique in the Whitworth experiment (transcriptional profiling by microarray), or it could reflect the differences in oocyte developmental stage and species-specific differences in expression profiles of the receptors (ovulated oocytes and mouse in our experiment, pre-ovulatory oocytes and pig in the Whitworth experiment).

Adiponectin was originally thought to be produced exclusively in adipose tissue, however, recent data indicate its expression in other tissues as well, including the female reproductive organs (Chen et al., 2006; Takemura et al., 2006; Archamco et al., 2007; Chabrolle et al., 2007; Schmidt et al., 2008; Maillard et al., 2010). The production of adiponectin by the early embryo itself has been demonstrated from the blastocyst stage. Schmidt et al. (2008) detected adiponectin mRNA and protein in rabbit blastocysts, which suggests the possibility of autocrine/paracrine regulation. On the other hand, these authors did not detect adiponectin in mouse blastocysts (although the protein was detected after embryo implantation, on Day 5 p.c.). Our results partially confirm this observation: we did not find adiponectin protein in mouse blastocysts, but we were able to detect adiponectin mRNA in the blastocyst (using different PCR primers than those used in Schmidt’s work). Taken together, these results indicate that mouse adiponectin gene is transcribed already at the blastocyst stage, but the mRNA is translated into protein only at later developmental stages.

To test the ability of adiponectin to influence the development of preimplantation embryos, we applied full-length adiponectin at physiologically relevant concentration (Nishizawa et al., 2002; Combs et al., 2003; Ledoux et al., 2006), and compared its effects with two other protein isoforms: truncated globular adiponectin and mutated full-length adiponectin (trimeric adiponectin) which can only form trimer, but not hexamer or high molecular weight forms. The full-length adiponectin administered to mouse preimplantation embryos in vitro significantly changed the embryo distribution, increasing the proportion of embryos with high cell numbers and decreasing the proportion of embryos with lower cell numbers. In contrast, administration of the trimERIC adiponectin decreased the proportion of embryos with higher cell numbers and increased the proportion of embryos with lower cell numbers. The analysis of cell number in embryos that reached the blastocyst stage confirmed the opposite effects of the two adiponectin isoforms. Incubation of embryos with the full-length adiponectin led to significant increase in the embryo cell number, whereas incubation of embryos with the trimERIC

Table II Analysis of cell death incidence in blastocysts after incubation with three different adiponectin isoforms.

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Normal cells</th>
<th>Dead cells</th>
<th>Number of blastocysts / number of cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>FL AD</td>
<td>97.68</td>
<td>2.32**</td>
<td>133/13 506</td>
</tr>
<tr>
<td>CTRL</td>
<td>97.35</td>
<td>2.65</td>
<td>160/15 069</td>
</tr>
<tr>
<td>GL AD</td>
<td>96.81</td>
<td>3.19**</td>
<td>148/12 987</td>
</tr>
<tr>
<td>CTRL</td>
<td>97.10</td>
<td>2.90</td>
<td>156/13 715</td>
</tr>
<tr>
<td>TR AD</td>
<td>96.79</td>
<td>3.21*</td>
<td>141/11 843</td>
</tr>
<tr>
<td>CTRL</td>
<td>97.34</td>
<td>2.66</td>
<td>139/12 829</td>
</tr>
</tbody>
</table>

The 4-cell mouse embryos were incubated in the presence of adiponectin (FL AD, full-length adiponectin; GL AD, globular adiponectin; TR AD, trimeric adiponectin) for 65 h, and then the cell number in embryos that reached the blastocyst stage was determined. Values are arithmetical means ± SEM. Black columns, adiponectin-treated groups; white columns, corresponding control groups. Total number of evaluated blastocysts in the experimental groups: FL AD 133, CTRL 160; GL AD 148, CTRL 156; TR AD 141, CTRL 139. Statistical difference between adiponectin-treated groups and corresponding control groups was assessed with unpaired Student’s t-test: **P < 0.01.

Figure 4 Mean cell number in blastocysts after incubation with three different adiponectin isoforms. The 4-cell mouse embryos were incubated in the presence of adiponectin (FL AD, full-length adiponectin; GL AD, globular adiponectin; TR AD, trimeric adiponectin) for 65 h, and then the cell number in embryos that reached the blastocyst stage was determined. Values are arithmetical means ± SEM. Black columns, adiponectin-treated groups; white columns, corresponding control groups. Total number of evaluated blastocysts in the experimental groups: FL AD 133, CTRL 160; GL AD 148, CTRL 156; TR AD 141, CTRL 139. Statistical difference between adiponectin-treated groups and corresponding control groups was assessed with unpaired Student’s t-test: **P < 0.01.
adiponectin led to significant reduction of the cell number in blastocysts. Moreover, the administration of trimeric adiponectin significantly increased the proportion of dead cells in the blastocysts. On the other hand, we found no significant effect of globular adiponectin on the examined parameters. Our results are in accordance with the findings of Chappaz et al. (2008), who showed the positive effect of full-length porcine recombinant adiponectin on in vitro development of parthenogenetically activated porcine embryos to blastocyst stage. These authors also showed the stimulatory effect of adiponectin on in vitro meiotic maturation of porcine oocytes. In contrast, Maillard et al. (2010) found no effect of human recombinant adiponectin on bovine oocyte maturation and preimplantation embryo development in vitro, which suggests some possible species-specific effects of adiponectin.

Several studies have demonstrated effects of adiponectin in various cell types (proinflammatory or anti-inflammatory effects, growth-inhibiting or growth-stimulating effects, activation of various signaling molecules), and these data clearly show that the effects of adiponectin are cell-type and isoform dependent (Tsao et al., 2002, 2003; Luo et al., 2005; Bub et al., 2006; Neumeier et al., 2006; Song et al., 2009; Grossmann et al., 2008). The different effects of the three adiponectin isoforms found in our experiment could result from different intracellular signaling cascades induced by particular adiponectin isoforms, or they could also relate to differences in the abundance of AdipoR1 and AdipoR2 receptor in the embryo. It has been demonstrated that AdipoR1 is a high-affinity receptor for globular adiponectin and a low-affinity receptor for full-length adiponectin, whereas AdipoR2 is an intermediate-affinity receptor for full-length and globular adiponectin (Yamauchi et al., 2003). We found a lower signal intensity for AdipoR1 protein than for AdipoR2 protein in all tested developmental stages, which suggests lower abundance of the AdipoR1 receptor in preimplantation embryos. This could, at least in part, explain the lower effectiveness of the globular adiponectin in our experiment. The opposite effects of the two full-length adiponectin isoforms suggest that the trimeric adiponectin and the adiponectin capable of forming higher multimers can trigger different signaling pathways in preimplantation embryo cells. The changes in cell numbers found in our study suggest an involvement of mitogen-activated protein kinases in the adiponectin functioning, as it has been demonstrated in other cell types (Luo et al., 2005; Benaitreau et al., 2009).

In contrast to leptin, adiponectin knock-out mice are fertile (Ma et al., 2002), which suggests that adiponectin is not absolutely essential for the reproductive process. However, several data indicate that adiponectin can play an important complementary role in the regulation of the female reproductive function. It has been demonstrated that adiponectin can play a role in the modulation of ovarian and endometrial function, influencing perivulatory remodeling of the ovarian follicle, steroid synthesis/secretion as well as energy supply and inflammatory response of endometrial cells (Ledoux et al., 2006; Takemura et al., 2006; Chabrolle et al., 2007; Pierre et al., 2009; Maillard et al., 2010). Our results indicate possible influences of adiponectin on the early embryo development which takes place in the oviduct. The source of adiponectin in the oviduct fluid can be maternal blood circulation (where adiponectin is present in relatively high concentrations) or oviduct epithelium (Leese, 1988; Buhi et al., 2000; Archanco et al., 2007). The distribution of adiponectin isoforms in the oviductal fluid is unknown, and there are results showing differences in the distribution of adiponectin isoforms between the serum and the follicular fluid of women undergoing treatment by ICSI (Bersinger and Wunder, 2010).

In any case, the above-mentioned data suggest that changes in maternal adiponectin levels or isoform distribution could influence the development of the preimplantation embryo. Changes in adiponectin levels have been shown to be associated with some pathologies of the female reproductive system such as polycystic ovarian syndrome (Ardawi et al., 2005), endometriosis (Takemura et al., 2005), endometrial cancer (Dal Maso et al., 2004), pre-eclampsia (Mazaki-Tovi et al., 2009), and breast cancer (Vona-Davis and Rose, 2007). Obesity associated with decreased adiponectin levels is considered as a risk factor for these disorders, although the decreased serum adiponectin level is not always connected with the increased body mass index in these diseases. Obesity is also considered to be a negative factor for fertility, contributing to anovulation, reduced conception rate, reduced response to fertility treatment and impaired pregnancy outcomes. Obese women have a high prevalence of infertility compared with the lean population, and there are data suggesting that some negative influences of obesity on reproductive health can be mediated by adipokines, including adiponectin (Fedorcsák et al., 2004; Norman et al., 2004; Mitchell et al., 2005; Campos et al., 2008). Some studies even suggest the potential of maternal adiponectin to serve as a marker for fertility (Bersinger et al., 2006; Bersinger and Wunder, 2010).

In summary, our results indicate that adiponectin can directly influence development of the preimplantation embryo, and the effects of adiponectin on the embryo are isoform dependent. These results suggest a possible way how maternal obesity could affect the embryo in very early pregnancy.

Authors’ roles
Š.C.: Experimental design, data analysis and interpretation, writing the article. J.B.: Acquisition and analysis of data from the immunohistochemical study, embryo culture and morphological evaluation, critical reading of the manuscript. A.B.: Acquisition and analysis of RT–PCR and real-time PCR quantification data, critical reading of the manuscript. D.F.: Analysis and interpretation of data from the immunohistochemical study and embryo morphological evaluation, critical reading of the manuscript. P.R.: Preparation of animals, isolation and culture of embryos, critical reading of the manuscript. J.K.: Experimental design, data interpretation, critical reading of the manuscript. All authors read and approved the final manuscript.

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