Title

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Journal

Diabetologia, 59(11)

ISSN

0012-186X

Authors

Qiao, Liping
Wattez, Jean-Sebastien
Lee, Samuel
et al.

Publication Date

2016-11-01

DOI

10.1007/s00125-016-4061-x

Peer reviewed

HHS Public Access

Author manuscript

Diabetologia. Author manuscript; available in PMC 2017 November 01.

Published in final edited form as:

Diabetologia. 2016 November ; 59(11): 2417–2425. doi:10.1007/s00125-016-4061-x.

Knockout maternal adiponectin increases fetal growth in mice: potential role for trophoblast IGFBP-1

Liping Qiao[1], **Jean-Sebastien Wattez**[1], **Samuel Lee**[1], **Zhuyu Guo**[1], **Jerome Schaack**[2], **William W. Hay Jr**[3], **Matteo Moretto Zita**[4], **Mana Parast**[4], and **Jianhua Shao**[1]

[1]Department of Pediatrics, University of California San Diego, 9500 Gilman Drive, MC 0983, La Jolla, CA 92093, USA

[2]Department of Microbiology, University of Colorado at Denver and Anschutz Medical Center, Aurora, CO 80045, USA

[3]Department of Pediatrics, University of Colorado School of Medicine, Aurora, CO 80045, USA

[4]Department of Pathology, University of California San Diego, La Jolla, CA 92093, USA

Abstract

Aims/hypothesis—The main objective of this study was to investigate whether maternal adiponectin regulates fetal growth through the endocrine system in the fetal compartment.

Methods—Adiponectin knockout (*Adipoq*$^{-/-}$) mice and in vivo adenovirus-mediated reconstitution were used to study the regulatory effect of maternal adiponectin on fetal growth. Primary human trophoblast cells were treated with adiponectin and a specific peroxisome proliferator-activated receptor α (PPARα) agonist or antagonist to study the underlying mechanism through which adiponectin regulates fetal growth.

Results—The body weight of fetuses from *Adipoq*$^{-/-}$ dams was significantly greater than that of wild-type dams at both embryonic day (E)14.5 and E18.5. Adenoviral vector-mediated maternal adiponectin reconstitution attenuated the increased fetal body weight induced by maternal adiponectin deficiency. Significantly increased blood glucose, triacylglycerol and NEFA levels were observed in *Adipoq*$^{-/-}$ dams, suggesting that nutrient supply contributes to maternal adiponectin-regulated fetal growth. Although fetal blood IGF-1 concentrations were comparable in fetuses from *Adipoq*$^{-/-}$ and wild-type dams, remarkably low levels of IGF-binding protein 1 (IGFBP-1) were observed in the serum of fetuses from *Adipoq*$^{-/-}$ dams. IGFBP-1 was identified in the trophoblast cells of human and mouse placentas. Maternal fasting robustly increased IGFBP-1 levels in mouse placentas, while reducing fetal weight. Significantly low IGFBP-1 levels

Corresponding author: J. Shao, jishao@ucsd.edu, Department of Pediatrics, University of California San Diego, 9500 Gilman Drive, MC 0983, La Jolla, CA 92093, USA.

Duality of interest statement

The authors declare that there is no duality of interest associated with this manuscript.

Contribution statement

JSh designed the study, and WWH and MP contributed to the study design and discussion; JSc designed and created viral vectors for in vivo transduction, LQ, J-SW, SL, ZG and MMZ contributed to data acquisition and analysis and drafting the article; JSh interpreted the data; and JSh wrote the manuscript, with a significant contribution from JSc. All authors approve the final version of this paper. JSh is the guarantor of this work and, as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

were found in placentas of *Adipoq*$^{-/-}$ dams. Adiponectin treatment increased IGFBP-1 levels in primary cultured human trophoblast cells, while the PPARα antagonist, MK886, abolished this stimulatory effect.

Conclusions/interpretation—These results indicate that, in addition to nutrient supply, maternal adiponectin inhibits fetal growth by increasing IGFBP-1 expression in trophoblast cells.

Keywords

Adiponectin; Fetus; Growth; IGF-binding protein; Placenta

Introduction

Obesity is an important risk factor of type 2 diabetes and cardiovascular diseases. Despite efforts to fight obesity in the last decade, the prevalence of adult obesity in the USA is still increasing [1]. Results from recent epidemiological studies have indicated that the origins of obesity can be traced to intrauterine fetal development and growth [2]. A new paradigm for the gestational origins of obesity is based on the strong association of a high prevalence of obesity and metabolic defects in later life with high or low birthweight [3–5]. Birthweight is a key marker of fetal growth and is routinely measured. Therefore, elucidating how fetal growth is controlled by the intrauterine metabolic environment will provide mechanistic insight into the gestational origins of obesity.

Nutrient supply is essential for fetal growth. However, the importance of the hormonal system in regulating fetal growth is also well documented [2, 4, 6]. Studies have demonstrated that the fetal IGF system plays a central role in regulating fetal growth [7, 8]. Severe intrauterine and postnatal growth retardation were observed in individuals with an *IGF1* mutation and in *Igf1* or *Igf2* knockout mice [8–12]. Unlike in adults, IGF expression in the fetal compartment is not controlled by growth hormone [7]. IGF-1 is expressed in several fetal tissues, where it stimulates cell growth through endocrine and paracrine effects. IGF-2 is mainly expressed in trophoblast cells, where it enhances fetal growth by promoting placental growth and nutrient transport via autocrine and/or paracrine pathways [8]. Therefore, IGF-1 is the main circulating hormone affecting fetal growth. A family of six IGF-binding proteins (IGFBPs) serves as carriers of IGF-1 in the circulation. Due to their high affinity for IGF, IGFBPs also control the activity of IGFs by modulating their bioavailability. During pregnancy, IGFBP-1 is the predominant binding protein for IGFs in the fetal circulation [13–15]. Fetal blood IGFBP-1 levels inversely correlate with birthweight [14, 16]. Maternal overexpression of *Igfbp1* inhibits mouse and rat fetal growth [13, 17–19]. Interestingly, although IGFs play an important role in intrauterine growth restriction and development of obesity in later life induced by maternal nutrient deficiency [7, 20], it is unknown whether the IGF system is involved in altered fetal growth resulting from maternal obesity.

Adiponectin is an adipocyte-secreted hormone that plays an important role in glucose and lipid metabolism [21]. Some human studies have found that maternal blood adiponectin levels are inversely correlated with offspring birthweight [22, 23]. In line with these correlative studies, the Jansson and Powell group showed that infusing full-length

recombinant adiponectin to increase maternal adiponectin inhibits fetal growth in mice [24].However, endogenous adiponectin forms multimers and maternal adiponectin is significantly reduced during late normal gestation [23, 25]. Furthermore, the correlation between maternal adiponectin level and birthweight was not identified in studies of pregnant women with and without gestational diabetes [26, 27]. Therefore, it is necessary to use other experimental systems to verify the regulatory effect of maternal adiponectin on fetal growth. In addition, it is not clear whether maternal adiponectin regulates fetal growth through the endocrine system in the fetal compartment.

Methods

Materials

Glucose oxidase/Peroxidase (PGO), WY14643, DNase I, collagenase, Percoll and Fao hepatoma cells were purchased from Sigma (St. Louis, MO, USA). Fao cells were derived from a rat hepatoma and no contamination was present. MK886 was obtained from Cayman Chemical (Ann Arbor, MI, USA). Rabbit anti-p-IGF-1Rβ and rabbit anti-IGF-1Rβ antibodies were obtained from Cell Signaling (Danvers, MA, USA). Rabbit anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and HRP-linked secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Goat anti-IGFBP-1 and the mouse anti-IGF-1 (total) Luminex kit were purchased from R&D Systems (Minneapolis, MN, USA). The mouse diabetes multiplex assay kit was obtained from Bio-Rad (Hercules, CA, USA). NEFA and triacylglycerol (TG) assay kits ware purchased from Wako Diagnostics (Richmond, VA, USA). FBS, NuPAGE gels, SuperScript III reverse transcriptase, Trizol, trypsin and the oligo(dT)$_{12–18}$ primer were obtained from Invitrogen (Carlsbad, CA, USA). Rabbit serum and diaminobenzidine were obtained from Vector Laboratories (Burlingame, CA, USA). All antibodies were validated by their manufacturers.

Experimental animals

Adiponectin knockout (*Adipoq*$^{-/-}$) mice were from a C57BL/6 background [28]. C57BL/6 mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Ten- to 12 week-old nulliparous female mice were randomly assigned for mating. *Adipoq*$^{-/-}$ and wild-type (WT) mice were cross mated (Fig. 1a); the point at which pregnancy was indicated by the presence of a vaginal plug was assigned embryonic day (E)0.5. To reconstitute adiponectin, 1×10^9 plaque-forming units of purified adenoviral vectors encoding adiponectin or green fluorescent protein (GFP) (named Ad-*Adipoq* and Ad-*gfp*, respectively) were injected into *Adipoq*$^{-/-}$ dams through the tail vein at E15.5 [29]. Placentas and fetuses were collected via Caesarean section at E14.5 or E18.5. Pregnant C57BL/6 mice were fasted overnight (~14 h) and fetal tissue samples were collected at E18.5. All mouse experiments were carried out under Association for Assessment and Accreditation of Laboratory Animal Care guidelines with approval from the University of California San Diego Animal Care and Use Committee. Blood glucose levels were determined using the glucose oxidase/peroxidase method with a standard curve, following manufacturer's instruction. Blood TG and NEFA concentrations were measured using kits from Wako, following manufacturer's instructions. Blood IGF-1 and insulin levels were determined using Luminex assay kits, as per

manufacturers' instruction. Mouse body composition was measured by Echo-MRI (Houston, TX, USA). Fetal blood insulin was measured using a mouse diabetes multiplex assay kit.

Human primary trophoblast cell culture

Healthy term human placentas were obtained from the University of California San Diego (UCSD) Perinatal Biobank for use in a UCSD Institutional Review Board approved protocol. All patients gave informed consent for the collection and use of placental tissue. Villous tissue was used for isolating trophoblast cells using a three-enzyme digestion and a Percoll gradient approach, as previously described [30]. Briefly, chorionic villi were minced, washed in PBS, and subjected to three sequential digestions: Digestion I: 300 U/ml DNase I, 150 U/ml collagenase and 50 U/ml hyaluronidase (Stemcell Technologies, Vancouver, BC, Canada); Digestions II and III: 0.25% trypsin (wt/vol.) and 300 U/ml DNase I. The pelleted cells from the second and third digests were pooled and resuspended in HBSS, and separated on a Percoll gradient. Purified primary trophoblast cells were seeded on 6-well culture plates overnight in Iscove's modified Dulbecco's medium containing 10% (vol./vol.) FBS. After culture for 24 h, cells were treated with the peroxisome proliferator-activated receptor α (PPARα) agonist WY14643 or the PPARα antagonist MK886, for 12 h. For adiponectin treatment, a Fao hepatoma cell and primary trophoblast co-culture system was used [31]. Fao cells were transduced with adenoviral vectors in insert wells for 12 h to induce *Adipoq* expression and adiponectin secretion. After co-culture, protein and mRNA were extracted from the primary trophoblast cells.

Plasmid constructs and generation of adenovirus vectors

Adenoviruses encoding mouse adiponectin or GFP were constructed using the pAd/CMV/V5-Dest vector (Invitrogen). Construction and purification of the viral vectors were carried out as previously described [32].

Immunohistochemistry

Human and mouse placenta biopsy samples were fixed in 10% (vol./vol.) neutral-buffered formalin, processed and paraffin embedded. After washing, samples were heated in 0.1 mol, pH 6.0 citrate buffer for 15 min at 95°C to induce antigen retrieval. They were further blocked with 2% (vol./vol.) normal rabbit serum with 1% (wt/vol.) BSA in PBS for 2 h in a humid chamber at room temperature (RT). Sections were incubated with 10 μg/ml anti-IGFBP-1 primary antibody or rabbit serum (negative control) overnight at 4°C. After washing, samples were overlaid with 1 μg/ml biotinylated rabbit anti-goat antibody (KPL, Gaithersburg, MD, USA) for 2 h at RT and then treated with an immunoperoxidase system for 1 h at RT. Sections were visualised by incubation with 3,3′-diaminobenzidine at RT for 1.5 min and counterstained with haematoxylin (Richard-Allan Scientific, Kalamazoo, MI, USA).

Western blotting and quantitative real-time PCR

Protein samples were extracted from placentas, livers or cultured cells and were separated by SDS-PAGE (using NuPAGE gels). After blotting, proteins were detected with anti-IGFBP1,

anti-IGF-1Rβ, anti-p-IGF-1Rβ and anti-GAPDH antibodies (most at 1:1000 dilution; see figure legends). Protein bands were quantified using Quantity One software (Bio-Rad).

Total RNA was prepared from tissues or cells using Trizol, and cDNA was synthesised using SuperScript III reverse transcriptase and oligo$(dT)_{12–18}$ primer. Quantitative real-time PCR (qPCR) was performed using an Mx3000p Real-Time PCR system (Stratagene, San Diego, CA, USA) with specific primers for *Igf-2Igf-2R and Igfbp1* (see electronic supplementary material [ESM] Table 1). Gene expression was normalised to 18S rRNA levels.

Statistical analysis

Data are expressed as means ± SEM. Statistical analyses were performed using Student's *t* tests or ANOVA, followed by Bonferroni post hoc testing using GraphPad Prism 6 (La Jolla, CA, USA). Statistical significance was set at $p<0.05$.

Results

Maternal adiponectin inhibits fetal growth

Adiponectin cannot pass through the placental barrier [33]. We mated WT dams with *Adipoq*$^{-/-}$ sires or *Adipoq*$^{-/-}$ dams with WT sires (Fig. 1a). All fetuses were therefore *Adipoq*$^{-/+}$. Pregravid body weights were similar in *Adipoq*$^{-/-}$ and WT dams (Fig. 1b). During pregnancy, all dams gained a similar amount of weight (Fig. 1c) and had comparable body fat at E18.5 (Fig. 1d). Litter sizes were similar in *Adipoq*$^{-/-}$ and WT dams (Fig. 1e). Body weight was significantly higher in fetuses from *Adipoq*$^{-/-}$ dams (Fakd) than in fetuses from WT dams (Fwtd) at both E14.5 (data not shown) and E18.5 (Fig. 1f). We used adenoviral vector-mediated in vivo gene transduction to reconstitute maternal adiponectin in *Adipoq*$^{-/-}$ dams [34]. Similar to in non-pregnant mice [34], adiponectin protein levels at 3 days after Ad-*Adipoq* viral vector injection were significantly increased in dam's blood (data not shown). Our results showed that the body weight was significantly lower in fetuses from adiponectin-reconstituted dams compared with the fetuses from Ad-*gfp*-treated *Adipoq*$^{-/-}$ dams (Fig. 1g). Together, these data demonstrate that maternal adiponectin inhibits fetal growth.

Nutrient supply is essential for fetal growth

Interestingly, blood glucose, NEFA and TG concentrations in *Adipoq*$^{-/-}$ dams were significantly higher than in WT dams (Fig. 1h–j). Our results also showed that fetal blood glucose (Fig. 1k), but not TG and NEFA (data not shown), was significantly increased in fetuses of *Adipoq*$^{-/-}$ dams. In contrast, adiponectin reconstitution robustly decreased maternal blood glucose, TG and NEFA concentrations and fetal blood glucose levels (ESM Fig. 1). These data indicate that adiponectin plays an important role in maintaining maternal energy homeostasis. Since maternal metabolism directly affects fetal nutrient supply, these results also lead us to postulate that reducing the fetal nutrient supply might be one of the underlying mechanisms through which maternal adiponectin inhibits fetal growth. In line with this hypothesis, the fetal/placental weight ratio, which represents placental nutrient transport efficiency, was significantly increased in Fakd (Fig. 1l). A separate project is

underway to investigate how adiponectin regulates maternal metabolism and the role of placental nutrient supply in fetal growth regulation by maternal adiponectin.

Adiponectin increases fetal blood IGFBP-1 protein levels

The fetal IGF system plays a central role in fetal growth [7, 8]. We measured blood total IGF-1 protein concentrations in both fetuses and dams, and levels of IGF-2 and its receptor in placentas from the cross-breeding studies described above. There was no significant difference in the total IGF-1 concentration of either fetal or maternal blood (Fig. 2a,b). *Igf-2* and *Igf-2* receptor (*Igf-2R*) mRNA levels in placentas were similar in Fakd and Fwtd (Fig. 2c). These results suggest that maternal adiponectin deficiency has no significant effect on fetal blood IGF-1 concentration or placental IGF-2 expression. Insulin is another hormone that regulates fetal growth [35]. However, despite increases in fetal blood glucose (Fig. 1k), no significant alteration in the fetal blood insulin concentration was detected in Fakd (Fig. 2d), which may be due to the lack of response by fetal beta cells to glucose [35].

IGF-1 bioactivity is also tightly controlled by IGFBP-1 in fetuses [13–15]. A remarkable decrease in IGFBP-1 protein levels was found in the serum of Fakd (Fig. 2e). Maternal adiponectin reconstitution significantly increased fetal blood IGFBP-1 protein levels (Fig. 2f). Since fetal blood IGFBP-1 levels inversely correlate with birthweight [14, 16] and transgenic overexpression of IGFBP-1 inhibits mouse and rat fetal growth [13, 17–19], we propose that IGFBP-1 mediates adiponectin-induced inhibition of fetal growth. Consistent with this notion, we observed a significant increase in IGF-1 receptor β phosphorylation in fetal livers from *Adipoq*$^{-/-}$ dams (Fig. 2g), suggesting increased IGF-1 bioavailability and activity in fetuses of *Adipoq*$^{-/-}$ dams.

Adiponectin increases IGFBP-1 expression in mouse placentas

The liver is reported to be the major fetal mouse tissue that secretes IGFBP-1 into the circulation; however, the study did not include placentas [36]. Our results showed that maternal adiponectin gene knockout had no significant effect on fetal liver IGFBP-1 protein (Fig.3a) and mRNA levels (ESM Fig. 2a). These results indicate that the decrease in blood IGFBP-1 levels in *Adipoq*$^{-/-}$, dams is likely caused by a mechanism independent of liver IGFBP-1 expression.

IGFBP-1 is also expressed in human decidual cells and the mouse endoderm yolk sac [37]. In the present study, immunohistochemical analysis detected IGFBP-1-positive syncytiotrophoblast cells in both mouse labyrinthine and human chorionic villi (Fig.3b–e). Furthermore, overnight maternal fasting robustly increased fetal blood IGFBP-1 protein levels and placental IGFBP-1 expression (Fig. 3f,g). Surprisingly, fetal liver IGFBP-1 protein (Fig. 3h) and mRNA levels (ESM Fig. 2b) were significantly reduced after maternal fasting. Most importantly, in contrast to the increased placental and blood IGFBP-1 protein levels, fetal body weight was remarkably reduced after maternal overnight fasting (Fig. 3i). Together, these results suggest that the placenta is an important source of fetal blood IGFBP-1. As shown in Fig. 3j,k, IGFBP-1 protein and mRNA levels were significantly decreased in placentas from *Adipoq*$^{-/-}$ dams. In contrast, maternal adiponectin

reconstitution significantly increased IGFBP-1 protein levels in placentas (Fig. 3l). These results indicate that adiponectin increases IGFBP-1 gene expression in mouse placentas.

Adiponectin increases IGFBP-1 gene expression through PPARα

PPARα is a member of the PPAR transcription factor family, and is expressed in both human and rodent placentas [38, 39]. PPARα binds to the promoter region and upregulates *IGFBP1* transcription in a variety of cell types [40, 41]. Adiponectin increases PPARα expression and transcriptional activity in both hepatocytes and trophoblasts [42, 43]. We therefore treated primary human trophoblast cells with the PPARα agonist WY14643 and antagonist MK886. The levels of IGFBP-1 protein were significantly increased in WY14643-treated cells and robustly decreased in MK886-treated trophoblast cells (Fig. 4a). These results indicate that PPARα upregulates IGFBP-1 gene expression in trophoblast cells.

Using the co-culture system and primary human trophoblast cells, we demonstrated that IGFBP-1 protein levels were robustly increased in adiponectin-treated cells (Fig. 4b), which supports our *in vivo* findings that maternal adiponectin increased IGFBP-1 expression in the placenta. Most importantly, this stimulatory effect was abolished by MK886 treatment (Fig. 4b). Therefore, these studies demonstrate that adiponectin increases IGFBP-1 gene expression in trophoblast cells via PPARα.

Discussion

Adiponectin is an adipocyte-derived hormone that has been suggested to mediate maternal obesity-altered fetal growth [23, 44]. Recent studies, including our work, demonstrated that adiponectin is not expressed in the placenta and cannot pass through the placental barrier [23, 33]. Furthermore, our animal study also revealed that adiponectin is not present within the fetal compartment in early life [33]. Although it is still unclear when and where adiponectin expression is initiated in the human fetus, a significant increase in fetal blood adiponectin levels occurs in the last trimester [27, 45]. In contrast, maternal adiponectin expression is significantly decreased in late pregnancy [25]. Therefore, at delivery, the neonatal blood adiponectin level is four- to sevenfold higher than the level in the mothers' blood [45]. The opposing changes in adiponectin expression in maternal and fetal tissues during late pregnancy may explain why birthweights are inversely correlated with maternal blood adiponectin concentrations but positively associated with cord blood adiponectin concentrations [22, 23, 27, 45]. These correlations also suggest that maternal and fetal adiponectin may regulate fetal growth via different mechanisms. Therefore, we designed the present study to address whether and, if so, how maternal regulation of fetal growth occurs. By crossing $Adipoq^{-/-}$ and WT mice, we created adiponectin-deficient and WT dams in which all fetuses were genetically identical. This system allowed us to exclude the possible effects of fetal adiponectin. We found that body weight was significantly higher in Fakd than in Fwtd at both E14.5 and E18.5. Furthermore, maternal adiponectin reconstitution reversed the change in fetal weight to within the normal range. Therefore, these results demonstrate that maternal adiponectin suppresses fetal growth, consistent with the results of maternal adiponectin infusion [24]. The effects of fetal adiponectin on fetal growth are beyond the scope of the current study. Although our previous study revealed that fetal adiponectin

increases hepatic *de novo* lipogenesis [33], which might provide substrates for the rapid expansion of fetal tissue at late gestation, studies designed to specifically verify the regulatory effects of fetal adiponectin on fetal growth are required.

Nutrient supply is essential for fetal growth. Previous studies showed that maternal adiponectin inhibits the expression and activity of several key amino acid transporters in trophoblast cells by suppressing insulin–mTOR signalling [24, 43]. The present study does not provide evidence that adiponectin affects placental amino acid transport. However, our results showed that adiponectin deficiency robustly increased maternal and fetal blood glucose concentrations and the fetal/placental weight ratio, supporting the idea that inhibiting placental nutrient transport might be one of the mechanisms through which maternal adiponectin suppresses fetal growth. The finding that maternal blood glucose, TG and NEFA levels in $Adipoq^{-/-}$ dams is significantly altered also indicates that maternal adiponectin plays a very important role in maintaining maternal metabolism homeostasis. We propose that maternal adiponectin limits fetal nutrient supply by reducing placental nutrient transport and nutrient availability at the placenta. This hypothesis is being tested in a separate study.

Fetal growth is rigorously regulated by fetal hormones, in addition to nutrient supply [7, 35]. The IGF system plays a central role in regulating fetal growth [9–12]. In addition to regulation via changes in protein levels, IGF activity is controlled by bioavailability via the high affinity binding of IGFBPs [7]. Although IGFBP-3 is the most abundant binding protein in human blood, the regulatory effects of IGFBP-3 on fetal growth are still uncertain [46–48]. In contrast to IGFBP-1, human studies have reported that cord blood IGFBP-3 concentrations do not inversely correlate, and may even positively correlate, with birthweight [46–49]. IGFBP-1 is the dominant IGF-binding protein in rodent fetal blood [13–15]. IGFBP-1 inhibits fetal growth by reducing IGF-1 bioavailability and activity [9, 12]. The results of our study indicate that maternal adiponectin increases fetal blood IGFBP-1 levels. Interestingly, although Fakd exhibited a higher growth rate, the blood total IGF-1 concentrations were similar in both Fakd and Fwtd (Fig. 2a). These results suggest that maternal adiponectin reduces IGF-1 bioavailability in the fetus. Increased IGF-1 receptor phosphorylation in Fakd livers supports this notion. Therefore, we propose that maternal adiponectin suppresses fetal growth by increasing IGFBP-1, thereby reducing IGF-1 bioavailability in fetal blood.

Another important finding of this study is the regulatory effect of maternal adiponectin on IGFBP-1 levels in trophoblast cells. To identify which fetal tissue mediates adiponectin-increased blood IGFBP-1 protein, we compared levels of IGFBP-1 in the livers of Fakd and Fwtd, because the liver is considered the main source of fetal blood IGFBP-1 [36]. However, no notable alteration of IGFBP-1 protein and mRNA levels was detected in fetal livers from an $Adipoq^{-/-}$ dam (Fig. 3a and ESM Fig. 2a) or after maternal adiponectin reconstitution (data not shown). These results indicate that the fetal liver is not responsible for the adiponectin-dependent increase in IGFBP-1 levels in fetal blood. These data also indicate that one or more other tissues secrete IGFBP-1 into fetal blood. We found that IGFBP-1 is expressed in trophoblast cells in both human and mouse placentas. Overnight fasting dramatically increased IGFBP-1 expression in placentas, accompanied by a fourfold

increase in fetal blood IGFBP-1 protein levels and a significant reduction in fetal body weight, indicating that the placenta is another source of fetal blood IGFBP-1. We also found a significant difference in IGFBP-1 expression in placentas from *Adipoq*$^{-/-}$ and WT dams. Elevated IGFBP-1 protein was detected in placentas of maternal adiponectin-reconstituted dams and adiponectin-treated primary human trophoblast cells. In addition, a PPARα-specific antagonist abolished adiponectin-induced IGFBP-1 expression in primary trophoblast cells. Adiponectin is known to increase PPARα-dependent transcriptional activity in various cells, including trophoblasts [42]. Together, our results demonstrate that adiponectin enhances IGFBP-1 expression in trophoblast cells via PPARα. Since trophoblast cells are the main component of the interface between the maternal and fetal compartments, our finding provides a new mechanism for fetal growth regulation by maternal hormones. The placenta is an endocrine organ that secretes several hormones into the maternal blood and plays a key role in maintaining pregnancy. The discovery of IGFBP-1 in trophoblast cells extends the endocrine function of the placenta into the fetal system. However, the mechanisms by which trophoblast IGFBP-1 affects fetal growth remain to be elucidated. We also appreciate that there are significant differences in placental tissue structure between mice and humans. Therefore, further studies are required to verify the physiological function of this new component of placental endocrine system in fetal growth and its origins. The questions of how much trophoblast cell-expressed IGFBP-1 can be secreted into fetal compartments, especially in humans, whether IGFBP-1 interacts with IGF-2 locally and how this interaction regulates placenta development and nutrient transport still need to be addressed.

In conclusion, our results provide further experimental evidence that maternal adiponectin suppresses fetal growth. Our study also reveals that maternal adiponectin increases IGFBP-1 protein levels in fetal blood by increasing IGFBP-1 expression in trophoblast cells. We propose that, in addition to modulating the fetal nutrient supply, maternal adiponectin inhibits fetal growth by reducing IGF-1 bioavailability in the fetal compartment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding

This work was supported by the National Institute of Health grants (HD069634 DK095132) and an American Diabetes Association grant (no. 1–16-IBS-272), which were awarded to JSh.

Abbreviations

Ad-*gfp*	Adenoviral vector encoding green fluorescent protein
Ad-*Adipoq*	Adenoviral vector encoding adiponectin
E	Embryonic day
Fakd	Fetuses from *Adipoq*$^{-/-}$ dams

Fwtd	Fetuses from wild-type dams
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
IGFBP-1	IGF-binding protein 1
PPARα	Peroxisome proliferator-activated receptor α
RT	Room temperature
TG	Triacylglycerol
WT	Wild-type

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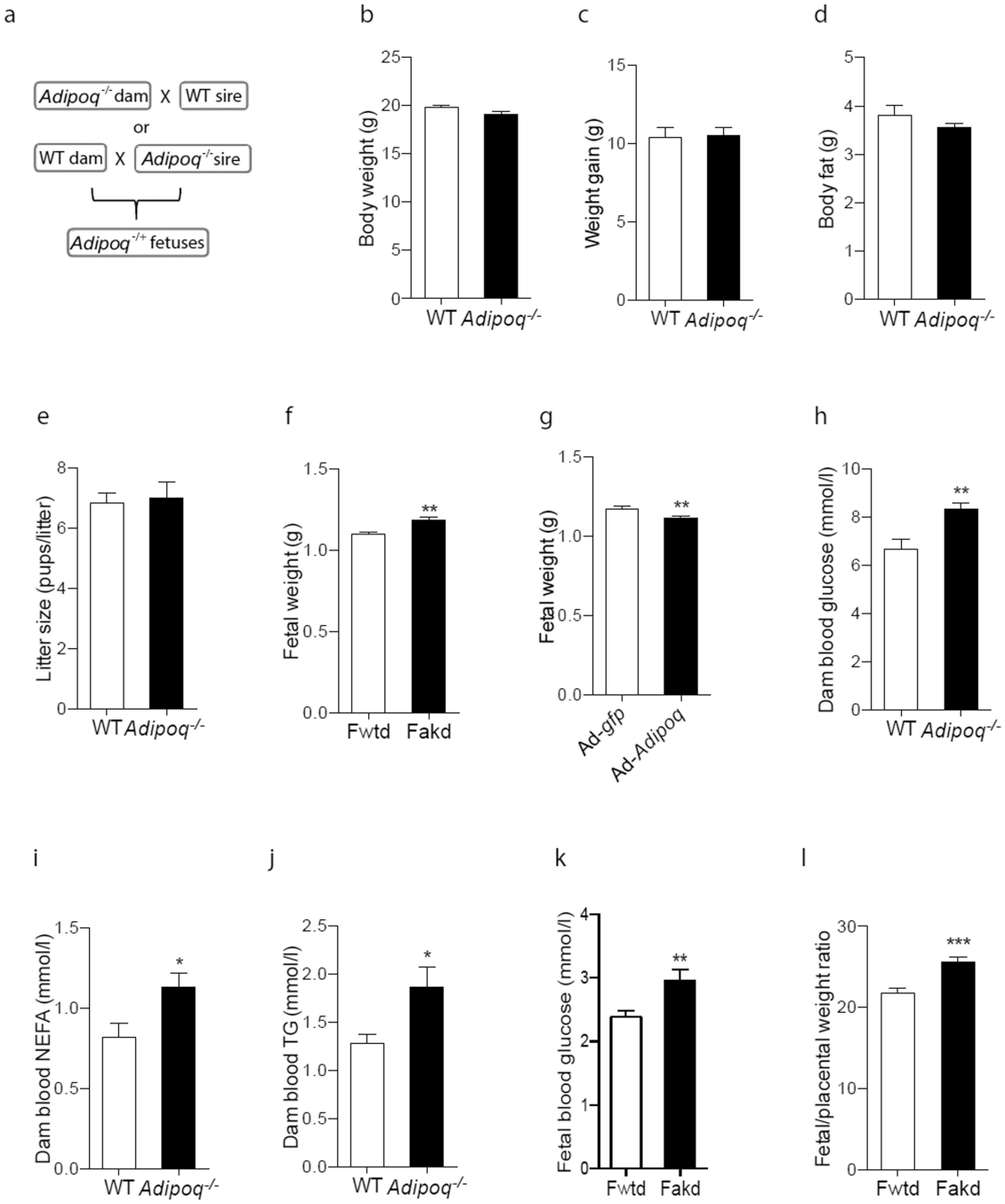

Fig. 1.

Effects of maternal adiponectin on fetal body weight. (**a**) Schematic diagram showing the production of *Adipoq*$^{-/+}$ fetuses. For this, 10–12 week-old nulliparous *Adipoq*$^{-/-}$ and WT female mice were mated with WT or *Adipoq*$^{-/-}$ male mice. (**b**) Maternal body weight. (**c**) Gestational weight gain was calculated by the difference in body weight between E0.5 and E18.5 (with pups). (**d**) Maternal body fat, (**e**) litter size and (**f**) fetal body weight were determined after removal of fetuses through Caesarean section at E18.5. (**g**) At E15, *Adipoq*$^{-/-}$ dams (n=6) were transduced with purified Ad-*gfp* or Ad-*Adipoq* through tail vein

injection. Fetal samples were collected and weighed at E18.5. (**h**,**k**) Blood glucose, (**i**) NEFA and (**j**) TG levels were measured using glucose oxidase or assay kits. (**l**) Fetal:placental weight ratios were calculated using samples at E18.5. (**b, d**) Maternal body composition was determined by EchoMRI. (**b**–**e, h**–**j**) n=8–10; (**f, g, l**) n=45–72; (**k**) n=12. *p<0.05, **p<0.01, ***p<0.001

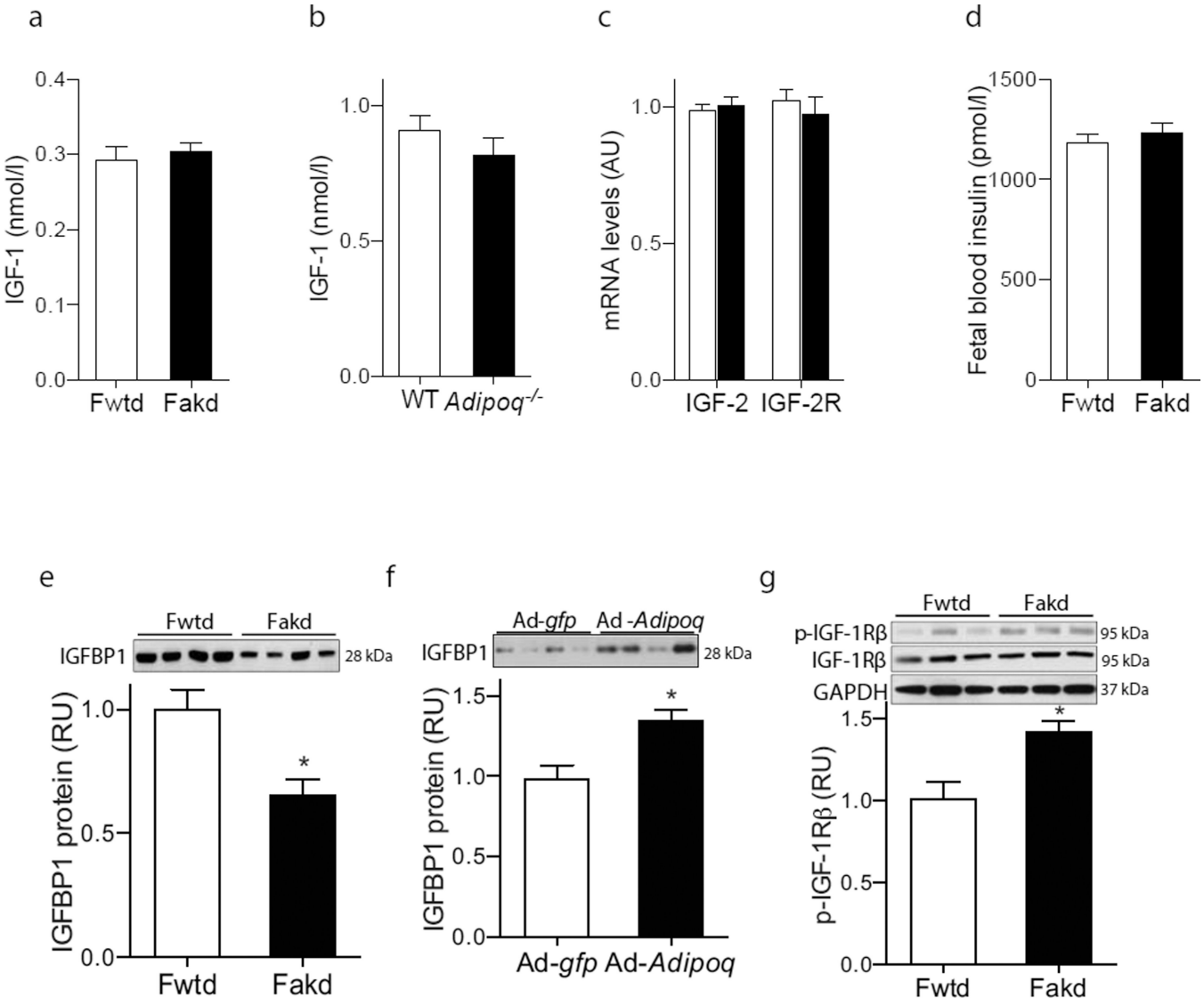

Fig. 2.

Maternal adiponectin has no effect on blood IGF-1 concentration but increases IGFBP-1 protein levels in fetal blood. (**a, b**) Blood total IGF-1 concentrations were analysed in (a) fetuses (n=12) and (**b**) dams (n=8). (**c**) mRNA levels of *Igf2* and *Igf2r* were determined using qPCR with a relative quantification assay (white bar, Fwtd; filled bar, Fakd). (**d**) Fetal blood insulin was measured using a mouse diabetes multiplex assay kit (n=12). (**e, f**) IGFBP-1 protein levels in fetal serum samples from $Adipoq^{-/-}$ or adiponectin-reconstituted and control dams (n=12) were measured by western blotting with specific antibodies. (**g**) Levels of phosphorylated or total IGF-1Rβ protein were determined in fetal livers at E18.5 (n=6) by western blotting. RU, relative units. *p<0.05

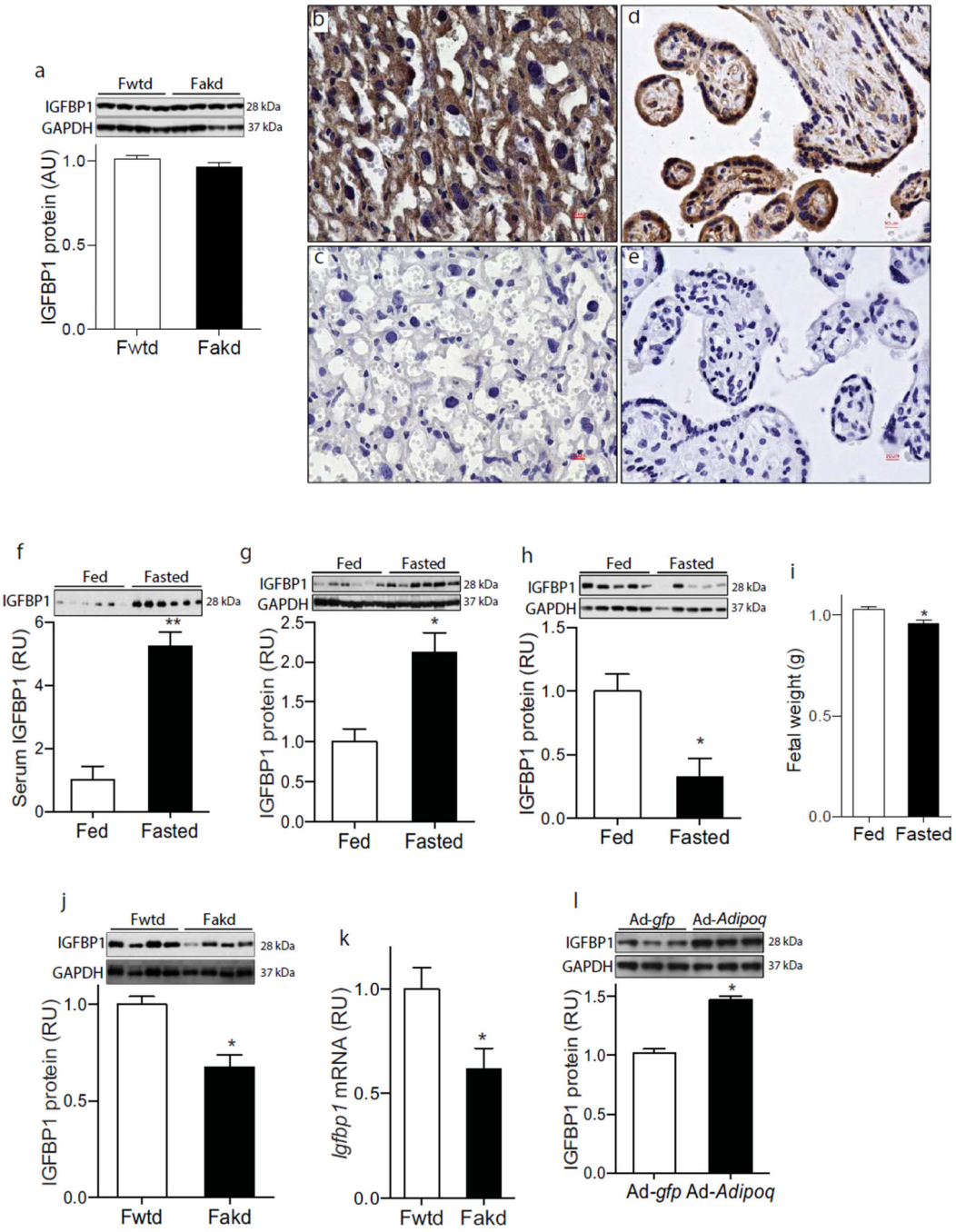

Fig. 3.

IGFBP-1 levels in trophoblast cells and the effect of maternal adiponectin on placental IGFBP-1 content. (**a**) IGFBP-1 protein levels were determined by western blotting of fetal liver samples (E18.5) from WT or *Adipoq*$^{-/-}$ dams obtained under normal feeding conditions (n=12). (**b, c**) The placental labyrinth of C57BL/6 mice at E18.5 and (**d, e**) the villous fraction of term human placentas were probed with (**b, d**; brown colour) an anti-IGFBP-1 antibody or (**c, e**) normal rabbit serum. Images were captured with magnification ×40. (**f**–**j**) Pregnant C57BL/6 mice were fasted overnight or fed before tissue collection at

E18.5 (n=8). IGFBP-1 levels were determined by western blotting in (**f**) fetal blood, (**g**) placental or (**h**) fetal liver samples. (**i**) Overnight fasting reduced fetal weight (n=54–68). (**j**) IGFBP-1 levels were measured by western blotting in placental tissue samples from WT or *Adipoq*$^{-/-}$ dams (n=8). (**k**) *Igfbp1* mRNA levels were measured by qPCR in placenta samples from WT or *Adipoq*$^{-/-}$ dams (n=8). (**l**) IGFBP-1 levels were measured by western blotting in placental tissue samples from Ad-*gfp* or Ad-*Adipoq*-transduced *Adipoq*$^{-/-}$ dams (n=8). RU, relative units. *p<0.05, **p<0.01

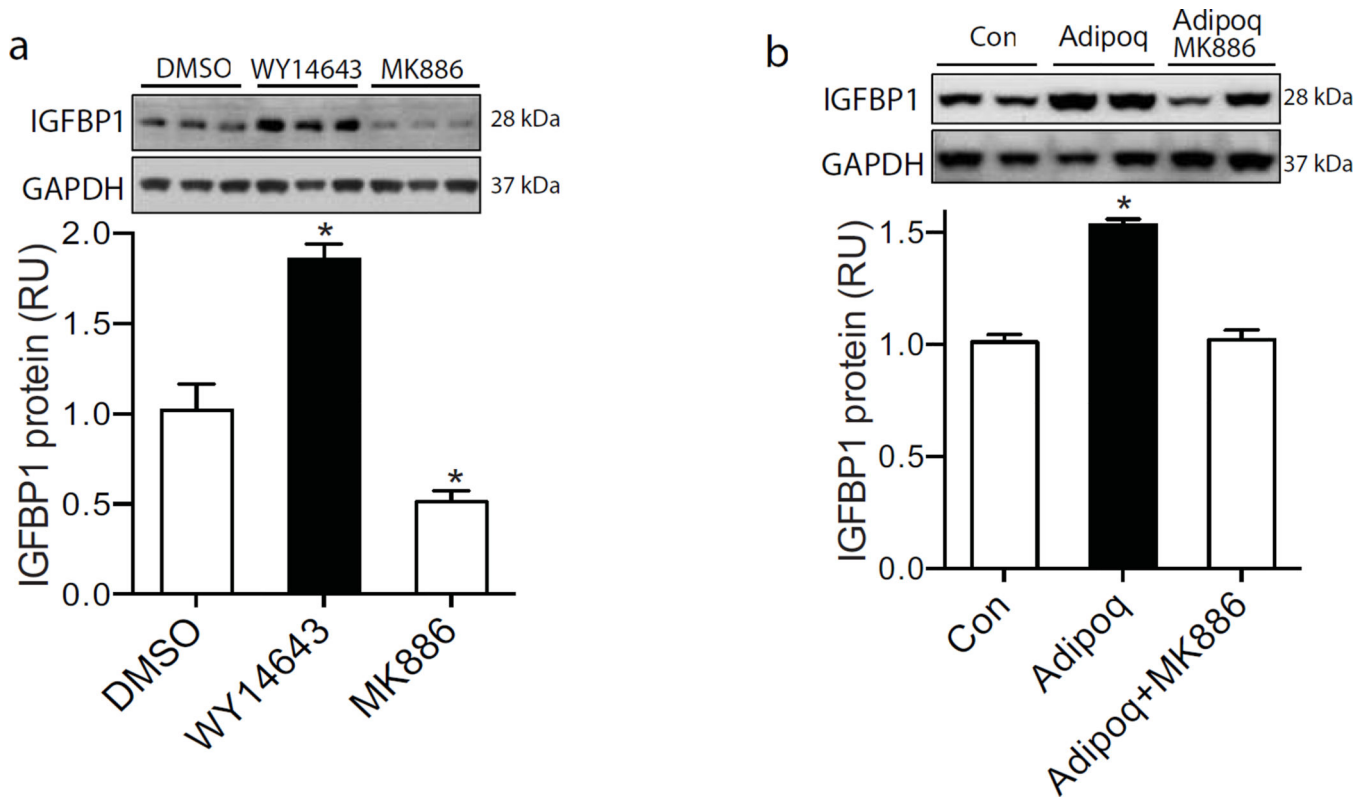

Fig. 4.

Adiponectin enhanced the IGFBP-1 expression in trophoblast cells via PPARα. (**a**) IGFBP-1 levels in primary trophoblast cells treated overnight with a PPARα agonist WY14643 (5 μmol/l) or antagonist MK886 (10 μmol/l) were analysed by western blotting. (**b**) Adiponectin was ectopically expressed in Ad-*Adipoq*-transduced Fao cells. Insert wells containing transduced Fao cells were co-cultured overnight with trophoblast cells. MK886 was then added to one group of adiponectin-treated cells (Adipoq). IGFBP-1 protein levels in trophoblast cells were analysed by western blotting (n=6). RU, relative units. *p<0.05 vs control cells (Con)