Adiponectin and adiponectin receptors in the mouse preimplantation embryo and uterus

S.T. Kim, K. Marquard, S. Stephens, E. Louden, J. Allsworth, and K.H. Moley*

Department of Obstetrics and Gynecology, Washington University in St Louis, 660 South Euclid Avenue, St Louis, MO 63110, USA

*Correspondence address. Tel: +1-314-362-1997; Fax: +1-314-747-4150; E-mail: moleyk@wustl.edu

Submitted on December 10, 2009; resubmitted on September 13, 2010; accepted on September 17, 2010

BACKGROUND: Adiponectin (Adipoq), a protein secreted by adipocytes in inverse proportion to the adipose mass present, modulates energy homeostasis and increases insulin sensitivity. Tissue Adipoq signaling decreases in settings of maternal diabetes, polycystic ovary syndrome (PCOS) and endometriosis, conditions which are associated with reproductive difficulty. Our objective was to define the expression and hormonal regulation of Adipoq and its receptors in the mouse preimplantation embryo and uterus.

METHODS AND RESULTS: By real-time quantitative PCR, mRNA transcripts for Adipoq, AdipoR1, AdipoR2, Ppara, Ppard, FATP1 (SLC27A1) and acyl CoA oxidase (Acox1) were identified in mouse 2-cell and 8-cell embryos, while blastocyst stage embryos and trophoblast stem (TS) cells expressed mRNA for all genes except Adipoq. Protein expression of Adipoq, AdipoR1, AdipoR2, the insulin sensitive transporters GLUT8 (Slc2A8), GLUT12 (Slc2A12) and p-PRKAA1 was identified by immunofluorescence staining in all stages of preimplantation embryos including the blastocyst. In situ hybridization demonstrated the presence of Adipoq, AdipoR1 and AdipoR2 mRNA in the mouse decidual cells of the implantation site and in artificially decidualized cells, and the expression of these proteins was confirmed by western blotting. Flow cytometry confirmed cell surface expression of AdipoR1 and AdipoR2 in TS cells and decidual cells.

CONCLUSIONS: These results suggest for the first time that Adipoq signaling may play an important role in preimplantation embryo development and uterine receptivity by autocrine and paracrine methods in the mouse. Implantation failures and pregnancy loss, specifically those experienced in women with maternal metabolic conditions such as diabetes, obesity and PCOS, may be the result of aberrant Adipoq and AdipoR1 and AdipoR2 expression and suboptimal decidualization in the uterus.

Key words: adiponectin / adiponectin receptor / preimplantation embryo / uterus / decidua

Introduction

Adiponectin (Adipoq) is a hormone secreted from the adipocytes, cells which highly express the Adipoq protein (Hu et al., 1996). Adipoq plays an important role in regulating energy homeostasis, specifically lipid and glucose metabolism (Berg et al., 2001; Fruebis et al., 2001; Yamauchi et al., 2001), by the activation of AMP-activated protein kinase (PRKAA1) (Yamauchi et al., 2002; Kahn et al., 2005) and peroxisome proliferator-activated receptors (PPARs) (Tsuchida et al., 2005; Nawrocki et al., 2006). In addition, Adipoq has anti-inflammatory, anti-angiogenic and anti-atherosclerotic effects (Yokota et al., 2002; Brakenhielm et al., 2004; Goldstein and Scalia, 2004). Administration of recombinant Adipoq to rodents increases glucose uptake and fatty acid oxidation and reduces fatty acid uptake (Fruebis et al., 2001; Heilbronn et al., 2003).

Recently, two Adipoq receptors (AdipoR1 and AdipoR2) have been identified (Yamauchi et al., 2003) and found to have functional differences. AdipoR1 is highly expressed in the skeletal muscle, while AdipoR2 is highly expressed in the liver. Targeted disruption of AdipoR1 shows the abrogation of Adipoq-induced PRKAA1 activation, whereas that of AdipoR2 increases inflammation and oxidative stress and decreases the activity of Ppara signaling (Yamauchi et al., 2007).

A few studies have demonstrated the relationship between Adipoq and female reproduction. Rat Adipoq, AdipoR1 and AdipoR2 are expressed in theca cells, corpus luteum and oocyte (Chabrolle et al., 2007). Plasma Adipoq levels are reduced in pre-eclampsia (Ouyang et al., 2007), and serum Adipoq level is low in women with polycystic ovarian syndrome (PCOS) (Sir-Petermann et al., 2007). Schmidt et al. (2008) showed that mouse and rabbit blastocysts express AdipoR1 and AdipoR2 mRNA, however only rabbit...
blastocysts express Adipoq mRNA. In the rabbit uterus, Adipoq, AdipoR1 and AdipoR2 are expressed in the glands at the preimplantation stage. Another study revealed high AdipoR1 and AdipoR2 expression in the mid-secretory phase of the human endometrium (Takemura et al., 2005). In addition, serum Adipoq levels are decreased in women with endometriosis (Takemura et al., 2006), anovulatory PCOS (Carmina et al., 2008, 2009), gestational diabetes (Ategbo et al., 2006) and endometrial cancer (Dal Maso et al., 2004). These studies suggest that Adipoq and AdipoR1/R2s may be hormonally regulated at critical times in the peri- and post-implantation period, and that abnormalities in expression of Adipoq and AdipoR1/R2s may occur in certain pathologic conditions associated with pregnancy loss and implantation pathologies. Although several different models of Adipoq, AdipoR1 and AdipoR2 deficiency in mice have been created, the reproductive phenotypes have not been consistently reported or investigated in a systematic fashion (Kubota et al., 2002; Ma et al., 2002; Maeda et al., 2002; Nawrocki et al., 2006). These ambiguities make it difficult to determine whether this ligand and its receptors are essential to female reproduction. In addition, several Adipoq paralogs have been identified in mouse and are called C1q/TNFα-related proteins and thus may compensate for Adipoq in null mice during the process of preimplantation development, implantation and decidualization (Wong et al., 2004). A formal investigation of the relationship between Adipoq signaling and female reproductive tract cross-talk during implantation and early pregnancy, however, has yet to be performed. The objective of this study was to examine the expression and hormonal regulation of Adipoq and AdipoR1/R2s in the preimplantation embryo and uterus of the mouse.

Materials and Methods

Animals and tissue preparation

Mice were housed according to Institutional Animal Care and Use Committee and National Institutes of Health guidelines. Adult C57BL6 female mice purchased from the National Cancer Institute (NIH, Bethesda, MD, USA) were mated with fertile male mice of the same strain to induce pregnancy (Day 1, vaginal plug). On Day 5 and Day 6 of pregnancy, implantation sites (ISs) were visualized by intravenous injection of Chicago Blue dye solution (Sigma, St. Louis, MO, USA). Uteri were isolated from three different pools of embryos which included 155 two-cell embryos (from 10 mice), 132 eight-cell embryos (from 10 mice) and 91 blastocysts (from eight mice). Q-RT PCR was performed in triplicate from each pool of RNA. For the uterine tissue quantification, the normalized Ct of each gene was compared with the Ct at the d5 inter-ISs value. Four animals were used at each time point and six implantation and inter-implantation paired samples were obtained from each animal. Each animal experiment was repeated three times for a total of 48 mice. See Table II for the experimental design and number of animals and IS/inter-IS pairs. Q-RT PCR experiments were repeated three times independently. Data are represented as the mean ± SEM.

Immunofluorescent microscopic detection of protein

Embryos were recovered, fixed and immunostained as described previously (Riley et al., 2005) for AdipoR1 and AdipoR2 (Alpha Diagnostics, Inc., San Antonio, TX, USA), Adipoq (Sigma), Slc2A8 and Slc2A12 (made by the Moley laboratory) (Carayannopoulos et al., 2000; Heilig et al., 2003) and phospho-PRKAA1 (Thr172) (Upstate Cell Signaling, Lake Placid, NY, USA). Nuclei were stained with TO-PRO-3 iodide dye (Molecular Probes, Eugene, OR, USA) by incubating them in 4 mM of the dye for 20 min. After washing three times in phosphate-buffered saline (PBS), fluorescence was observed under a confocal microscope.

Table I Primer sequences.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5’ to 3’)</th>
<th>Size (bp)</th>
<th>GeneBank No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adipoq</td>
<td>F: tgg tcc tct taa tcc tgc cca 144</td>
<td>NM_009605</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: cca acc tgc aca agt cct cct 133</td>
<td>NM_028320</td>
<td></td>
</tr>
<tr>
<td>AdipoR1</td>
<td>F: acg tgg gag agt cat ccc gca t 140</td>
<td>NM_179785</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: ctc tgt ggg gat ggc gaa gat 153</td>
<td>NM_011144</td>
<td></td>
</tr>
<tr>
<td>Ppara</td>
<td>F: gga ggg ttc ggg ggc taa gg 109</td>
<td>NM_011145</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: gca gct cgg gga atg agg 120</td>
<td>NM_011977</td>
<td></td>
</tr>
<tr>
<td>Acox1</td>
<td>F: aga gcc cca tct gtc ctc tc 286</td>
<td>NM_015729</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: act ggg agt ctc caa aac caa a 196</td>
<td>NM_001088</td>
<td></td>
</tr>
<tr>
<td>Prp</td>
<td>F: tcc atc gtc aac aaa gac ggg 123</td>
<td>NM_008084</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: act tgg gct caa tga tgt cac</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C: tgg tct ctt cct gtg ctc</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>F: cgc ttt ctg cgt atc gtc tg</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: gat gca cgg gat cgt ggc t</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>T: fcc aga ctt cca aca tga gga</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C: ctg gtc gta ggt gcc aat ta</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>F: gtg gca tca att ccc gaa tgt ca</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: cct cat cac gtc tat aca tgc g</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>F: aga tgg ggg tga agc gat tgt</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: tggagccttagagtgggtca</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

F, forward; R, reverse.
with Nikon EZ 7.1 software (Nikon Eclipse E800; Nikon Instruments Corp., Melville, NY, USA). Each slide contained 10–15 embryos at each stage. Five mice at each time point for each experiment were used to obtain the embryos at different stages. These experiments were repeated three times for a total of 60 mice.

**Immunohistochemistry**

Frozen sections (10 mm) of ISs and inter-ISs were fixed in 3% paraformaldehyde in PBS for 15 min and washed two times. After blocking of the endogenous peroxidase activity with 3% H2O2 in methanol for 10 min and blocking of background with 10% non-immune goat serum for 1 h, the sections were incubated with the primary antibody (10 ng/ml) at 4°C overnight, washed three times for 5 min with PBS and incubated with biotinylated secondary antibody (Zymed, San Francisco, CA, USA) for 30 min with enzyme conjugate (streptavidin peroxidase; Zymed) for 30 min. Colouring reaction was done using 3,3′-diaminobenzidene (DAB), and sections were counterstained with Hematoxylin (Zymed). Four animals were used at each time point and six implantation and inter-implantation paired samples were obtained from each animal. This experiment was repeated three times (Table II).

**Western blot analysis**

Western blot analysis was performed as described previously (Archanco et al., 2007). Briefly, total protein extract (~15 mg) was separated in a 10% sodium dodecyl sulphate-polyacrylamide gel and then transferred onto nitrocellulose membranes. After blocking with 5% non-fat dry milk powder in 1 x Tris-buffered saline and 0.05% Tween 20 (TBS-T) for 1 h at room temperature, the membranes were incubated with primary antibody (0.25 mg/ml) or preimmune serum at 4°C overnight, washed three times with TBS-T, and incubated with horse-radish peroxidase-conjugated goat anti-rabbit (40 ng/ml; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h. The source and preparation of the primary antibodies are described above. After washing, the signals were visualized using enhanced chemiluminescence western blotting detection reagents (Amersham, Piscataway, NJ, USA) or SuperSignal West Dura Extended Duration Substrate (Thermo Scientific, Rockford, IL, USA). Four animals were used at each time point and six implantation and inter-implantation paired samples were obtained from each animal. This experiment was repeated three times (Table II).

<table>
<thead>
<tr>
<th>Table II Experimental design for Figs 4 and 6.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exp. 1 n = 16 mice</td>
</tr>
<tr>
<td>4 mice x 6 IS/inter-IS pairs</td>
</tr>
<tr>
<td>4 mice x 6 IS/inter-IS pairs</td>
</tr>
<tr>
<td>4 mice x 6 IS/inter-IS pairs</td>
</tr>
<tr>
<td>4 mice x 6 IS/inter-IS pairs</td>
</tr>
<tr>
<td>4 mice x 6 IS/inter-IS pairs</td>
</tr>
<tr>
<td>4 mice x 6 IS/inter-IS pairs</td>
</tr>
<tr>
<td>Total mice = 48</td>
</tr>
<tr>
<td>n = number of uterine pairs</td>
</tr>
<tr>
<td>72 total pairs of IS/inter-IS at d5</td>
</tr>
<tr>
<td>72 total pairs of IS/inter-IS at d5</td>
</tr>
<tr>
<td>72 total pairs of IS/inter-IS at d5</td>
</tr>
<tr>
<td>72 total pairs of IS/inter-IS at d5</td>
</tr>
</tbody>
</table>

In situ hybridization

Sense and antisense 35S-labeled cRNA probes were generated using Sp6 and T7 7T polymericas. Probes had specific activities of ~2 x 106 cpm/ml. Frozen sections (10 μm) were fixed in cold 4% paraformaldehyde solution in PBS, acetylated and hybridized with a 35S-labeled cRNA probe at 45°C overnight. After hybridization, sections were incubated with RNase A (20 mg/ml) at 37°C for 20 min, rinsed, and detected by autoradiography using NTB-2 liquid emulsion (Eastman Kodak Co., Rochester, NY, USA). Sections were counterstained with DAPI after fixation and development. Sections hybridized with the sense probes served as negative controls and showed no positive signals. Sections were taken from the uteri of four different animals for each time point and six implantation and inter-implantation paired samples were obtained per animal. Each experiment was repeated three times (Table II).

**Delayed or activated implantation**

To induce conditions of delayed implantation, six mice per experiment were ovariectomized (OVX) on the morning of Day 4 of pregnancy and maintained with daily injections of progesterone (2 mg/mouse in 0.1 ml of sesame oil, sc) from Day 5 to Day 7. To terminate delayed implantation and to induce blastocyst activation, a single subcutaneous injection of estradiol (E2) (25 mg/mouse in 0.1 ml of sesame oil, sc) was given to one group of three mice at the same time as progesterone injection on Day 7, while the second group of three mice received only progesterone. Whole uteri were collected from each group 12 h after the last injection of steroid. Uteri from three different animals per group (delayed = 3 mice versus activated = 3 mice) were prepared for immunohistochemistry. All experiments (six mice each) were performed in triplicate with three independent sets of animals for a total of 18 mice (Table III).

**Artificial decidualization**

Induction of artificial decidualization was performed as described elsewhere (Deb et al., 2006). In brief, six OVX mice were allowed 20 days of recovery and then injected subcutaneously with E2 (100 ng/mouse) for 3 days. After 2 days rest, E2 (10 ng/mouse) and progesterone (1 mg/mouse) were injected subcutaneously for 3 days. At the third day of E2 + progesterone injection, 20 μg of oil was infused intraluminally into the one uterine horn, and the other side horn was used as control. After progesterone (1 mg/mouse) injection for 4 days, uteri were collected and frozen. β-Actin was used as internal control. Immunoblots were used to confirm the control as a relative density of 1. Both uterine hornes from three different animals (control versus artificial decidualization) were prepared for western immunoblot and compared after normalization by Student’s t-test. All experiments were performed three times with three independent sets of animals for a total of nine mice (Table III).

**Isolation and culture of endometrial stromal cells**

Isolation and culture of artificial decidualization was performed as described elsewhere (Li et al., 2007). In brief, uteri from five to eight mice were dissected at pregnant day 4 and blastocysts were flushed to confirm pregnancy. After washing with Hank’s balanced salt solution (HBSS), uterine tissues were cut into 1–3 mm pieces, pooled and placed in Dulbecco’s modified Eagle’s medium (DMEM)/Ham’s F12 without phenol red (1:1 vol/vol; Invitrogen Co., Grand Island, NY, USA),
containing 0.2% collagenase (type I), with gentle pipetting every 15 min. After digestion, the cell suspension was left in an upright position for 5 min. Then, the supernatant, the stromal cell-rich fraction, was serially transferred onto 70 and 40 μm cell strainers (Falcon, Franklin Lakes, NJ, USA) and centrifuged for 5 min. The purity of stromal cells obtained by this method was usually >90%, as determined by immunostaining against vimentin and cytokeratin. The purified stromal cells were washed, and the number of unattached cells were removed by washing several times with HBSS, and cell culture was continued after addition of fresh DMEM/Ham’s F12 without phenol red medium supplemented with 2% charcoal-stripped fetal bovine serum (FBS) (Hyclone Laboratories, South Logan, UT, USA), 100 IU/ml penicillin, 100 mg/ml streptomycin. In vitro decidualization was achieved with progesterone (1 μM) and estrogen (10 nM) for 3 days. For inhibition experiments, cells were incubated for 10 min with different concentrations of cytochalasin B (Sigma), an inhibitor of facilitative glucose transporters or Slc2As. Decidualization was quantified by the level of decidual/trophoblast prolactin-related protein (PRP, NM_010088) mRNA expression. Five to ten pregnant mice were used to collect stromal cells for each experiment. Experiments were repeated five times with five different sets of animals (six, six, five, seven and eight mice sequential experiments) for a total of 32 mice (Table III).

<table>
<thead>
<tr>
<th>Figure 7</th>
<th>Figure 8</th>
<th>Figure 9</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong># Mice</strong></td>
<td><strong>Act/delay implant</strong></td>
<td><strong># Mice</strong></td>
</tr>
<tr>
<td>Exp. 1</td>
<td>6</td>
<td>3/3</td>
</tr>
<tr>
<td>Exp. 2</td>
<td>6</td>
<td>3/3</td>
</tr>
<tr>
<td>Exp. 3</td>
<td>6</td>
<td>3/3</td>
</tr>
<tr>
<td>Exp. 4</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Exp. 5</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Total no. mice</td>
<td>18</td>
<td></td>
</tr>
</tbody>
</table>

**Table III** Experimental design for Figs 7–9.

**Figure 1** RT-PCR of Adipoq signaling in the preimplantation embryo. GAPDH was used as internal control to normalized cycle threshold (Ct). The normalized Ct of each individual gene was compared with the Ct at a 2-cell stage and expressed as fold change compared with 2-cell mRNA. Numbers of embryos pooled: 155 two-cell embryos (10 mice), 132 eight-cell embryos (10 mice) and 91 blastocysts (8 mice). Experiments were conducted three times in triplicate from each pool of RNA. (A) AdipoQ, AdipoR1 and AdipoR2 expression at different time points in development. Note the differences between different developmental stages for each gene [AdipoR2 (8-cell and blastocyst) versus 2-cell; AdipoR1 (8-cell versus 2-cell)], U.D., undetected; (B) Expression of Ppara and Ppard, both downstream and upstream components respectively of the adiponectin signaling cascade, in embryos from different stages. Note the decrease in expression of both with development to a blastocyst; (C) Expression of Slc27A1 and Acox1, two downstream components of adiponectin signaling. Note the increase in expression of the fatty acid transport protein, Slc27A1 between the 2-cell and 8-cell stage, however this drops to near negative expression at the blastocyst stage.
Cell culture

Trophoblast stem (TS) cell culture and isolation and culture of endometrial stromal cells (ESCs) were performed as described previously and above (Rossant, 2001; Li et al., 2007). The TS cell line was a generous gift from Dr Janet Rossant (Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Canada). TS lines were maintained in the presence of mouse-embryonic fibroblast (MEF) conditioned media (MEF-CM). MEF-CM was generated as described previously (Rossant, 2001). TS cells were cultured in 70% MEF-CM and 30% TS medium supplemented with 25 ng/ml fibroblast growth factor-4 and 1 μg/ml heparin (Sigma).

Flow cytometry

TS cells and ESCs were harvested with Cell Dissociation Solution (1×) Non-enzymatic (Sigma), washed with PBS/10% FBS, and incubated in blocking solution (PBS/10% FBS/5% normal rabbit sera). Alexa 488 labeled-primary antibodies or rabbit immunoglobulin G for isotype control were treated for 30 min on ice. Acquisition and analysis were performed on the fluorescence-activated cell sorter Calibur cytometer using Cell Quest software (Becton Dickinson, Rockville, MD, USA). The experiment with TS cells was performed twice with three sets of cell lysates/group. The experiment with ESCs was performed three times with three sets of cell lysates/group.

Statistical analysis

Differences in RT-PCR data between IS and inter-IS samples were analyzed with Student’s t-test for comparison of two different groups. Protein quantification from western immunoblotting was performed via normalization to β-actin for each gel. Student’s t-test was used for comparison between two groups. A P-value of <0.05 was considered statistically significant.

Results

Expression of Adipoq signaling molecules in preimplantation embryo

To determine whether Adipoq and Adipoq signaling molecules are expressed in the preimplantation embryo, RT-PCR was performed.
RT-PCR demonstrated the presence of Adipoq mRNA in the 2-cell and 8-cell embryo, however, Adipoq mRNA was not detected at the blastocyst stage. AdipoR1 and AdipoR2 mRNA was detected at all stages of the preimplantation embryo, although levels were lowest at the blastocyst stage. Adipoq and AdipoR1/R2 levels peaked at the 8-cell embryo stage (Fig. 1A). Ppara and Ppard are involved in Adipoq signaling as downstream and upstream molecules respectively (Kadowaki and Yamauchi, 2005; Choi et al., 2007). RT-PCR demonstrated the presence of mRNA for both Ppara and Ppard at all stages of the preimplantation embryo (Fig.1B). In addition, mRNA transcripts for fatty acid transport protein (Slc27A1) and acyl CoA oxidase (Acox1), which are downstream proteins of the Adipoq signaling pathway (Yamauchi et al., 2001; Palanivel et al., 2007), were also detected at all stages of the preimplantation embryo (Fig. 1C). Interestingly, by immunofluorescence staining, Adipoq protein was detected from the 1-cell to blastocyst stage, despite the lack of Adipoq mRNA at the blastocyst stage (Fig. 2A). AdipoR1 (Fig. 2B) and AdipoR2 (Fig. 2C) proteins were also detected at all stages of the preimplantation embryo. Next, we analyzed glucose transporters (Slc2A) and p-PRKAA1 protein expression in the blastocyst, since Adipoq signaling affects downstream glucose metabolism via PRKAA1 activation and increased glucose uptake (Kadowaki and Yamauchi, 2005; Palanivel et al., 2007). Slc2A8 and Slc2A12, two facilitative glucose transporters, and p-PRKAA1, a direct target of Adipoq signaling, were all expressed at the blastocyst stage (Fig. 2D).

To confirm the expression and localization of Adipoq signaling molecules in the blastocyst, we analyzed TS cells, which are originally derived from the blastocyst. RT-PCR did not detect Adipoq mRNA in the TS cells, however it did demonstrate the presence of AdipoR1 and AdipoR2 mRNA (Fig. 3A). Ppara and Ppard mRNA transcripts were detected in TS cells although the level of Ppara mRNA was extremely low (Fig. 3B). Slc27A1 and Acox1 mRNA transcripts were also detected in the TS cells (Fig. 3C). TS cell surface expression of AdipoR1 and AdipoR2 was confirmed by flow cytometry (Fig. 3D).

Expression of Adipoq signaling molecules in uterus during peri-implantation periods

To determine whether the expression of Adipoq signaling molecules differ in pregnancy, we analyzed ISs and inter-ISs in pregnant uteri from Day 5 to Day 8 of pregnancy. As shown in Fig. 4A–C, mRNA levels of Adipoq, AdipoR1 and AdipoR2 were all significantly higher at the ISs than at the inter-ISs. Adipoq and AdipoR1 gradually increased at the ISs from Day 5 to Day 8. Ppara mRNA was also significantly higher at the ISs compared with the inter-ISs, and gradually increased at the ISs from Day 5 to Day 8 (Fig. 4D). Ppard mRNA was increased less dramatically at the ISs compared with the inter-ISs (Fig. 4E); however, the difference was significant. Slc27A1 and Acox1 mRNA were also significantly increased at the ISs compared with the inter-ISs. These transcripts gradually increased at the ISs from Day 5 to Day 8 (Fig. 4F and G).

To verify the localization of Adipoq, AdipoR1 and AdipoR2 mRNA, in situ hybridization was performed. Adipoq mRNA mainly localized to the decidual cells of the ISs and to the luminal epithelia of the inter-ISs. From Day 5 to Day 8, Adipoq mRNA levels appeared to increase in the decidual cells of the ISs. In addition, Adipoq mRNA was detected in the embryo at Day 7 and Day 8 (Fig. 5A). AdipoR1 mRNA was highly localized in the decidual cells and embryo of the ISs and in the luminal...
AdipoR1 was widely localized in the decidual cells (Fig. 5B), whereas AdipoR2 was highly focused in the decidual cells and embryo of the ISs and in the luminal epithelia of the inter-ISs. AdipoR2 was more strongly localized in the primary decidual cells than in the secondary decidual cells (Fig. 5C).

Next, protein expressions of Adipoq, AdipoR1 and AdipoR2 in the pregnant uterus were compared by western immunoblotting. Adipoq, AdipoR1 and AdipoR2 protein expressions were significantly higher at the ISs than at the inter-ISs from Day 5 to Day 8 (Fig. 6A, Supplementary data, Fig. S1), and followed the same general trend as the mRNA. Immunohistochemistry was used to detect AdipoR1 and AdipoR2 proteins. Both were localized in the decidual cells of the ISs and in the glandular and luminal epithelia, but not in the stromal cells of the inter-ISs at Day 7 (Fig. 6B and C). These results were consistent with the mRNA results.

Decidualization effects on Adipoq, AdipoR1 and AdipoR2 expressions in the delayed implantation model

To determine whether decidualization affects the expressions of Adipoq, AdipoR1 and AdipoR2 in the uterus during the period of implantation, we analyzed protein expression by immunofluorescent microscopy in sections from the uteri of mice subjected to delayed and activated implantation. After the termination of delayed implantation by the injection of E2, the expressions of Adipoq, AdipoR1 and AdipoR2 all increased (Fig. 7).

Expressions of Adipoq, AdipoR1, AdipoR2 and GLUT during decidualization

To confirm that the increase in Adipoq, AdipoR1 and AdipoR2 expression seen in Fig. 6 was in the decidual cells, we used both an artificial decidualization model and performed in vitro decidualization of harvested ESCs. As shown in Fig. 8, Adipoq, AdipoR1 and AdipoR2 protein expression in the uteri of an artificial decidualization model was significantly higher than in the control uteri. Next, to determine if decidual cells secreted Adipoq, we isolated ESCs from the uteri at Day 4 of pregnancy and cultured them with estrogen (E2) and progesterone to induce in vitro decidualization. Decidual cells, not control ESCs, secreted Adipoq to the media (Fig. 9A). By flow cytometry, cell surface expressions of AdipoR1 and AdipoR2 were found to be higher in the decidual cells than in the control ESCs (Fig. 9B). Decidualization was confirmed by the mRNA level of decidual/trophoblast PRP, one of the markers of decidual cells. Abundant PRP expression was only detected in the decidual cells (Fig. 9C).
In this study, we have investigated the role of Adipoq in the embryo–maternal interaction by mapping out the expression pattern of this ligand and its receptors in the murine reproductive tract from fertilization through implantation. Adipoq plays an important role in regulating whole body energy homeostasis by increasing insulin sensitivity (Berg et al., 2001; Fruebis et al., 2001; Yamauchi et al., 2001), both at the level of the liver and of skeletal muscle. Although tissue-specific functions of Adipoq vary depending on the cell type, the main mechanism of action of Adipoq is activation of PRKAA1 (Yamauchi et al., 2002; Kahn et al., 2005) and induction of the PPARs (Tsuchida et al., 2005; Nawrocki et al., 2006). In this study, we determined that mRNA of Adipoq, AdipoR1/R2s and downstream signaling

**Discussion**

In this study, we have investigated the role of Adipoq in the embryo–maternal interaction by mapping out the expression pattern of this ligand and its receptors in the murine reproductive tract from fertilization through implantation. Adipoq plays an important role in regulating whole body energy homeostasis by increasing insulin sensitivity (Berg et al., 2001; Fruebis et al., 2001; Yamauchi et al., 2001), both at the level of the liver and of skeletal muscle. Although tissue-specific functions of Adipoq vary depending on the cell type, the main mechanism of action of Adipoq is activation of PRKAA1 (Yamauchi et al., 2002; Kahn et al., 2005) and induction of the PPARs (Tsuchida et al., 2005; Nawrocki et al., 2006). In this study, we determined that mRNA of Adipoq, AdipoR1/R2s and downstream signaling
components such as the PPARs and the fatty acid oxidation proteins, Slc27A1-1 and Acox1, are present in the preimplantation mouse embryo from the 2-cell to 8-cell stages, most likely due to persistence of maternal mRNAs. At the blastocyst stage, mRNA for all of the above, with the exception of Adipoq, was also detected. This discrepancy may be due to embryonic genomic activation and thus expression of all these mRNAs except Adipoq. Alternatively, the embryos could experience selective degradation of Adipoq message.
Adipoq mRNA at the blastocyst stage had previously been described more quickly than the other mRNAs. Although this lack of Adipoq and AdipoR1/R2s in mouse embryos and uterus (Mao et al., 2007). Taken together, Adipoq by an autocrine (produced by the embryo itself), a paracrine (secreted by the endometrium or oviduct) and/or an endocrine source (mouse adipose tissue) may play important roles in preimplantation mouse embryo development and implantation. Adipoq signaling during the preimplantation period may function to promote fatty acid oxidation for energy substrate via PRKAA1 activation and ACOX1/SLC7A1 induction (Campos et al., 2008; Dupont et al., 2008). In recent studies we demonstrated that PRKAA1 activity is decreased in poor quality embryos from diabetic mice, and that PRKAA1 activation both rescues the embryo and stimulates fatty acid oxidation (Eng et al., 2007). We hypothesize that Adipoq via AdipoR1 and/or AdipoR2 may act via the transcription factor APPL1 to trigger PRKAA1 activation and downstream fatty acid oxidation, and to replete energy stores in the developing embryo as it travels down the oviduct (Mao et al., 2006). Future studies will be designed to test this hypothesis using allogenic mating pairs as opposed to syngeneic pairs as done in this study. Although it is a limitation of this study, our conclusions are valid. Using allogenic mating, however, is more clinically relevant and may reveal additional findings not detected in this study.

In this study, we also examined the dynamic expression and hormonal regulation of Adipoq and AdipoR1/R2s during early pregnancy. A recent study reported that AdipoR1 and AdipoR2 are highly expressed in the mid-secretory phase of the human endometrium, which is equivalent to the window of implantation (Takemura et al., 2006). In addition, serum Adipoq levels are decreased in women with endometriosis (Takemura et al., 2005), PCOS (Carmina et al., 2008, 2009) and obesity and type 2 diabetes (Weyer et al., 2001), which are all associated with high rates of implantation failure and pregnancy loss. These prior studies suggest that paracrine secretion from the endometrium may be timed to optimize both uterine receptivity and blastocyst activation. To determine whether the expression of Adipoq and downstream signaling molecules are regulated in the pregnant uterus, we analyzed the implantation (IS) versus inter-IS expression of these protein in the pregnant uterus from Day 5 to Day 8. Adipoq signaling was dramatically higher in the ISs than in the inter-IS, and Adipoq and AdipoR1/R2s were mainly localized in the decidual cells and embryo of the IS and in the luminal epithelia in the inter-IS. These findings suggest that Adipoq is important for initiation and/or maintenance of implantation but is also basally expressed in luminal epithelium as well. Whereas AdipoR1 was widely distributed among all the decidual cells, AdipoR2 was more strongly localized in the primary decidual cells than in the secondary decidual cells. AdipoR1 is highly expressed in the skeletal muscle, and AdipoR2 is highly expressed in the liver. Targeted disruption of AdipoR1 shows the abrogation of Adipoq-induced PRKAA1 activation, whereas that of AdipoR2 increases inflammation and oxidative stress and decreases the activity of Ppara signaling (Yamauchi et al., 2007). This suggests that AdipoR1 and AdipoR2 may have different functions in regard to decidualization in the pregnant uterus.

Estrogen injection for termination of delayed implantation increased the expression of Adipoq and AdipoR1/R2s in the luminal epithelia. This suggests that Adipoq and AdipoR1/R2 expression in the luminal epithelia before implantation is important for blastocyst activation and uterine receptivity. Moreover, increased expressions of Adipoq and AdipoR1/R2s were confirmed by using artificial

**Figure 7** Immunofluorescence of Adipoq and AdipoRs in the uterus of delayed or activated implantation model. Paraformaldehyde-fixed tissue slices were incubated with primary antibodies. Slides were then incubated with a secondary antibody, Alexa Fluor 488 goat anti-rabbit immunoglobulin G (green fluorescence). TO-PRO-3 iodide was used to stain the nuclei (blue fluorescence). Uteri from three different animals per group (delayed versus activated) were prepared for immunohistochemistry. All experiments were performed three times with three independent sets of animals for a total of 18 mice.

more quickly than the other mRNAs. Although this lack of Adipoq mRNA at the blastocyst stage had previously been described (Schmidt et al., 2008), protein expression had not been measured. In this study, evaluation of Adipoq and AdipoR1/R2s protein expressions during the preimplantation stages revealed the presence of all these proteins by immunofluorescence staining. This suggests that the Adipoq protein detected in the blastocyst may still be present from message produced from the 8-cell or morula stage embryo. Alternatively, Adipoq in the blastocyst may be derived from another source such as the uterine epithelia, since Adipoq exists in the luminal and glandular epithelia of uterus before implantation and it is a secretory protein. Moreover, the oviduct also expresses Adipoq, and may secrete Adipoq into the oviductal fluid. A recent study in the rat also suggests that the rat oviduct expresses Adipoq (Archanco et al., 2007).
decidualization and an in vitro decidualization system. This suggests that Adipoq signaling by autocrine and paracrine mechanism may play an essential role in embryo development and decidualization in the IS of pregnant uterus during the peri-implantation phase. These findings also suggest that progesterone and E2 may cross-talk with Adipoq signaling or modulated Adipoq effects via different receptors. Several studies using breast cancer cells lines have reported conflicting results. One study found expression of both receptors and Adipoq in MCF-7 breast cancer cells. Expression of AdipoR1 decreased in response to E2 exposure; a finding that was inhibited by adding Adipoq. In addition, Adipoq plus E2 resulted in increased proliferation in E2 responsive MCF-7 cells but decreased proliferation and increased apoptosis in non-E3 responsive NDA-MB-231 breast cancer cell lines (Pfeiler et al., 2008). Alternatively, Grossmann and colleagues reported that Adipoq inhibited growth and increased apoptosis in MCF-7 cells (Grossmann et al., 2008). Studies in the ovary have demonstrated that Adipoq added to porcine granulosa cells modulates the expression of steroidogenic proteins such as StAR and cytochrome P450 via thiomimogen activated protein kinase (MAPK) pathway (Ledoux et al., 2006). Interestingly, Adipoq alone did not affect steroid production in rat granulosa cells, however, it did increase progesterone production in response to insulin-like growth factor-I by 2-fold (Chabrolle et al., 2007).

PRKAA1 is activated by an increase in the AMP/ATP ratio and plays a key role in energy metabolism including fatty acid oxidation and synthesis, glucose uptake and cholesterol synthesis (Corton et al., 1994; Kemp et al., 1999; Hardie, 2003). Additionally, Scl2A4 translocation is mediated by PRKAA1 signaling in the heart (Russell et al., 1999; Goldstein and Scalia, 2004) and recent evidence suggests that Scl2A8 also translocates in blastocysts and TS cells in response to PRKAA1 activation (Eng et al., 2007; Louden et al., 2008). Adipoq may act via PRKAA1 activation to promote adequate glucose transport in the preimplantation embryo at the blastocyst stage. Other studies have demonstrated that Adipoq in the heart induces cyclooxygenase-2 (COX-2)-dependent prostaglandin E2 synthesis by p-PRKAA1 pathway (Shibata et al., 2005). This report, together with other findings in heart (Takahashi et al., 2005; Ikeda et al., 2008) and adipose tissue (Yokota et al., 2002), suggest that Adipoq signaling may play an important role in uterine receptivity and decidualization through the COX-2 pathway.

Our study also demonstrates for the first time that ESCs secrete Adipoq when decidualized. A recent study found that Adipoq exerts anorexigenic and insulin/leptin-like actions in the rat hypothalamus, and AdipoR1 mediates these actions through IRS1/2-Akt/FOXO1, Erk and JAK2-STAT3 signaling pathway (Coope et al., 2008). This suggests that AdipoR1 may mediate the decidualization induced by Adipoq through Akt/Erk/PRKAA1 signaling and through glucose utilization by affecting GLUT localization. We also showed that AdipoR2 is mainly localized in the primary decidual cells of the IS. Major fluctuations in oxygen concentrations occur at the fetomaternal interface.
During normal pregnancy (Gellersen et al., 2007). This suggests that AdipoR2 may mediate Adipoq function by decreasing oxidative stress through PPAR signaling to prevent embryo and fetal stress.

In conclusion, Adipoq signaling may play an important role in preimplantation embryo development and uterine receptivity in an autocrine/paracrine/endocrine manner. The reproductive tract expression pattern of the ligand and receptors suggests embryo–uterus cross-talk occurs via Adipoq signaling before and during decidualization in order to maintain pregnancy during the peri-implantation period.

Supplementary data
Supplementary data are available at http://humrep.oxfordjournals.org/.

Funding
This work was supported by funding from the NIH ROI HD040810-06 and ROI HD065435 to K.H.M. and T32 HD049305 to E.L.

Conflict of interest: none declared.

References
Carayannopoulos MO, Chi MM, Cui Y, Pingsterhaus JM, McKnight RA, Mueckler M, Devaskar SU, Moley KH. GLUT8 is a glucose transporter responsible for insulin-stimulated glucose uptake in the blastocyst. Proc Natl Acad Sci USA 2000; 97:7313–7318.


