CpG Oligodeoxynucleotides Downregulate Placental Adiponectin and Increase Embryo Loss in Non-Obese Diabetic Mice

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Introduction
The maternal–fetal interface refers to an area of direct contact between maternal (decidua) and fetal (trophoblast) tissues, and effective maternal–fetal cross talk promotes successful fetal antigen exposure to the maternal environment, thus influencing normal fetal growth. During pregnancy, bacterial infections may damage the local molecular and cellular microenvironment, and aberrant cellular interactions at the maternal–fetal interface can lead to serious complications of pregnancy, such as implantation failure, embryo loss, spontaneous abortion, premature delivery, pre-eclampsia, and intrauterine growth restriction.

Toll-like receptor 9 (TLR9) recognizes unmethylated CpG dinucleotides, a characteristic feature of microbial DNA. CpG oligodeoxynucleotides (ODNs) are short single-stranded synthetic DNA molecules containing unmethylated CpG motifs that mimic microbial DNA and can therefore produce an immunostimulatory response. We have previously demonstrated that intraperitoneal ODN injection in non-obese diabetic (NOD) mice caused a series of immunological changes at the maternal–fetal interface. The NOD mouse strain is a model of type 1 diabetes.

Problem
CpG oligodeoxynucleotides (ODNs) can induce immunological changes in non-obese diabetic (NOD) mice and increase embryo loss, but little is known about the mechanism. This study aimed to determine the role of adiponectin in CpG ODN-induced pregnancy failure.

Method of study
Oligodeoxynucleotide 1826 was intraperitoneally injected to NOD mice, and ODN 2216, ODN 2006, and ODN 2395 were used to stimulate human trophoblast cell lines to investigate adiponectin expression patterns and its possible effects on trophoblast function.

Results
CpG ODNs downregulated adiponectin via the cJun N-terminal kinase signaling pathway and led to increased embryo loss (from 6.9 to 33.3%). ODN 2006 impaired human trophoblast cell migration, which was successfully rescued by adiponectin treatment.

Conclusion
CpG ODNs decreased placental adiponectin expression in NOD mice and impaired human trophoblast function and was associated with increased embryo loss. Adiponectin may therefore play an important protective role in the prevention of bacteria-induced pregnancy failure.
diabetes, which has natural killer (NK) cell deficits, and is prone to embryo loss. Our studies identified the ODN-induced immunological changes to be taking place via the TLR9 pathway. These changes included abnormal proliferation of uterine macrophages and neutrophils, and tumor necrosis factor-α (TNF-α) and mouse keratinocyte-derived cytokine (mKC) production by uterine CD11b+ F4/80+ cells, which led to increased fetal loss and premature delivery. Depletion of F4/80+ cells or transplantation of induced Treg (iTreg) cells rescued CpG-mediated pregnancy failure.6,7

In addition to immunological factors, metabolism at the maternal–fetal interface is emerging as an important factor to ensure normal pregnancy. As the most abundant circulating adipokine, adiponectin plays a key role in metabolic disorders. Adiponectin is a 30-kDa protein, which exists as two forms (glomerular adiponectin and full-length adiponectin), and mediates its actions mainly via two molecules; adiponectin receptor 1 and adiponectin receptor 2.8 Adiponectin is involved in various physiological activities, including energy homeostasis, insulin sensitivity, glucose and lipid metabolism, inflammation, immunity, and angiogenesis, and can act as an antidiabetic, anti-atherogenic, or anti-inflammatory adipokine.9 Furthermore, adiponectin also plays an important role in maintaining normal reproductive function, and its receptors are expressed in several reproduction-related organs, including the pituitary gland, hypothalamus, testis, ovary, oviduct, uterus, endometrium, and placenta.10–12 Circulating adiponectin levels have been widely documented as being markedly lower in patients with obesity, diabetes mellitus, and metabolic syndrome compared to healthy individuals (in both pregnant and non-pregnant women), and abnormal levels of adiponectin are associated with a series of pregnancy complications, including gestational diabetes mellitus and pre-eclampsia. For example, low circulating adiponectin levels in pregnant women have been associated with an increased risk of gestational diabetes mellitus,10,13 and in pregnant women with normal weight, circulating adiponectin has been correlated negatively with the gestational age.14 In addition, it has been suggested that pregnant women with a lower level of serum adiponectin are more likely to develop pre-eclampsia.15,16 However, some studies have reported higher serum adiponectin concentrations in pre-eclamptic patients compared to women with normal pregnancies.17,18 While metabolic dysfunctions are frequently linked to reproductive abnormalities, the relationship between the anti-inflammatory role of adiponectin and CpG ODN-induced embryo loss in NOD mice remains unclear.

In this study, we hypothesized that adiponectin may play a key role in resisting CpG-induced damage to trophoblast function. CpG ODNs were used to mimic bacterial infections during pregnancy. Placental adiponectin expression was significantly decreased after treatment with ODN, and this decreased adiponectin expression was correlated with increased embryo loss in NOD compared to wild-type (WT) mice. We also investigated adiponectin expression patterns and its potential effects on trophoblast function and pregnancy outcome using human trophoblast cell lines, to explore the relationship between CpG ODN-induced embryo loss and adiponectin function.

Materials and methods

Treatment of Pregnant Mice

Mouse treatment methods are shown in Figure S1. Healthy WT female BALB/c and female NOD mice with BALB/c background and male C57BL/6 mice aged 8–10 weeks were purchased from Beijing HFK Bioscience Co. Ltd. (Beijing, China). All mice were housed in a pathogen-free facility. All animal procedures followed national animal care guidelines and were approved by the Institutional Animal Care and Use Committee of Shanghai Jiao Tong University. The day on which the vaginal plug was detected was designated as gestational day 0.5 (E0.5). ODN 1826 is specific for mouse tissues and cells19,20 and was therefore used for all animal experiments conducted in this study. Pregnant mice were intraperitoneally injected with ODN 1826 control or ODN 1826 (InvivoGen, San Diego, CA, USA) at the dose of 25 μg/dam dissolved in 200 μL phosphate-buffered saline (PBS) (Gibco BRL Co.Ltd., Gaithersburg, MD, USA) at the dose of 25 μg/dam dissolved in 200 μL phosphate-buffered saline (PBS) (Gibco BRL Co.Ltd., Gaithersburg, MD, USA) on E6.5. Each group consisted of at least 6 mice. Pregnant mice were killed on E10.5.6,7,21 Embryos with a smaller size (20% smaller than the average size), hemorrhage (at the implantation site), and necrosis were identified as resorbed embryos.22,23 Placentas were immediately collected and frozen in liquid nitrogen.

Cell Culture

BeWo (cell model to mimic syncytialization of placent villous trophoblast in vivo) and JAR (human...
choriocarcinoma cell line used as in vitro model of cytotrophoblasts) cell lines (China Infrastructure of Cell Line Resources, Beijing, China) were routinely grown at 37°C with 5% CO₂ in phenol-red DMEM/F12 medium (Gibco) supplemented with 15% fetal bovine serum (FBS) (Gibco) and phenol-red DMEM medium (Gibco) with 10% FBS, respectively, along with streptomycin (10 μg/mL) and penicillin (100 U/mL) (Gibco). HTR-8, a human extravillous trophoblast cell line, was kindly provided by P. K. Lala (University of Western Ontario, Ontario, Canada) and cultured in phenol-red DMEM/F12 medium supplemented with 10% FBS, streptomycin (10 μg/mL), and penicillin (100 U/mL) at 37°C with 5% CO₂.

Cells were seeded onto plates and incubated overnight at 37°C with 5% CO₂. The following day, BeWo and HTR8 cells were cultured in DMEM/F12 medium, and JAR cells were cultured in DMEM medium, all supplemented with 1% FBS in the presence of various agents, such as forskolin (25 nM; Sigma Aldrich, St. Louis, MO, USA), adiponectin (400 ng/mL; Peprotech, Rocky Hill, NJ, USA), TLR9 agonists (5 μM), SP600125, PD98059 (Cell Signaling Technology, Danvers, MA, USA), or corresponding controls.

Quantitative Real-time PCR (qRT-PCR)

Total RNA was extracted using TRIzol reagent (TaKaRa Bio Inc., Tokyo, Japan), and 1 μg of total RNA was used to synthesize first-strand cDNA with the PrimeScript™ II 1st Strand cDNA Synthesis Kit (TaKaRa Bio Inc.) using random or oligo-dT primers. Thereafter, qRT-PCR was performed using a SYBR Green kit (TaKaRa Bio Inc.) according to the manufacturer’s instructions. Primer sequences for all genes are listed in Table I. All samples were amplified in triplicate, and the mean was used for analysis. The \(2^{-\Delta\Delta C_T}\) method was applied to calculate the relative expression normalized to the internal controls β-actin or GAPDH.

Western Blotting

Total protein from WT or NOD mouse placenta or BeWo cells was isolated using radioimmunoprecipitation assay buffer (Pierce, Waltham, MA, USA) and then centrifuged at 12,000 \( \times g \) for 15 min at 4°C. Equal amounts of protein samples were subjected to 10% polyacrylamide gel electrophoresis under denaturing conditions (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF; Bio-Rad Laboratories, Richmond, CA, USA) membranes. After transfer, the PVDF membranes were blocked with 5% non-fat milk in TBST for 1 hr at room temperature and then incubated overnight at 4°C with primary antibodies against adiponectin (1:1000 dilution; Abcam, Cambridge, UK) or α-Tubulin (1:500 dilution; Boster, Wuhan, China). At room temperature, the membranes were washed three times with TBST for 5 min each time and then probed with a secondary antibody for 1 hr. Signals were detected using the Pro-light HRP Chemiluminescent Kit (Tiangen Biotech, Beijing, China) according to the manufacturer’s instructions. Gel Pro software was used to obtain quantitative data from Western blots. Detection of α-Tubulin was used as a loading control.

Immunofluorescence

Bicolor immunofluorescence was used to identify and localize proteins in sections of WT and NOD mouse placenta or BeWo cells.
mouse placenta tissues. Placentas were fixed in 4% paraformaldehyde, embedded in optimal cutting temperature compound, and sectioned at 5 μm. After deparaffinization, rehydration, and unmasking, slides were blocked with 5% FBS for 1 hr at room temperature. After the blocking buffer was aspirated, sections were incubated with a primary antibody [rabbit anti-adiponectin polyclonal antibody (1:50 dilution; Abcam) or mouse anticytokeratin 7 monoclonal antibody (1:100 dilution; Abcam)] overnight at 4°C. Cytokeratin 7 was used as a trophoblast marker as previously described. After being washed three times with PBS, specimens were incubated with secondary fluorescent antibodies for 1 hr at room temperature in the dark. Sections were finally mounted with fluoroshield mounting medium with 4',6-diamidino-2-phenylindole (DAPI; Abcam) and then observed using a fluorescence microscope. For the BeWo cells, identification and localization of proteins were conducted using an immunofluorescence assay as previously reported. Rabbit anti-adiponectin polyclonal antibody (1:50 dilution; Abcam) and E-Cadherin rabbit monoclonal antibody (1:200 dilution; Cell Signaling Technology) were used as primary antibodies.

Human Sample Characteristics

Ten women aged 22–35 years (mean age, 28.2 ± 2.9) with normal pregnancies were recruited. All of these women had had at least one successful pregnancy, without chromosomal abnormality, obesity, spontaneous abortion, preterm labor, or pre-eclampsia in any pregnancy. These patients underwent artificial abortion (dilatation and curettage) to their unwanted pregnancies at 8–12 gestational weeks and samples of villus and decidual tissues were collected during pregnancy termination and stored in liquid nitrogen. The protocol of this study was approved by the Medical Ethics Committee of the International Peace Maternity & Child Health Hospital of China Welfare Institute, Shanghai. Informed written consent was obtained from all participants before enrollment.

Immunohistochemistry

Placenta tissues of WT and NOD mice, as well as human villus and decidual tissues were collected from terminations (at 8–12 gestational weeks) of normal pregnancies, and subsequently embedded, sliced, deparaffinized, rehydrated, and unmasked using standard immunohistochemical techniques, using the BB-SA-1021 detection kit (Boster) according to the manufacturer’s instructions, with mouse anti-adiponectin as the primary antibody (1:500 dilution; Abcam).

Transfection

Small interfering RNA (siAdipoq; GenePharma, Shanghai, China) was transfected into cells at a final concentration of 100 nmol/L using Oligofectamine reagent (Invitrogen, Carlsbad, CA, USA). To generate the adiponectin overexpression construct, the coding region sequence of human adiponectin was cloned into pEX-2 vector (GenePharma) and transfected into the cells using Lipofectamine 3000 (Invitrogen).

Migration Assay

Migration of JAR or HTR-8 cells was measured using a Transwell assay. JAR or HTR-8 cells (1 × 10⁵ cells) in DMEM/F12 (200 μL) with ODN 2006 control (5 μM), ODN 2006 (5 μM) or ODN 2006 (5 μM) plus adiponectin (400 ng/mL) were placed into the upper chambers of a 24-well cell culture chamber (0.8 μm pore size; Corning, New York, NY, USA). The lower chambers were filled with 800 μL DMEM/F12 containing 15% FBS. Following incubation at 37°C for 24 hr, the inserts were removed, washed in ice-cold PBS, fixed in 4% paraformaldehyde, stained with crystal violet, wiped with a cotton bud, and observed using an inverted phase-contrast microscope.

Gelatin Zymography

Matrix metalloproteinase-2 (MMP-2) enzyme activity in JAR cell culture medium was tested by standard gelatin zymography techniques as previously reported. Briefly, cells were grown to approximately 80% confluency in complete growth media and then washed twice with sterile PBS to remove the serum completely. The cells were then incubated in serum-free Opti-MEM medium with ODN 2006 control, ODN 2006, or ODN 2006 plus adiponectin. After 16 hr, cell culture media were standardized according to standard procedures and subjected to 10% SDS-PAGE with 0.1% w/v gelatin. After electrophoresis, the gel was incubated at room temperature for 30 min in 100 ml diluted renaturing solution and then rinsed at least once with 300 mL.
dH₂O to remove the SDS. The gel was incubated with gentle agitation at room temperature for 30 min in 100 mL developing buffer, which was exchanged with 100 mL fresh developing buffer for further incubation at 37°C for approximately 16 hr. After the incubation, the gel was immersed in staining solution for 1 hr, followed by destaining solution. Results were observed under a UV transilluminator.

Statistical Analyses

Experiments were performed in technical duplicates of at least three biological replicates. Data are represented as the mean ± standard error of the mean (S.E.M.). Two-way ANOVA followed by post hoc Bonferroni test was used to compare drug treatments with corresponding vehicle controls in animal experiments (Figs 1a,b,d and S2). One-way ANOVA followed by post hoc Tukey’s test was used to compare treatment groups with corresponding control groups in cell experiments when more than two groups were compared (Figs 2c,d,f, 3b, 4a–c,e,h, and 5b,c), and student’s t-test was used when two groups were compared (Figs 3d–f, and 4g). Differences were considered to be statistically significant when P-values were <0.05, and sufficient statistically significant when P-values were <0.01.

Results

ODN 1826 Increases Embryo Loss in NOD Mice

To investigate whether adiponectin could rescue the CpG ODN-induced damage during pregnancy, NOD mice were chosen as mouse models. qRT-PCR analysis of mouse placentas showed that placental Tlr9 (encoding the ligand of CpG ODN) expression levels were significantly increased in WT mice that were injected with ODN 1826 compared with control mouse groups (Fig. 1a). When only the ODN 1826 control was intraperitoneally injected, the embryo loss rate was higher in NOD mice than in WT mice, but the difference was not statistically significant [6.9% (7/102) versus 3.8% (4/106)]. ODN 1826 caused a further increase in the rate of fetal resorption in NOD mice [33.3% (33/99) versus 6.9% (7/102); P < 0.05], but did not impair pregnancy in WT mice [5.3% (5/99) versus 3.8% (4/106); Figure S2]. ODN 1826 thus increased the rate of embryo loss in NOD but not WT mice (Figure S2).

ODN 1826 Decreases Adiponectin Expression in NOD Mouse Placenta

To investigate the changes of adiponectin expression in ODN 1826-injected NOD mice, qRT-PCR and
Western blot analyses of mouse placentas were performed. qRT-PCR results demonstrated that adiponectin expression was downregulated in ODN 1826-injected mice but did not reach statistical significance (Fig. 1b). Western blot and immunohistochemical analyses showed that adiponectin was significantly downregulated in NOD mice but not in WT mice after injection with ODN 1826, while in control groups, placental adiponectin expression was significantly higher in the NOD compared to WT mice (Fig. 1c–e).

Adiponectin Is Expressed by Syncytiotrophoblasts

Immunohistochemical analyses of mouse placental tissue, and human first-trimester villus and decidual tissues, and bicolor immunofluorescence analysis of mouse placental tissue were used to determine the adiponectin expression patterns in both mouse and human placentas. Mouse immunohistochemistry results showed that adiponectin was expressed in the decidua (DE), trophoblast giant cells (TG), and labyrinth (LA; Fig. 1e). Cytokeratin 7 was used as a trophoblast marker, and bicolor immunofluorescence assay also showed that adiponectin was expressed in the mouse trophoblast cells (Fig. 2a). Immunohistochemical analysis of decidual and villus tissues collected from terminated pregnancies demonstrated that adiponectin expression was localized mainly in syncytiotrophoblast and decidual glands (Fig. 2b). BeWo cells can be fused by forskolin and were therefore chosen as a model of syncytiotrophoblast formation to study adiponectin expression and function. qRT-PCR (Fig. 2c,d) and Western blot (Fig. 2e,f) analyses showed that increased β-HCG secretion (a marker of syncytialization) and adiponectin expression was consistent with increased forskolin concentration, demonstrating that adiponectin expression rises with increasing degree of syncytialization.

ODN 2006 Reduces Expression of Adiponectin in Fused BeWo Cells via the JNK Signaling Pathway

CpG ODNs (also known as human TLR9 agonists) were used to investigate adiponectin expression.
There are three classes of stimulatory CpG ODNs: class A (ODN 2216), class B (ODN 2006), and class C (ODN 2395), each with their own control. ODN 2216 (induce high IFN-α production), 2006 (strongly active B cells), and 2395 (combine features of both ODN 2216 and ODN 2006) are specific for human tissues. Following stimulation with 25 nM forskolin for 24 hr, the medium (25 nM forskolin, 1% FBS) was refreshed, and each of the three classes of CpG ODNs and their respective controls were added to at a concentration of 5 μM according to the manufacturer’s instructions for another 48 hr of culture. Western blotting analysis showed that ODN 2216 and ODN 2395 did not affect adiponectin expression (Fig. 3a,c,d,f), while ODN 2006 significantly downregulated adiponectin expression in fused BeWo cells (Fig. 3b,e). To investigate the mechanism involved in the ODN 2006-mediated decrease of adiponectin expression, the inhibitors SP600125 and PD98059 were used to block the c-Jun N-terminal protein kinase (JNK) and mitogen-activated protein kinase (MEK) signaling pathways, respectively. Western blotting (Fig. 3g–h) and immunofluorescence analyses showed SP600125 partially rescued the ODN 2006-induced decreased adiponectin expression. **P < 0.01. adipokin, adiponectin; 2216, ODN 2216; 2216 ctrl, ODN 2216 control; 2006, ODN 2006; 2006 ctrl, ODN 2006 control; 2395, ODN 2395; 2395 ctrl, ODN 2395 control; SP, SP600125; PD, PD98059; DAPI, 4′,6-diamidino-2-phenylindole. ODN, oligodeoxynucleotides.

ODN 2006 Disrupts Syncytialization of BeWo Cells

To investigate the influence of ODN 2006 on the syncytialization of syncytiotrophoblasts and the effect of adiponectin on this influence, the BeWo cell line was used as a human syncytiotrophoblast cell model, and leptin, syncytin-2, and β-HCG were used as indicators of syncytialization. qRT-PCR analysis showed that ODN 2006 decreased the leptin, syncytin-2, and β-HCG mRNA expression (Fig. 4a–c). However, knockdown (Fig. 4d,e) or overexpression (Fig. 4f,g)
of adiponectin did not alter ODN 2006-induced syncytiotrophoblast endocrine dysfunction, and β-HCG expression was not rescued (Fig. 4h). Similar results were observed by immunofluorescence analysis. Following incubation of BeWo cells with forskolin and ODN 2006 control, E-cadherin distribution indicated the syncytial formation of BeWo cells, while incubation with forskolin and ODN 2006, resulted in the E-cadherin being localized on the BeWo cell borders indicating that they were not fused. Following knockdown or overexpression of adiponectin, the localization of E-cadherin was not significantly different from that in cells only incubated with forskolin and ODN 2006 (Fig. 4i).

Adiponectin Rescues ODN 2006-Induced Impairment of Trophoblast Cell Migration

Transwell assays were used to investigate the migration of JAR and HTR-8 cells, as migration is a key component of trophoblast invasion. ODN 2006 disrupted HTR-8 and JAR cell migration, and adiponectin rescued this impairment (Fig. 5a). The numbers of migrating JAR (Fig. 5b) and HTR-8 (Fig. 5c) cells...
were significantly decreased when incubated with ODN 2006 compared to ODN 2006 control or with ODN 2006 plus adiponectin. ODN 2006 reduced latent MMP-2 and active MMP-2 enzyme activity, but this could be rescued by addition of adiponectin (Fig. 5d), indicating that CpG ODN-induced impairment of MMP-2-mediated cytotrophoblast migration can be neutralized by adiponectin.

Discussion

Adiponectin is an important modulator of insulin action and glucose metabolism, influences gonadotropin release and fetal growth, and maintains normal pregnancy. However, its anti-inflammatory role in bacterial infections during pregnancy is not well understood. In this study, we demonstrated, for the first time, that CpG downregulate adiponectin expression in mouse placenta and increase embryo loss in NOD mice.

NOD mice are known to be prone to embryo loss.27 The embryo was implanted on E4.5 or E5.5 and is finished on E6.5. On E10.5, the number of immune cells reached to the highest level. So we injected the pregnancy mice with ODN 1826 on E6.5 and killed the mice on E10.5 to investigate the influence of ODN 1826 on embryo loss, referenced to previous experimental methods.21 In control groups, the absolute number of lost embryos in NOD mice was only slightly but not significantly higher than that in WT mice. By contrast, when injected with ODN 1826, the fetal resorption rate in NOD mice was statistically higher than that in WT mice with the same treatment, and Tlr9 was upregulated in both WT and NOD mice. The NOD mouse is an immunodeficiency model mouse characterized by a functional deficit in NK cells but not in T and B lymphocytes and other immune cells.28,29 The results further confirmed that ODN 1826 may activate the immune system to disrupt the pregnancy outcome. Thus, we selected ODN 1826 to challenge NOD mice as a mouse model of the infection-induced increase of embryo loss rate to investigate the anti-inflammatory role of adiponectin during pregnancy.

In women, metabolic disorders increase the risk of menstrual cycle abnormalities, ovulatory dysfunction, and decreased fecundity, which can cause a number of pregnancy complications, including hypertension, pre-eclampsia, gestational diabetes, and fetal distress.30,31 Adiponectin is the most abundant adipokine expressed in reproductive organs. It can suppress macrophage production of pro-inflammatory cytokines, inhibit the phagocytic activity of macrophages, inhibit the activation of NF-κB, and resist TNF-α-

Fig. 5 Counteractive effects of adiponectin on ODN 2006-mediated impairment of migration ability of JAR and HTR-8 cells. (a) Migration abilities of JAR and HTR-8 cells were impaired by ODN 2006, but could be rescued by adiponectin. Numbers of migrating (b) JAR and (c) HTR-8 cells were both significantly decreased by ODN 2006. (d) ODN 2006 impaired both latent MMP-2 and active MMP-2 enzyme activity in JAR cells, but this could be rescued by adiponectin. **P < 0.01. adipq, adiponectin; 2006, ODN 2006; 2006 ctrl, ODN 2006 control. ODN, oligodeoxynucleotides.
Adiponectin is thought to function at the intricate interface between metabolism and inflammation in pregnant women, being broadly associated with various obesity-related diseases including infertility. Adiponectin in the NOD mouse was significantly higher than that in the WT mouse placenta in our control groups. As adiponectin promotes insulin sensitivity, a compensatory increase of expression of adiponectin may occur in the early stage of low insulin sensitivity of NOD mice. With development of autoimmune sialadenitis, the serum adiponectin level has been shown to decrease in NOD mice. Our results showed that, in response to ODN 1826 stimulation, placental adiponectin expression was downregulated in the NOD mouse but not in the WT mouse placenta. The reasons for the stable adiponectin expression in the placenta of WT mice remain to be explored.

While the presence of syncytiotrophoblast adiponectin expression has been previously debated, the present study demonstrated adiponectin expression in both syncytiotrophoblasts and decidual glands. In the in vitro model cell experiments, we also found that adiponectin was expressed in syncytial BeWo cells. Moreover, TLR9 agonists (ODN 2216, ODN 2006, and ODN 2395) and their controls were given to fused BeWo cells to investigate whether CpG ODN could influence adiponectin expression in human syncytiotrophoblasts. The expression of adiponectin decreased only after ODN 2006 stimulation for 48 hr. Then, we used ODN 2006 to stimulate cells in the following experiments. Furthermore, JNK and MEK signaling pathways have been previously reported as two signaling pathways that could reduce adiponectin expression during inflammation. In our current study, only specific inhibitors of the JNK signaling pathway could rescue the ODN 2006-mediated decrease of adiponectin expression, indicating JNK as the signaling pathway regulating adiponectin expression in syncytiotrophoblasts in response to ODN 2006 (or bacterial) stimulation.

In the current study, pregnant women underwent artificial abortion (dilatation and curettage) to their unwanted pregnancies at gestational week 8–12 were defined as normal pregnancies, and samples of villus and decidual tissues were collected during pregnancy termination. All of these women had had at least one successful pregnancy, without chromosomal abnormality, obesity, spontaneous abortion, preterm labor, or pre-eclampsia in any pregnancy. However, there are some potential limitations of using tissue from these subjects. Potential limitation may include the following: (i) As uterus ultrasonic examination is not performed, we have no idea whether the fetus is alive or dead. Thus, it is possible that the fetus is dead, but we may still consider it a normal pregnancy by mistake. (ii) Fetal chromosome examination is not performed at the present gestation. Thus, it is possible that we may define some patients with abnormal fetal chromosome as normal pregnancies by mistake. (iii) Other limitation: Taken together, the effect of adiponectin in protecting pregnancy may be slightly overestimated when these samples are used. In future researches, it is better to perform more detections including but not limited to uterus ultrasonic examination before pregnancy termination and fetal chromosome examination right at the end of pregnancy termination.

Trophoblast proliferation, migration, invasion, and endocrine secretion play important roles in a successful pregnancy, and it has been reported that adiponectin promotes the syncytialization of BeWo cells and primary trophoblast cells. However, no reports have been made about the effect of adiponectin on trophoblast syncytialization under infectious conditions. In the current study, ODN 2006 impaired the syncytialization of BeWo cells, but neither knockdown nor overexpression of adiponectin could neutralize this effect, indicating that while it can promote trophoblast syncytialization, adiponectin cannot rescue impairment of syncytialization caused by CpG ODNs or bacterial infection. Furthermore, in addition to supporting syncytialization, adiponectin can also promote the human trophoblast migration. However, little is known about the effect of adiponectin on trophoblast migration under infectious conditions. Our results showed that ODN 2006 impaired the migration of both JAR and HTR-8 cells, and adiponectin could mitigate this effect by regulating MMP-2 activity.

In conclusion, our findings provide evidence to demonstrate the anti-inflammatory function of adiponectin in countering the effect of CpG ODN (and bacterial infections) during pregnancy. CpG ODNs downregulated syncytiotrophoblast adiponectin...
expression via the JNK signaling pathway and induced embryo loss in NOD mice. CpG ODNs also impaired trophoblast syncytialization of syncytiotrophoblasts and cytotrophoblast migration. Although adiponectin could not prevent the CpG ODN-induced syncytiotrophoblast dysfunction, it was able to rescue the CpG ODN-induced impairment of cytotrophoblast migration. Our study provides further understanding of the anti-inflammatory role of adiponectin in response to specific unmethylated CpG dinucleotides (infectious bacterial unmethylated dinucleotides) during placental development. These results indicate that adiponectin may be a beneficial factor in providing protection in bacteria-induced pregnancy failure and providing a new direction for the clinical treatment of pregnancy failure caused by bacterial infections.

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Conflict of interest

All authors declared that they have no conflict of interests.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. Animal treatment.
Figure S2. Increase of embryo loss in NOD mice by ODN 1826.