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# Adiponectin and adiponectin receptors in the mouse preimplantation embryo and uterus

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**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/TNFa-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 immediately frozen in cold Friendly Freeze'it (Curtin Matheson Scientific, Houston, TX, USA) and stored at −75°C until protein extraction or cryosection.

## RNA extraction and quantitative real-time PCR

Total RNA from harvested cells was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcription with oligo(dT) priming was performed from 1 mg of total RNA using Superscript III (Invitrogen). The relative expression of each transcript was determined by quantitative real-time PCR (Q-RT PCR) in an ABI 7000 Sequence Detection System (Applied Biosystems, Forrest City, CA, USA). Each well of the 96-well reaction plate contained a total volume of 25 ml with Power SYBR Green PCR Master Mix (Applied Biosystems). The threshold cycle (Ct) was used for determining the relative expression level of each gene, by normalizing to the Ct of *Gapdh* as described previously (Ratchford *et al*., 2008). The method of delta-delta cycle threshold (ddCT) was used to calculate the relative fold-change of each gene. Because SYBR Green binding is not sequence specific, a dissociation curve analysis was performed at the end of the amplification process, and the PCR products were subjected to agarose gel electrophoresis to verify the specificity of the PCR products.

For the embryo mRNA quantification, the normalized Ct of each gene was compared with the Ct at a 2-cell stage and expressed as fold-change compared with 2-cell mRNA. Primer sequences are listed in Table I. RNA was 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.**

| Gene | Sequence (5′ to 3′) | Size (bp) | GeneBank Acc. No. |
|---|---|---|---|
| *Adipoq* | F: tgt tcc tct taa tcc tgc cca | 144 | NM_009605 |
| *AdipoR1* | R: cca acc tgc aca agt tcc ctt | 133 | NM_028320 |
| *AdipoR2* | F: acg ttg gag agt cat ccc gta t | 140 | NM_197985 |
| *Ppara* | R: ctc tgt gtg gat gcg gaa gat | 153 | NM_011144 |
| *Ppard* | F: gga gtg ttc gtg ggc tta gg | 109 | NM_011145 |
| *Slc27a1* | R: gca gct ccg gtg ata tag agg | 120 | NM_011977 |
| *Acox1* | F: aga gcc cca tct gtc ctc tc | 286 | NM_015729 |
| *Prp* | R: act ggt agt ctg caa aac caa a | 196 | NM_010088 |
| *Gapdh* | F: tcc atc gtc aac aaa gac ggg | 123 | NM_008084 |
| | R: act tgg gct caa tga tgt cac | | |
| | F: cgc ttt ctg cgt atc gtc tg | | |
| | R: gat gca cgg gat cgt gtc t | | |
| | F: tcc aga ctt cca aca tga gga | | |
| | R: ctg ggc gta ggt gcc aat ta | | |
| | F: gct gca tca att cct gaa tgt ca | | |
| | R: cct cat cac gtc tat aca tgc g | | |
| | F: agg tcg gtg tga acg gat ttg | | |
| | R: tgtagaccatgtagttgaggtca | | |

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% $H_2O_2$ 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 mg/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 and 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× 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 for a total of 48 mice.

**Table II Experimental design for Figs 4 and 6.**

| | Gest day 5 | Gest day 6 | Gest day 7 | Gest day 8 |
|---|---|---|---|---|
| Exp. 1 $n = 16$ mice | 4 mice × 6 IS/inter-IS pairs | 4 mice × 6 IS/inter-IS pairs | 4 mice × 6 IS/inter-IS pairs | 4 mice × 6 IS/inter-IS pairs |
| Exp. 2 $n = 16$ mice | 4 mice × 6 IS/inter-IS pairs | 4 mice × 6 IS/inter-IS pairs | 4 mice × 6 IS/inter-IS pairs | 4 mice × 6 IS/inter-IS pairs |
| Exp. 3 $n = 16$ mice | 4 mice × 6 IS/inter-IS pairs | 4 mice × 6 IS/inter-IS pairs | 4 mice × 6 IS/inter-IS pairs | 4 mice × 6 IS/inter-IS pairs |
| Total mice = 48 | 24 pairs at d5 for each exp. | 24 pairs at d6 for each exp. | 24 pairs at d7 for each exp. | 24 pairs at d8 for each exp. |
| $n$ = number of uterine pairs | 72 total pairs of IS/inter-IS at d5 | 72 total pairs of IS/inter-IS at d6 | 72 total pairs of IS/inter-IS at d7 | 72 total pairs of IS/inter-IS at d8 |

## *In situ* hybridization

Sense and antisense $^{35}$S-labeled cRNA probes were generated using *Sp6* and *T7* polymerases. Probes had specific activities of ~$2 \times 10^6$ cpm/ml. Frozen sections (10 μm) were fixed in cold 4% paraformaldehyde solution in PBS, acetylated and hybridized with a $^{35}$S-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 ($E_2$) (25 ng/mouse in 0.1 mM 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 $E_2$ (100 ng/mouse) for 3 days. After 2 days rest, $E_2$ (10 ng/mouse) and progesterone (1 mg/mouse) were injected subcutaneously for 3 days. At the third day of $E_2$ + progesterone injection, 20 μ. 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 using normalized using 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),

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

| | Figure 7 | | Figure 8 | | Figure 9 | |
|---|---|---|---|---|---|---|
| | # Mice | Act/delay implant | # Mice | Con/ART decidual horn | # Mice | # Stromal cells |
| Exp. 1 | 6 | 3/3 | 6 | 6/6 | 6 | Pooled |
| Exp. 2 | 6 | 3/3 | 6 | 6/6 | 6 | Pooled |
| Exp. 3 | 6 | 3/3 | 6 | 6/6 | 5 | Pooled |
| Exp. 4 | | | | | 7 | Pooled |
| Exp. 5 | | | | | 8 | Pooled |
| Total no. mice | 18 | | 18 | | 32 | |

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).

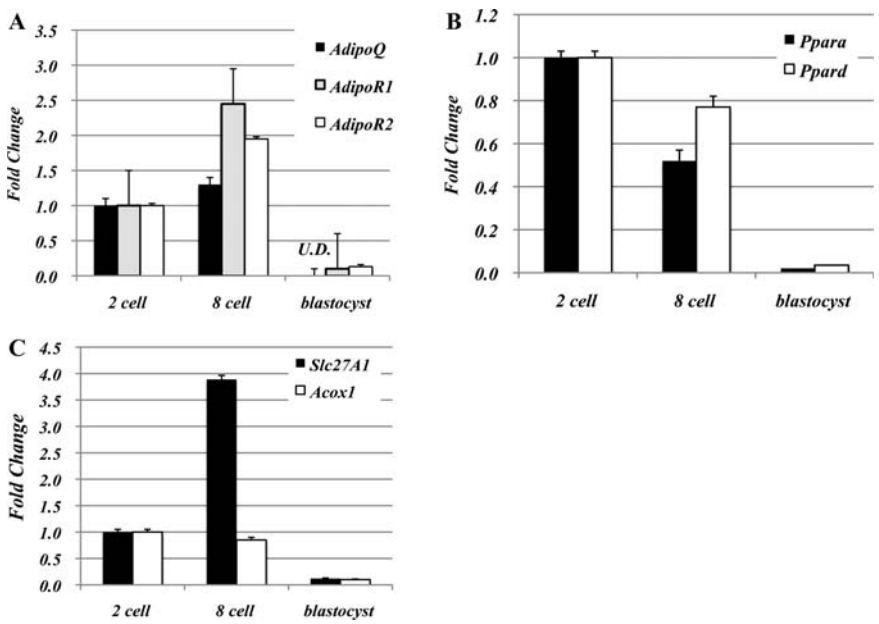


**Figure 1** RT-PCR of Adipoq signaling in the preimplantation embryo. GAPDH was used as internal control to normalized cycle threshold (Ct). The normalized Ct of each individual gene was compared with the Ct at a 2-cell stage and expressed as fold change compared with 2-cell mRNA. Numbers of embryos 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.

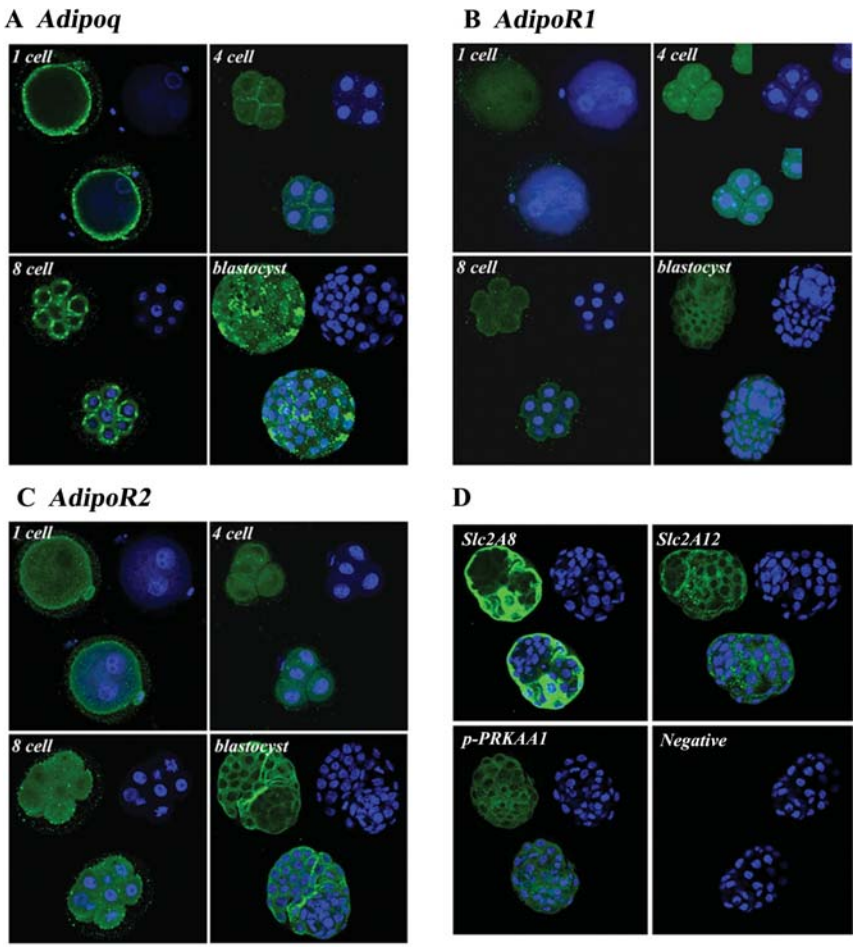


**Figure 2** Immunofluorescence of Adipoq (**A**), AdipoR1 (**B**), AdipoR2 (**C**) and Slc2A/p-PRKAA1 (**D**) in the embryo. Paraformaldehyde-fixed embryos were incubated with primary antibodies. Slides were then incubated with a secondary antibody, Alexa Fluor 488 goat anti-rabbit immunoglobulin G (green fluorescence). TO-PRO-3 iodide was used to stain the nuclei (blue fluorescence). 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 with three different sets of animals for a total of 60 mice.

## 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.

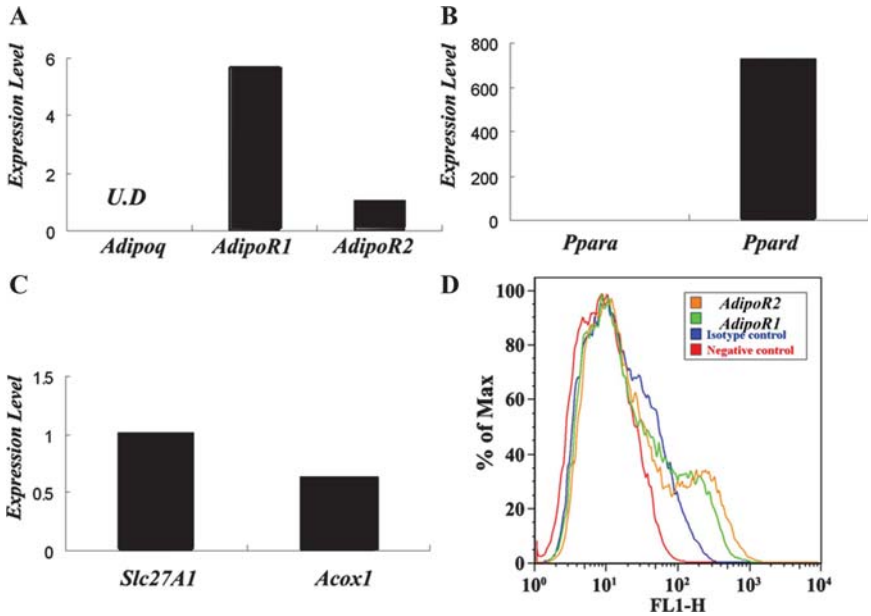


**Figure 3** Adipoq signaling expression in TS cells. (**A**–**C**) RT-PCR of transcripts for Adipoq signaling molecules in TS cells. The Ct was used for determining the relative expression level of each gene, by normalizing to the Ct of *Gapdh*. The method of ddCT was used to calculate the relative fold change of each gene. Note that *Adipoq* mRNA was not detected in TS cells, but mRNAs for *AdipoRs* and other Adipoq signaling molecules were detected. The mean of two RT-PCRs is shown. (**D**) Cell surface expression of AdipoR1 and AdipoR2 in TS cells by flow cytometry. This experiment was repeated twice with different sets of TS cells lysates. Data samples were run in triplicate. U.D, undetected.

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

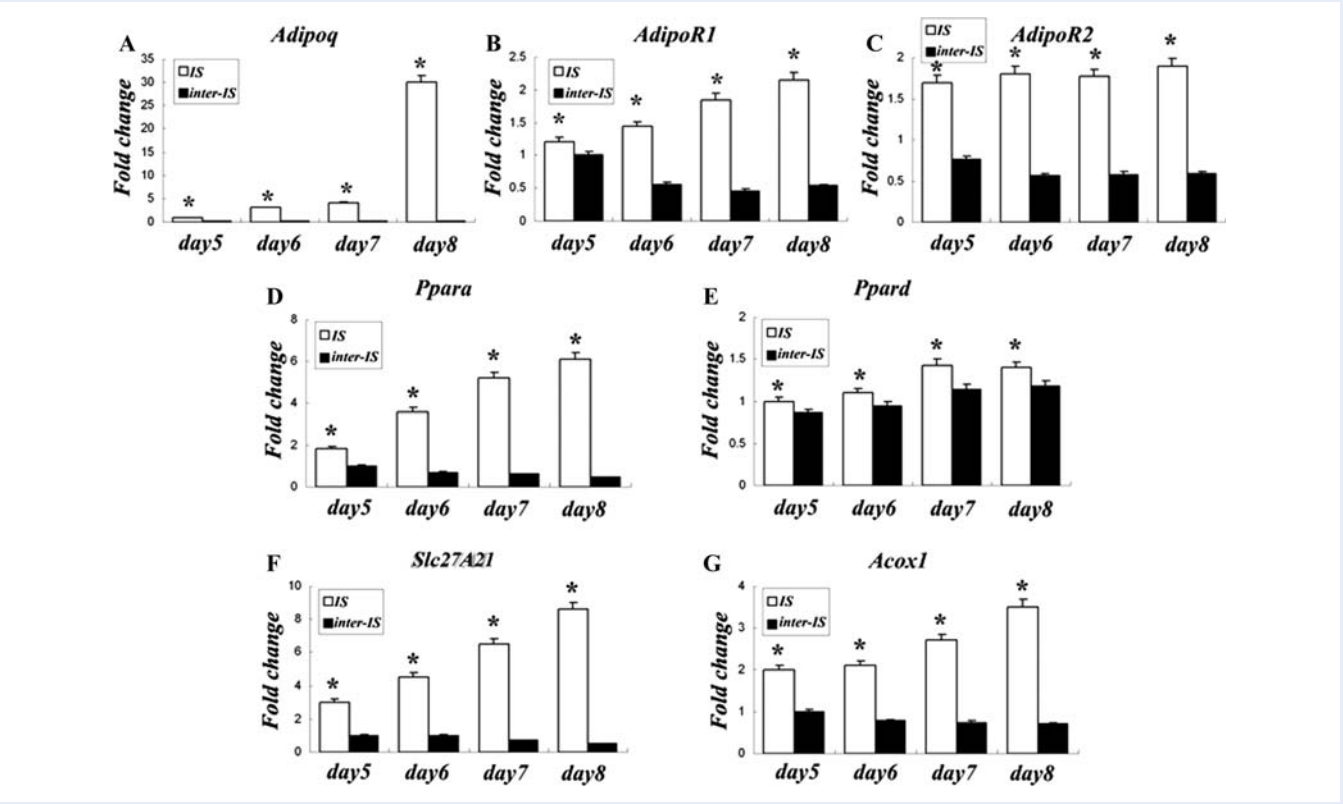


**Figure 4** RT-PCR of Adipoq signaling in the uterus during pregnancy. *Gadph* was used as internal control to normalized Cycle threshold (Ct). The normalized Ct of each gene was compared with the Ct at the d5 inter-IS value since this was considered the control state. Asterisks indicate significant differences between different day IS samples and their inter ISs by Student's *t*-test. ($P < 0.01$) IS, implantation sites; inter-IS, inter-implantation sites. 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 with three different sets of mice for a total of 48 mice. Q-RT PCR experiments were repeated at least three times independently. Data are represented as the mean ± SEM.

epithelia of the inter-ISs. *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 $E_2$, 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 ($E_2$) 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).

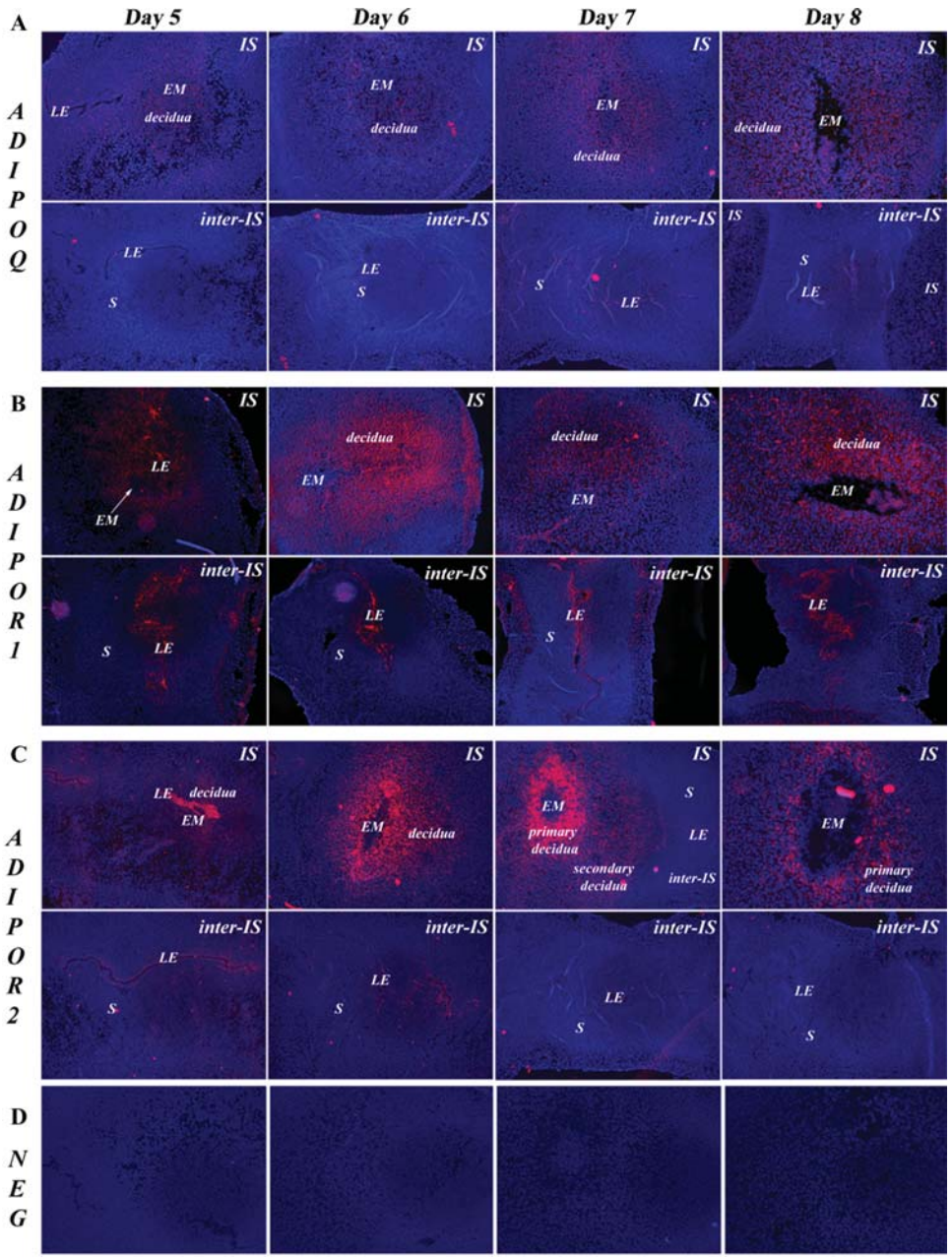


**Figure 5** *Adipoq* (**A**), *AdipoR1* (**B**) and *AdipoR2* (**C**) mRNA localization in the uterus during peri-implantation periods. Longitudinally, sectioned uterus were fixed on paraformaldehyde and hybridized with a $^{35}$S-labeled cRNA probe. Sections were then incubated with RNase A, rinsed and detected by autoradiography (red fluorescence). Sections were counterstained with DAPI (blue fluorescence). (**D**) Negative controls for each embryonic day. EM, embryo; IS, implantation sites; LE, luminal epithelia; S, stromal cells. 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 as described in Fig. 4. Each experiment was repeated three times with three different sets of animals for total of 48 mice.

# 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

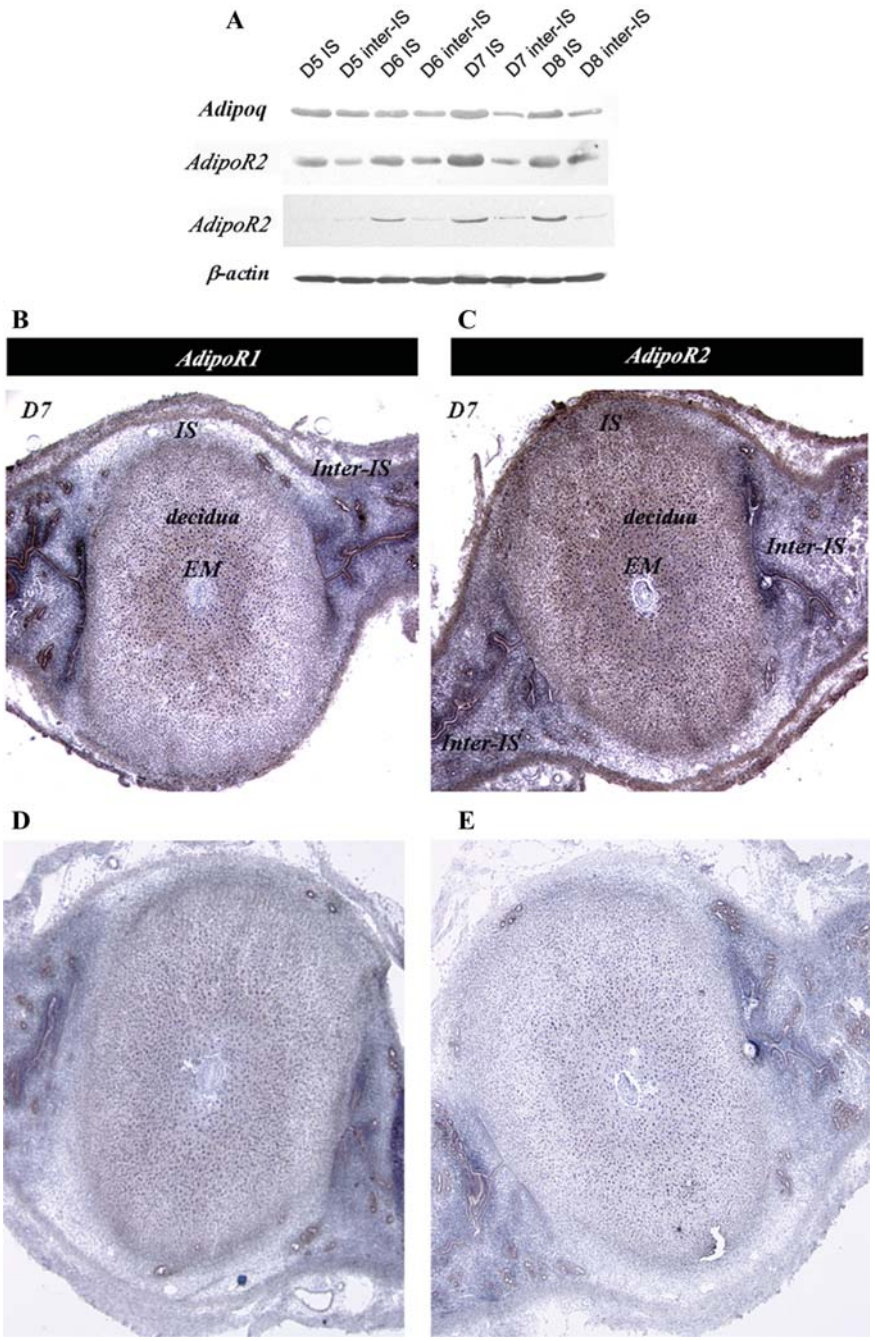


**Figure 6** Adipoq and AdipoRs protein expression in the uterus during pregnancy. (**A**) Western blot analysis of Adipoq and AdipoRs in the pregnant uterus. β-Actin was used as internal control. Note increased expression of Adipoq, AdipoR1 and AdipoR2 in the IS. See Supplementary data, Fig. S1 for quantification. Immunohistochemistry of AdipoR1 (**B**) and AdipoR2 (**C**) in the uterus at pregnant day 7. Longitudinally sectioned uterus were fixed on paraformaldehyde and incubated with primary antibodies. Slides were then incubated with biotinylated secondary antibody and with enzyme conjugate. Colouring reaction was done using DAB, and sections were counterstained with Hematoxylin. (**D**) and (**E**) are negative controls using non-immune immunoglobulin G for each experiment. EM, embryo; IS, implantation sites. 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 with three different sets of animals for a total of 48 mice.

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

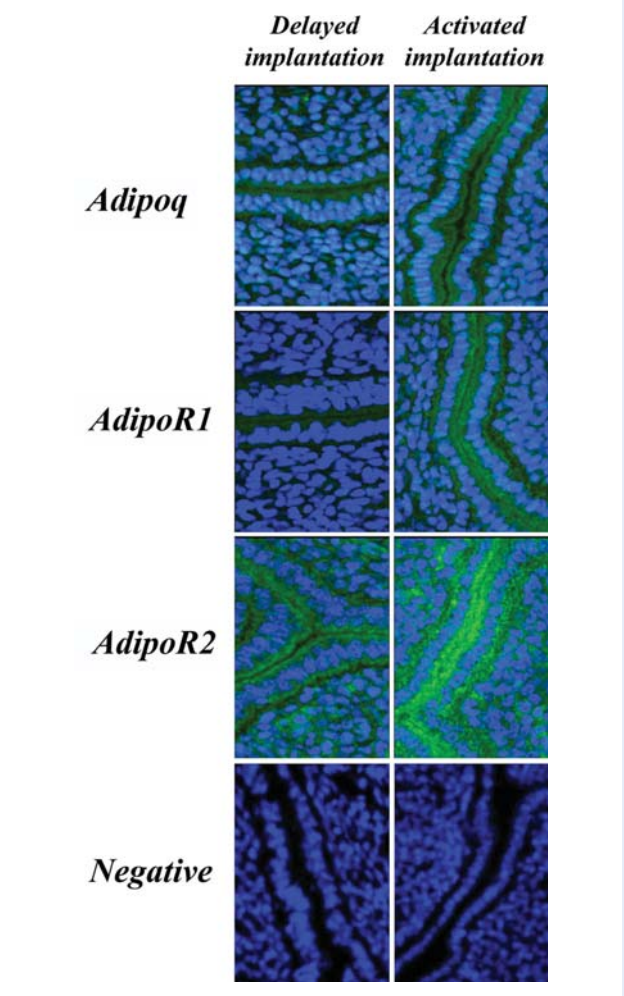


**Figure 7** Immunofluorescence of Adipoq and AdipoRs in the uterus of delayed or activated implantation model. Paraformaldehyde-fixed tissue slices were incubated with primary antibodies. Slides were then incubated with a secondary antibody, Alexa Fluor 488 goat anti-rabbit immunoglobulin G (green fluorescence). 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). 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 uteri 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

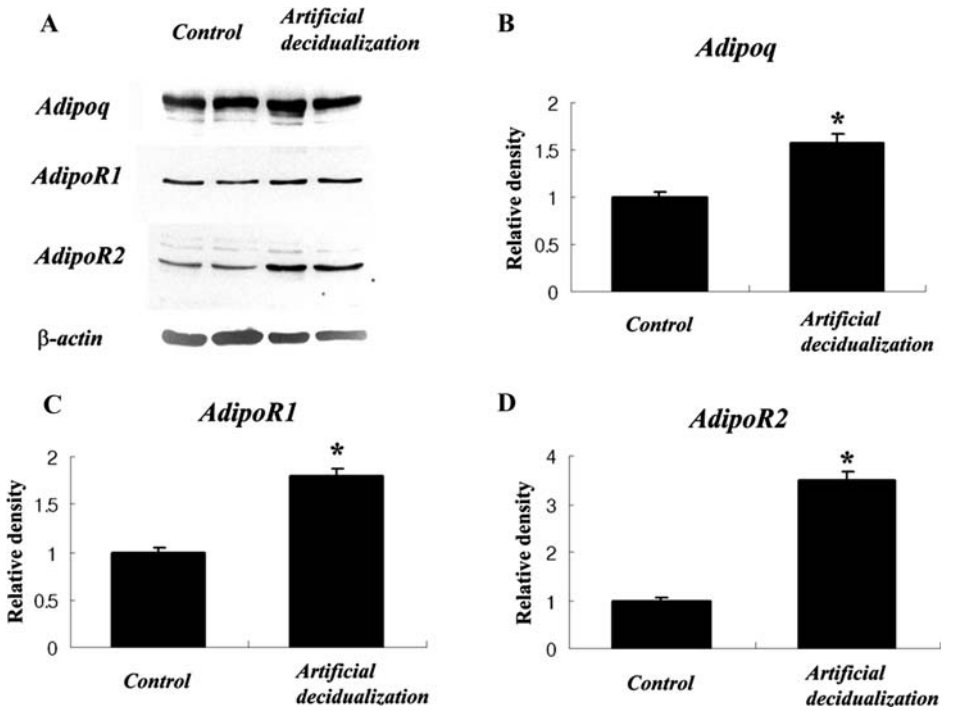


**Figure 8** Western blot analysis of Adipoq and AdipoRs in the uterus of artificial decidualization model. (**A**) Oil infused uterine horn was used as the uterus of artificial decidualization model, and the other side horn, which is no oil infused horn, was used as control. Two experiments are shown. β-Actin was used as internal control. (**B**–**D**) All represent the quantification of the immunoblots using normalized control as a relative density of 1. Uteri from three different animals per group (control versus artificial decidualization) were prepared for western immunoblot and compared after normalization by Student's *t*-test. Asterisks indicate significant differences ($P < 0.001$). All experiments were performed three times with three independent sets of animals for a total of 18 mice.

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 $E_2$ 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 $E_2$ exposure; a finding that was inhibited by adding Adipoq. In addition, Adipoq plus $E_2$ resulted in increased proliferation in $E_2$ 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 themitogen 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, Slc2A4 translocation is mediated by PRKAA1 signaling in the heart (Russell *et al.*, 1999; Goldstein and Scalia, 2004) and recent evidence suggests that Slc2A8 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 $E_2$ 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

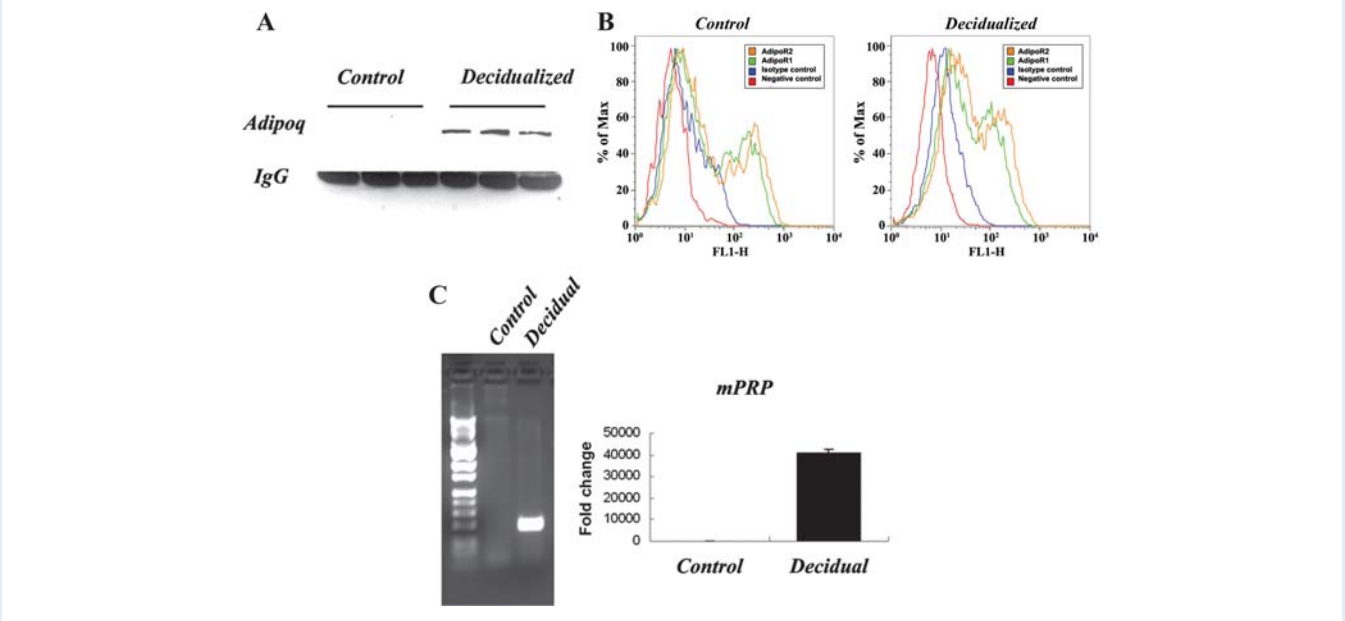


**Figure 9** Adipoq signaling expression in ESCs. ESCs isolated from the uterus at pregnant day 4 were decidualized for 3 days by stimulation with estrogen ($E_2$) and progesterone. Control ESCs were cultured without $E_2$ or progesterone treatment. Five to ten pregnant mice were used to collect stromal cells for each experiment. Experiments were repeated five times with five different sets of animals or a total of 32 mice. (**A**) Secreted Adipoq detection by western blot analysis of supernatant media. (**B**) Cell surface expression of AdipoR1 and AdipoR2 in ESCs by flow cytometry. (**C**) Decidual/trophoblast PRP mRNA expression by RT-PCR in ESCs during *in vitro* decidualization. *Gapdh* was used as internal control.

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/.

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