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Adiponectin is expressed in the brown adipose tissue and surrounding immature tissues in mouse embryos

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Abstract

Adiponectin is one of the adipocytokines, which are adipose-specific secretory factors. We examined its expression during embryogenesis. Transcripts of adiponectin were detected at a late stage of embryogenesis on embryonic (E) day E16.5. In situ hybridization showed that adiponectin transcripts were localized in brown adipose tissues (BATs) and surrounding immature tissues in mouse embryos. Immunohistochemistry using a specific anti-adiponectin antibody showed that the distribution of adiponectin closely parallels that of its mRNA. Adiponectin was also detected in serum at day E16.5, and its concentration peaked at birth. By contrast, transcripts of both the adiponectin receptors 1 and 2 were already expressed by day E12.5 in many tissues. Thus, their expression profile differed from that of adiponectin itself. Furthermore, experiments using primary cultures of brown adipocytes showed that adiponectin is regulated in brown adipocytes by various modulators, similar to its regulation in white adipose tissues (WATs). These data indicate that adiponectin has important roles in glucose and lipid metabolism during the perinatal period.

Keywords: Adiponectin; Brown adipose tissue; White adipose tissue; Gene expression; Embryogenesis; Adiponectin receptor

1. Introduction

Adipose tissue was originally considered to be a storage site of excess energy in the form of triglycerides. However, adipose tissue has recently been implicated as a possible endocrine mediator, linking obesity and diabetes. It induces the secretion of several hormones called adipocytokines, which are adipocyte-specific secretory factors. They include leptin, adiponectin, and tumor necrosis factor (TNF)-α [1].

Adiponectin, also called Acrp30, adipoQ, and GBP28, was originally identified independently by different groups [2–5]. It has been shown to influence glucose and lipid homeostasis and insulin sensitivity. Several groups have demonstrated that the administration of adiponectin increases fatty acid oxidation in muscle and decreases hepatic glucose production, resulting in an amelioration of insulin resistance and an improvement in glucose metabolism in diabetic mice [6–9]. Furthermore, activation of adenosine monophosphate kinase followed by inhibition of acetyl coenzyme A carboxylase, and stimulation of the peroxisome proliferator-activated receptor α, possibly constitute the mechanisms underlying the insulin-sensitizing effects of this adipocytokine [10,11].

Leptin, which is one of the well-known adipocytokines [12], was originally thought to be secreted only by adipocytes. However, leptin production has recently been demonstrated in a variety of tissues [13–17]. Leptin is also involved in the development of the metabolic complications observed in obesity, diabetes, and insulin resistance. Leptin and adiponectin are inversely correlated in these pathological conditions [18,19]. Leptin levels in the blood rise, whereas adiponectin level fall in obesity. Leptin is well characterized, but data on adiponectin are limited.

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In this study, as a first step in determining the function of adiponectin in the fetus, we examined the expression of the adiponectin gene and its protein product during embryogenesis using in situ hybridization and immunohistochemistry. The data show that adiponectin is expressed in BAT and surrounding immature tissues. The adiponectin is secreted into the blood, and its concentration peaks at birth. On the other hand, the expression patterns of adiponectin receptors 1 and 2, genes of which have now been cloned [20], were spatially and temporally different, appearing at least 4 days earlier. Experiments using primary cultures of brown adipocytes showed that adiponectin expression is regulated by various modulators, similar to its regulation in WATs.

2. Materials and methods

2.1. Animals

ICR mice and rabbits were purchased from Seac Yoshitomi (Fukuoka, Japan) and Kyudo (Saga, Japan), respectively. The mice were housed under a 12 h light/dark cycle with free access to standard chow. The animals used were treated in accordance with the Oita University Guidelines for the Care and Use of Laboratory Animals based on the National Institute of Health Guide for the Care and Use of Laboratory Animals.

2.2. Reagents

Insulin, dexamethazone, triiodothyronine, and isoproterenol were purchased from Sigma-Aldrich (St. Louis, MO). Sodium ascorbate bisphosphate purchased from Wako (Osaka, Japan), and troglitazone was purchased from Sankyo Junyaku (Tokyo, Japan). Anti-albumin antibody was purchased from Inter-Cell Technology (Jupiter, FL).

2.3. Northern blot analysis

Total RNA was extracted from mouse embryos at E12.5, E14.5, E16.5, and E18.5, and from adult mouse tissues and differentiated brown adipocytes using Isogen reagent (Nippon Gene, Tokyo, Japan). RNA was quantified by optical density at 260 nm.

Probes were synthesized by RT-PCR. The amplified fragments were subcloned into the pGEM-T Easy vector (Promega, Madison, WI) and the fragments digested with *Eco*RI were used as probes.

Total RNA (10–20 μg) was denatured in formamide and formaldehyde at 65 °C for 5 min and separated in formaldehyde-containing 1% agarose gels. RNA was blotted onto Hybond-N nylon membranes and hybridized at 42 °C for 16 h with an [α-^{32}P]dCTP-labeled cDNA probe prepared using the Megaprime DNA Labeling Kit (Amersham, Piscataway, NJ). The membrane was subsequently washed in 2× saline sodium citrate (SSC)/0.1% sodium dodecyl sulfate (SDS) and 0.1× SSC/0.1% SDS at 55 °C. Relative levels of adiponectin and β-actin mRNAs were quantified using a BAS2000 Phosphor Imaging System (Fuji Film, Tokyo, Japan). Adiponectin expression after treatment with various reagents was expressed as a ratio relative to the level in untreated control cells. Results are given as means ± S.D. of three to five independent experiments.

2.4. RT-PCR analysis

Total RNA (2 μg) from mouse embryos was reverse-transcribed in 20 μl of reaction mixture containing 5′ reverse transcription buffer, 10 mM dithiothreitol, 0.5 mM of each dNTP, 50 ng of random hexamers, 100 units of RNase inhibitor, and 200 units of MMLV reverse transcriptase (Invitrogen, Carlsbad, CA) at 37 °C for 1 h. The reaction was then heated at 70 °C for 10 min. After reverse transcription, PCR was performed in a 25-μl mixture containing 1 μl of reverse transcription reaction product, 10× PCR buffer, with 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 μM of each primer, and 1.0 unit of *Taq* DNA polymerase (Promega). Twenty-eight cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min were performed for adiponectin and β-actin, and 30 cycles for leptin and UCP1. PCR products (12 μl) were resolved electrophoretically on 1.5% agarose gel, and the gel was stained with ethidium bromide and photographed under UV light. The primer sets used in the experiments are shown in Table 1.

2.5. In situ hybridization

UCP1, UCP2, adipoR1, and adipoR2 cDNAs were generated by RT-PCR using the primers shown in Table 1. The amplified fragments were subcloned into the pGEM-T Easy vector (Promega). Mouse adiponectin cDNA inserted into the pBluescript vector was used for the experiment. All constructs were sequenced on an ABI310 sequencer (Applied Biosystems, Foster, CA). After linearization at the appropriate restriction sites, sense and antisense probes were generated by in vitro transcription with T3, T7, or SP6 polymerases in the presence of [^{35}S]-dUTP. In situ hybridizations were performed on 6 μm sections, as previously described [21,22]. Briefly, the sections were deparaffinized, treated with 20 μg/ml proteinase K, incubated with [^{35}S]-labeled antisense or sense cRNA riboprobe at 52 °C for 16 h, and washed several times with increasing stringency. Finally, slides were dipped in Kodak NBT-2, dried for 1 h, and exposed to film for 7 days at 4 °C. Sections were counterstained with hematoxylin. Photographs were taken using an Olympus BX-50 or Keyence VB-6000/6010 microscope.

2.6. Production of GST- or S-His-tagged adiponectin fusion proteins

cDNAs encoding a portion of mouse adiponectin (Fig. 4A) was generated by RT-PCR. The amplified fragment

Table 1
Primers used for cloning and RT-PCR

Gene	GeneBank accession no.	Sequence of primer	Size of product (bp)
Adiponectin	U37222	F: 5′-gaagatgacgttactacaac-3′ R: 5′-ggtagttgcagtcagttggt-3′	704
UCP1	MMU6341	F: 5′-tacacggggacctacaatgct-3′ R: 5′-tcgcacagcttggtacgctt-3′	307
UCP2	MMU69135	F: 5′-cctacagatgtggtaaaggtccgcttcc-3′ R: 5′-gagtcatcagtacagaggcacagggagg-3′	668
leptin	MMU18812	F: 5′-aatgtgctggagaccctgt-3′ R: 5′-tcagcattcagggctaacat-3′	505
β-actin	MM007393	F: 5′-aagagaggtatcctgaccct-3′ R: 5′-tacatggctggggtgttgaa-3′	218
Adipo R1	XM129394	F: 5′-aggatccagccagatgtctttccc-3′ R: 5′-ctctgtgtggatgcggaagat-3′	408
Adipo R2	MX132831	F: 5′-tcccaggaagatgaagggttttat-3′ R: 5′-tccctggttcctggagagta-3′	978

was subcloned into the pGEM-T Easy vector (Promega). Following digestion at the appropriate restriction sites, the fragment was subcloned into pGEX-4T (Amersham) or pET-30a (Novagen, Darmstadt, Germany). Recombinant mouse adiponectin was expressed and prepared according to the manufacturer's instructions. S-His-tagged adiponectin fusion protein was digested with 0.1 units of EK Max (Invitrogen) to remove the S-His tag from the fusion protein.

2.7. Production of antibody

Recombinant S-His-tagged adiponectin protein (0.5 mg) was mixed with Freund's Complete Adjuvant (Difco, Detroit, MI), and subcutaneously injected into female rabbits. The animals were boosted twice with the same amount of protein mixed with Freund's Incomplete Adjuvant (Difco), and the blood was collected 1 week after the last booster. The antibody was purified using GST-adiponectin affinity column chromatography.

2.8. SDS-PAGE and Western blot analysis

Culture medium (25 μl) or 1 μl of serum (original serum—1:100 dilution) was used for the experiments. Samples were dissolved in Laemmli buffer containing 5% mercaptoethanol and incubated at 100 °C for 5 min. The samples were resolved electrophoretically on 12.5% polyacrylamide gels.

The proteins on the gels were transferred to polyvinylidene fluoride (PVDF) membrane using a semidry blotting apparatus (Bio-Rad, Hercules, CA). The membrane was blocked with 4% skim milk in phosphate-buffered saline (PBS) at 4 °C overnight. It was then incubated with the primary antibody, purified anti-mouse-adiponectin rabbit polyclonal antibody (1:10,000 dilution), at room temperature for 1 h. Horseradish-peroxidase-conjugated anti-rabbit IgG goat polyclonal antibody (1:10,000 dilution) was used as the secondary antibody. The signal was detected with ECL-Plus reagent (Amersham Pharmacia), according to the manufacturer's protocol.

2.9. Immunohistochemistry

ICR mouse E12.5, E14.5, E16.5, and E18.5 embryos and adult tissues were fixed in 4% paraformaldehyde in PBS overnight at 4 °C. Whole embryos and adult tissues were dehydrated in a graded series of ethanol and embedded in paraffin. Consecutive sections were cut to 6 μm [23,24]. The deparaffinized sections were pretreated for antigen retrieval by autoclaving (121 °C, 110 kPa) in 10 mM citrate buffer (pH 6.0) for 5 min. Endogenous peroxidase activity in the sections was blocked with 3% H_2O_2 in methanol for 30 min, and the sections were then washed in PBS. The sections were then immersed in 5% normal goat serum in PBS for 30 min and incubated with primary antibody, purified anti-mouse-adiponectin rabbit polyclonal antibody (1:10,000 dilution) overnight at 4 °C. Immunoreactions were performed using the Vectastain ABC peroxidase kit (Vector Laboratories, Burlingame, CA). The antigenic sites were detected by reacting the sections with a solution of 0.05% 3,3′-diaminobenzidine tetrahydrochloride (Dojin Chemicals, Tokyo, Japan) in 0.05 M Tris–HCl buffer (pH 7.6) containing 0.01% H_2O_2 for 7 min. Sections were counterstained in 0.5% methyl green in 0.1 M sodium acetate for 5 min, washed, dehydrated in 100% ethanol, rinsed in xylene, and mounted with Permount (Fisher Scientific).

2.10. Cell isolation from tissues

Primary brown adipocytes were isolated from the interscapular brown adipose tissue of 2- to 4-week-old ICR mice, according to previously described procedures [25,26]. Brown adipose tissue was dissected out and then cut into small pieces in HEPES buffer (pH 7.4) containing 0.2% (w/v) crude collagenase (Wako). The tissue was digested for 30 min at 37 °C in a shaking water bath with mixing by vortex for 10 s every 5 min. After incubation, the

tissue remnants were filtered through 250 μm nylon mesh. The cell suspension was placed on ice for 20 min to allow the mature brown adipocytes and lipid droplets to float. The infranatant was collected and filtered through a 40-μm cell strainer (Falcon). The precursor cells were pelleted by centrifugation at 2000 rpm for 10 min. The pellet was washed once with Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) and the cells were finally resuspended in culture medium.

2.11. Cell culture

The cells were cultured in DMEM containing 10% fetal bovine serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, 0.2 mM sodium ascorbate biphosphate, and 50 nM triiodothyronine, and were grown to subconfluence at 37 °C in a atmosphere of 5% CO_2. Subconfluent cells were cultured for 2 days in induction medium further supplemented with 10 μg/ml insulin and 2.5 μM dexamethasone. After induction, the cells were cultured in maturation medium supplemented with 10 μg/ml insulin for 5–7 days.

Differentiated cells were incubated for 8 h in serum-free DMEM before treatment for 18 h with various hormones and agents, such as 100 nM insulin, 100 nM dexamethasone, 1 μM triiodothyronine, 1 μM isoproterenol, or 100 nM troglitazone.

2.12. Analysis of adiponectin secretion

Quantification of adiponectin protein in the supernatants of cell cultures was performed using a commercially available sandwich ELISA kit (Otsuka Pharmacy, Tokyo, Japan), according to the manufacturer's instructions. The sensitivity of the adiponectin assay was 0.25 ng/ml, and the coefficient of variation among assays was 10–15%. Concentration of adiponectin in the medium was expressed as a ratio relative to the expression in untreated control cells. Results are given as means ± S.D. of three independent experiments.

2.13. Statistical analysis

Data were analyzed with a *t*-test using Stat Views J-5.0 (Abacus Concepts, Berkeley, CA). A P value of < 0.05 was deemed statistically significant.

3. Results and discussion

3.1. Transcripts of adiponectin

To investigate how the adiponectin gene is regulated in the course of development, we initially used Northern blot analysis and reverse transcription-polymerase chain reaction (RT-PCR) (Fig. 1A and B). Transcripts of adiponectin were detected in the late stage of embryogenesis on day E16.5. However, no specific signal was detected in E12.5 or E14.5

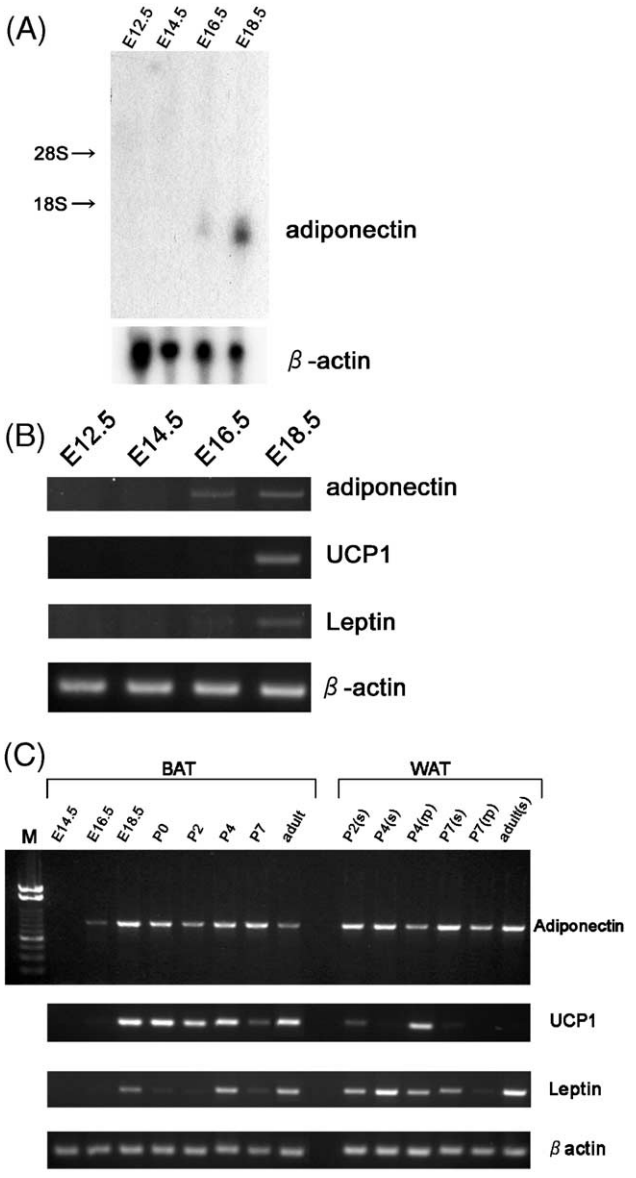


Fig. 1. Adiponectin gene expression at different stages of embryonic mouse development. (A) Northern blot analysis. Total RNA (20 μg) isolated from mouse embryos at different stages was separated in each lane. (B and C) RT-PCR analysis. RT-PCR was performed using specific primers (Table 1) for mouse adiponectin, UCP1, leptin, and β-actin mRNAs. The sizes of the various PCR products are shown in Table 1. β-actin was used as the internal control for total mRNA content. s, subcutaneous WAT; rp, retroperitoneal WAT.

mouse embryos. This result is consistent with previous studies using Northern blot analysis [27]. Uncoupling protein 1 (UCP1), which acts in thermogenesis in BAT, was slightly expressed at E16.5. Leptin was also detectable at E16.5. After birth, adiponectin is expressed in both BAT and WAT in the subcutaneous and retroperitoneal regions (Fig. 1C). Expression continued during adulthood. The level of adiponectin is lower in BAT than it is in WAT. Transcripts of UCP1 were transiently detected in WAT, as well as in BAT, a few days after birth. However, UCP1 expression in WAT might be due to the heterogeneity of immature adipose

tissue. Scattered brown adipocytes may be present in WAT [28]. The expression of leptin was completely different from that of adiponectin. Transcripts peaked around 4 days after birth had disappeared at 7 days and were again detected in adulthood (Fig. 1C).

We used in situ hybridization to determine the precise expression of these genes in various tissues at various developmental stages. Transcripts of adiponectin were localized in regions that were morphologically recognizable as BATs in the interscapula, cervix, and axilla on day E16.5

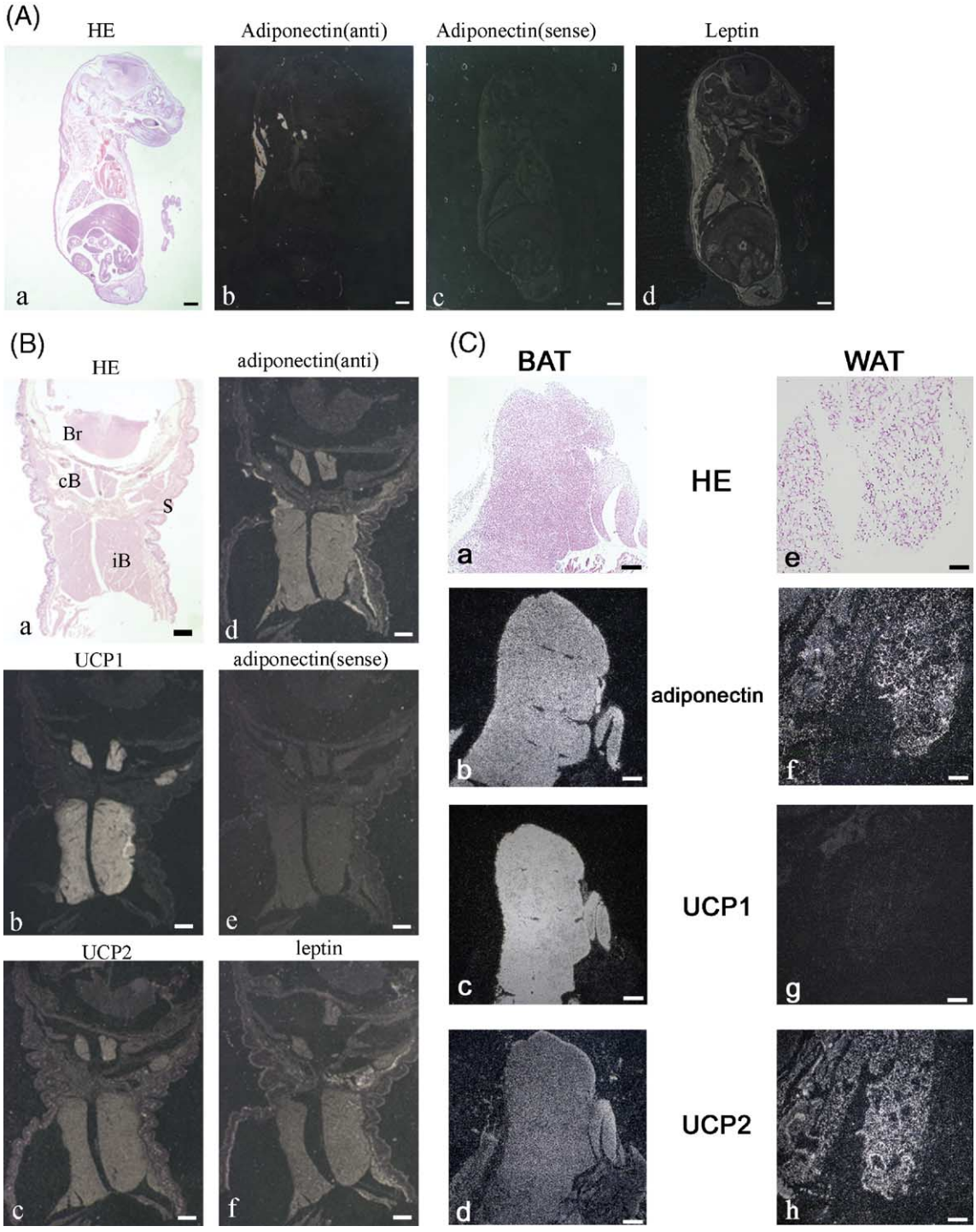
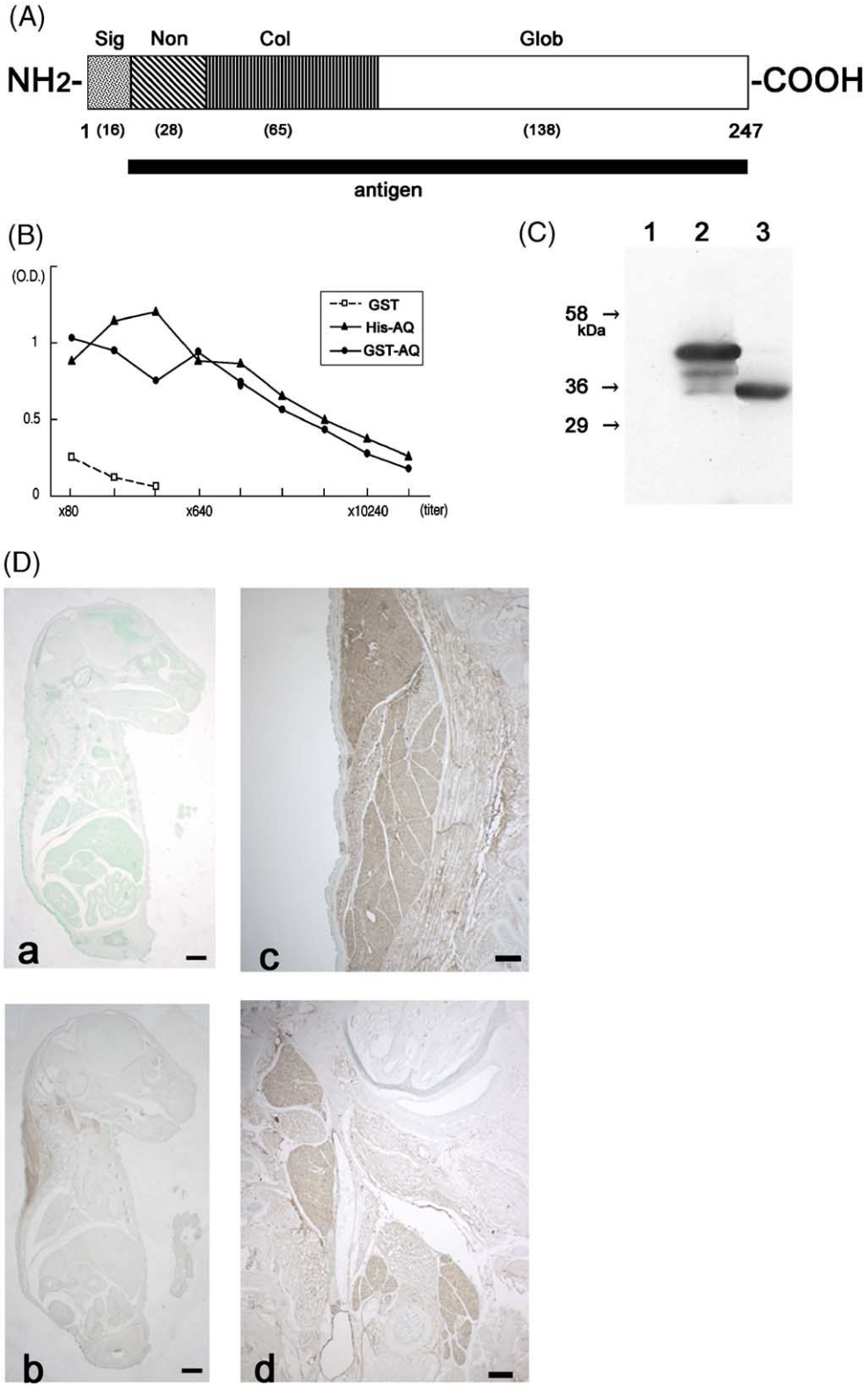


Fig. 2. In situ hybridization of E16.5 (A) and E18.5 (B) mouse embryos, and BAT and WAT of adult mouse (C). Dark-field photomicrographs of 6 μm sections hybridized with antisense RNA for adiponectin (panels b in A, d in B, and b and f in C), leptin (panels d in A, and f in B), UCP1 (panels b in B, c and g in C), UCP2 (panels c in B, and d and h in C), or sense RNA for adiponectin (panels c in A, and e in B) using ^{35}S-labeled cRNA probes. Hematoxylin–eosin staining was also performed (panels a in A, B and C). cB, cervical BAT; iB, intrascapular BAT; B, brain; S, skin. Scale bars: 1 mm in A, and 500 μm in B and C.

of gestation (Fig. 2A, panel b). However, the distribution of the leptin signal was completely different from that of adiponectin. Leptin was strongly expressed in the fetal cartilage and bone, and weakly in BAT and other tissues, including hair follicles, heart, liver, cochlear duct, and nasal turbinate (Fig. 2A, panel d). At E18.5, adiponectin was expressed in BAT and the surrounding tissue that would become adipose tissue (Fig. 2B, panel d). UCP1 was expressed in BAT, but not in the surrounding tissue (Fig. 2B, panel b). The expression of UCP2 and leptin was also



detected in both BAT and the surrounding tissue (Fig. 2B, panels c and f). In adult tissues, adiponectin was expressed strongly in BAT and moderately in WAT (Fig. 2C, panels b and f). The expression of UCP1 was restricted to BAT (Fig. 2C, panels c and g), whereas that of UCP2 was strong in WAT and only moderate in BAT (Fig. 2C, panels d and h).

3.2. Detection of adiponectin protein

To investigate the expression of adiponectin protein during development, a polyclonal antibody was raised against recombinant S-His-tagged adiponectin. The antibody was purified by affinity column chromatography, and its specificity was confirmed by enzyme-linked immunosorbent assay (ELISA) and immunoblotting (Fig. 3B and C). The antibody reacted with S-His-adiponectin and glutathione S-transferase (GST)-fused adiponectin, but did not cross-react with GST. We performed immunohistochemistry using serial embryonal E16.5 mouse sections (Fig. 3D). Immunostaining for adiponectin was observed in BATs, including the cervical and interscapular regions. The distribution of adiponectin protein closely paralleled that of adiponectin mRNA determined by in situ hybridization.

Adiponectin is abundant in the serum of the adult. To determine whether newly synthesized adiponectin is secreted into the blood of the fetus, we investigated serum adiponectin levels during the perinatal period using immunoblotting (Fig. 4). Adiponectin was detected in serum at E16.5 and its concentration peaked at birth. These results are consistent with the data from in situ hybridization and immunohistochemical analyses. The concentration of serum adiponectin was higher in the fetus than in the mother at birth (Fig. 4). After birth, adiponectin decreased gradually, but was moderately expressed as previously reported in humans [29]. In contrast, the concentration of leptin decreased rapidly after birth. Leptin is synthesized in the placenta during pregnancy in humans [29–32]. However, no expression of adiponectin was detected in the mouse placenta by RT-PCR (data not shown).

The differentiation of adipocytes varies greatly among species. Little or no lipid accumulation is observed at birth in the mouse. Morphologically, adipocytes develop rapidly after birth, within the first 24 h [33]. BATs are observed from E16.5 in the mouse embryo, but little or no WAT is observed at birth [33]. The WAT initially appeared in the subcutaneous region 2 days after birth and in the retroperitoneal region 4 days after birth (data not shown). Our results suggest that fetal BAT contributes to the high levels of adiponectin in the blood, and that BAT has an important function as an endocrine organ, as well as a thermogenic organ, in the perinatal period.

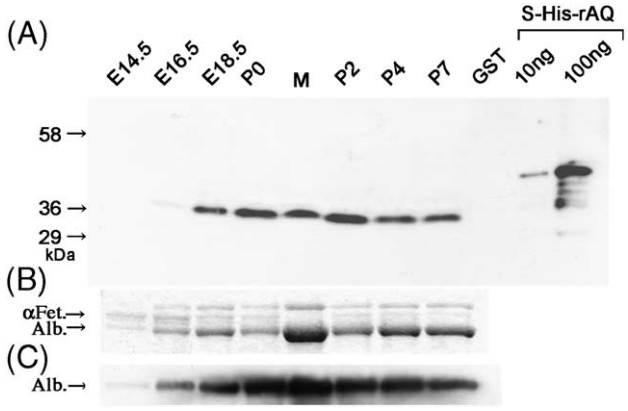


Fig. 4. Western blot analysis of serum adiponectin levels during mouse development. (A) Serum (1 μl) was boiled for 5 min in sample buffer and analyzed by SDS-PAGE on 12.5% gel. A Western blot was probed with anti-mouse-adiponectin rabbit polyclonal antibody. The antibody was detected with an anti-rabbit-IgG antibody coupled to horseradish peroxidase, and visualized with ECL-Plus. Molecular sizes are shown on the left. M, maternal sample. (B) The gel was stained with Coomassie Brilliant Blue. Serum (1 μl, 1:10 dilution) in sample buffer was applied in each lane. Alb, albumin; α-Fet, α-fetoprotein. (C) A Western blot was probed with anti-albumin antibody (1:1000 dilution). Serum (1 μl, 1:100 dilution) in sample buffer was applied to each lane.

3.3. Expression of adiponectin receptors

The genes for the adiponectin receptors 1 and 2 have now been cloned [20]. To compare their expressions with that of adiponectin in embryogenesis, we performed in situ hybridization. Transcripts of both adiponectin receptors were already detected in early stage of embryogenesis on day E12.5 (Fig. 5A). Receptor 1 was strongly expressed in the heart, and weakly in the liver (Fig. 5A, panels a and b) whereas receptor 2 was strongly expressed in the liver and intestines, and weakly in the heart (Fig. 5A, panels c and d). On day E16.5, receptor 1 was expressed strongly in skeletal muscle, the heart and the placenta, moderately in the lung, and weakly in the intestine and BAT (Fig. 5B, panels a–d, and Fig. 5C, panels a and b). Receptor 2 was expressed strongly in BAT and in the liver and intestines, moderately

Fig. 3. (A) Domain structure of adiponectin. The domains consist of a signal peptide (Sig), and nonhomologous (Non), collagen-like (Col), and globular (Glob) domains. The horizontal bar and numbers in parentheses indicate the portion of the protein used to generate the antibody and the amino acid numbers of each domain, respectively. Numbers 1 and 247 represent the beginning and end of the protein, respectively. (B) ELISA using anti-adiponectin antibody. The antigens coated on the wells were S-His-tagged adiponectin (His-AQ: closed triangles), GST-fused adiponectin (GST-AQ: closed circles), and GST (open squares). (C) Western blot probed with anti-adiponectin antibody. The antigens are GST (lane 1), S-His-tagged adiponectin (lane 2), and adiponectin (lane 3). Molecular sizes are shown on the left. (D) Immunohistochemical staining with an anti-mouse-adiponectin rabbit polyclonal antibody on an E16.5 mouse embryo. Panels a and b, sagittal section at low magnification. In panel a, the anti-mouse-adiponectin rabbit polyclonal antibody was preabsorbed with recombinant mouse adiponectin before incubation with the section. Panel c; A portion of cervical BAT. Panel d; A portion of interscapular BAT. Scale bars = 1 mm in panels a and b, and 100 μm in panels c and d.

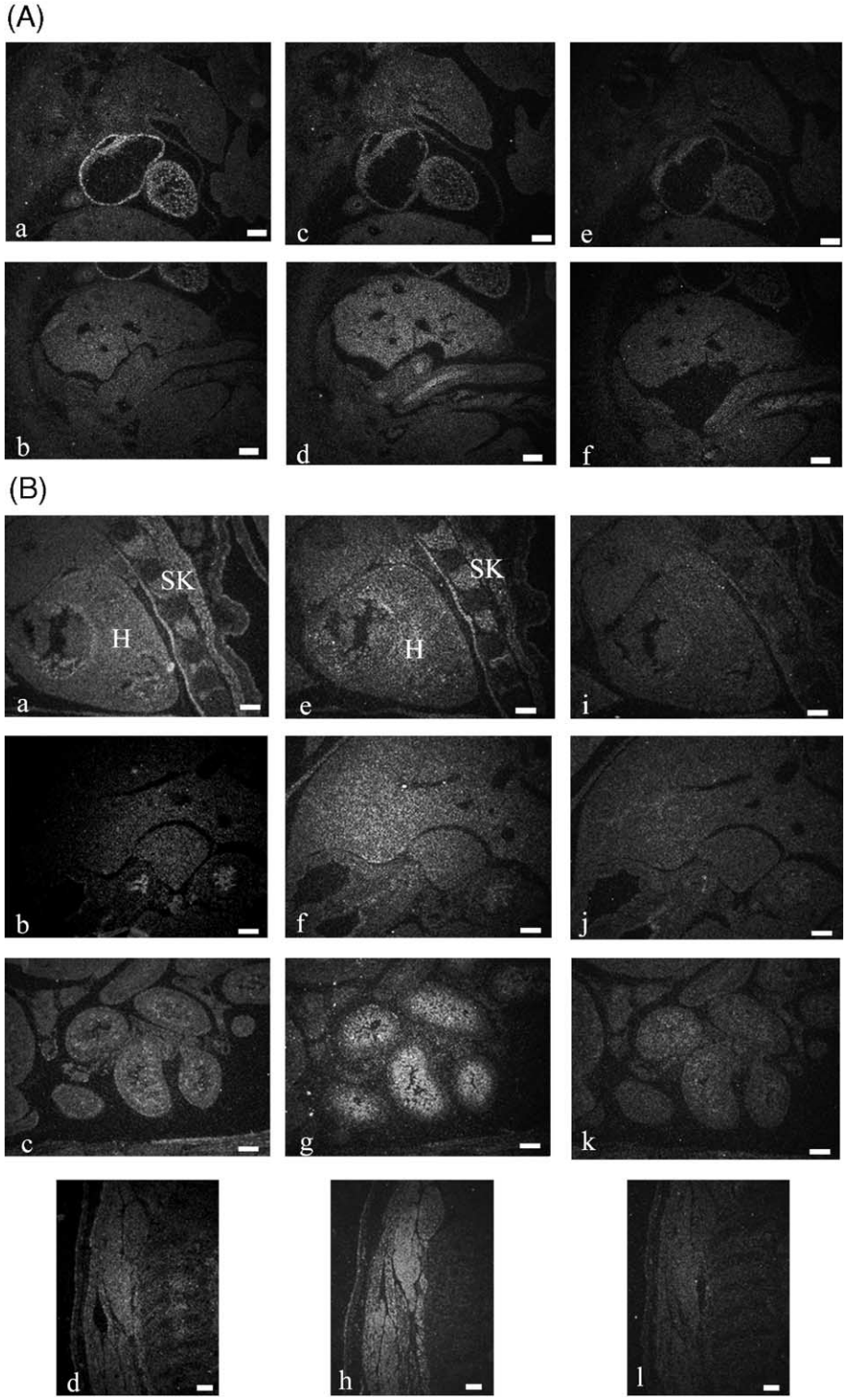


Fig. 5. In situ hybridization to detect adiponectin receptor 1 and 2 mRNAs in E12.5 (A) and E16.5 (B) mouse embryos, and E16.5 placenta (C). Dark-field photomicrographs of 6 μm sections hybridized with antisense RNA for adiponectin receptor 1 (panels a and b in A, a–d in B, and a in C), receptor 2 (panels c and d in A, e–h in B, and c in C), and sense RNA for receptor 1 (panels e and f in A, i–l in B, and b in C) and receptor 2 (panel d in C) using ^{35}S-labeled cRNA probes. In A, panels a, c, and e show heart tissue, and panels b, d, and f show the liver and intestine. In B, panels a, e, and i show heart tissue; panels b, f, and j show the liver; panels c, g, and k show intestine; panels d, h, and l show interscapular BATs. Specific regions are labeled H (heart) and SK (skeletal muscle). In C, note that non-specific signals derived from blood cells are just visible in panel c. Scale bars: 200 μm.

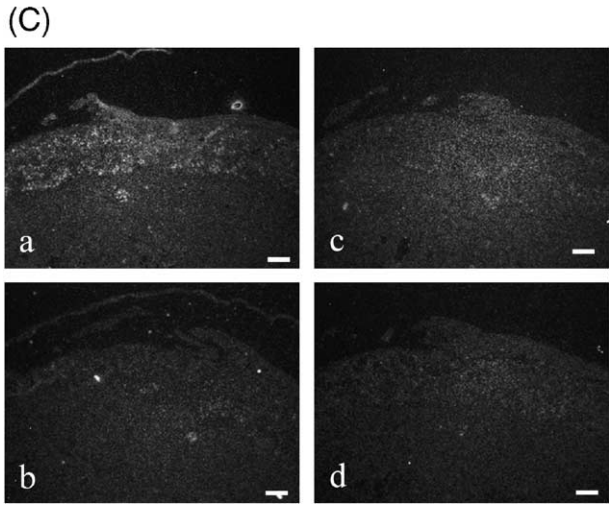


Fig. 5 (*continued*).

in the heart, and weakly in skeletal muscle (Fig. 5B, panels e–h). The tissue distribution in fetus was essentially consistent with that in adults using Northern blot analysis [20]. Thus, the signals of the adiponectin receptors differed from that of adiponectin both temporally and spatially. These results suggest that adiponectin might have multiple functions, which are still unknown, through these two receptors expressed in various tissues.

3.4. Adiponectin expression in primary cultures of brown adipocytes

Regulation of adiponectin expression in WAT has been well studied [12,34,35], but little information is available on the expression of adiponectin in BAT [19,36]. We used primary cultures of brown adipocytes to investigate the regulation of adiponectin in BAT. About 2–3 days after induction, brown preadipocytes undergo differentiation into mature brown adipocytes. This transition is identified by the capacity of the cells to display multilocular intracytoplasmic lipid droplets. Expression of adiponectin started on about day 2 after induction, peaked on day 8, and remained relatively high even on day 14 (Fig. 6A). Transcripts of UCP1 were detected on day 6 (a little later than those of adiponectin), peaked on day 8, and then decreased dramatically. Expression of leptin showed a pattern similar to that of adiponectin.

On day 8, differentiated brown adipocytes were incubated for 18 h with various modulators. Transcripts of adiponectin were then quantified by Northern blot analysis (Fig. 6B). Treatment with 100 nM insulin or 1 μM isoproterenol significantly decreased adiponectin mRNA levels, by approximately 50% and 66%, respectively ($P<0.05$). Treatment with dexamethasone tended to reduce adiponectin mRNA expression, whereas troglitazone and triiodothyronine tended to stimulate it.

The release of adiponectin induced by various modulators was assessed in cultured brown adipocytes. Brown adipocytes released half as much adiponectin at 24 h after stimulation than at 48 h (data not shown). Therefore, we examined the concentration of adiponectin in the medium at 48 h. Isoproterenol significantly inhibited the secretion of adiponectin by 42% ($P<0.05$), which is consistent with the data for mRNA (Fig. 6C and D). Experiments on WAT have shown that this effect is mediated via the activation of stimulatory guanine nucleotide-binding protein/protein-kinase-A-dependent pathway [37,38]. Dexamethasone, troglitazone, and triiodothyronine affected the release of adiponectin as they affected mRNA levels: dexamethasone reduced adiponectin release, whereas troglitazone and triiodothyronine stimulated it. Troglitazone, which is a kind of thiazolidinediones and is used as an antidiabetic drug, enhances both adiponectin mRNA and secretion via the activation of its promotor [2,39]. Stimulation with 100 nM insulin increased adiponectin secretion, which is inconsistent with the mRNA data. Many researchers have examined the effect of insulin on the expression of adiponectin in adipocytes. However, their results are controversial [34,36,40,41]. They used different adipocytes, a WAT cell line [34,41], a primary culture derived from human visceral adipocytes [40], or the BAT cell line T37i [36]. The conditions of cell culture also differed in these experiments. The results reflect the kinds of adipocytes used and/or the culture conditions. Jonathan et al. showed that insulin accelerates adiponectin secretion via the activation of phosphatidylinositol-3-kinase in its secretary pathway [41]. This secretion may change in a time-dependent manner, increasing at the beginning of stimulation and inhibited at a later stage after the decrease in mRNA. This complex regulatory mechanism seems to act in response to stimulation by insulin.

Except for these data on insulin, our data are basically consistent with those from experiments using WAT and brown adipocyte cell lines [34,36]. This suggests that the adiponectin gene is regulated by various modulators in brown adipocyte tissue, similar to its regulation in WAT. Adiponectin has important roles in glucose and lipid

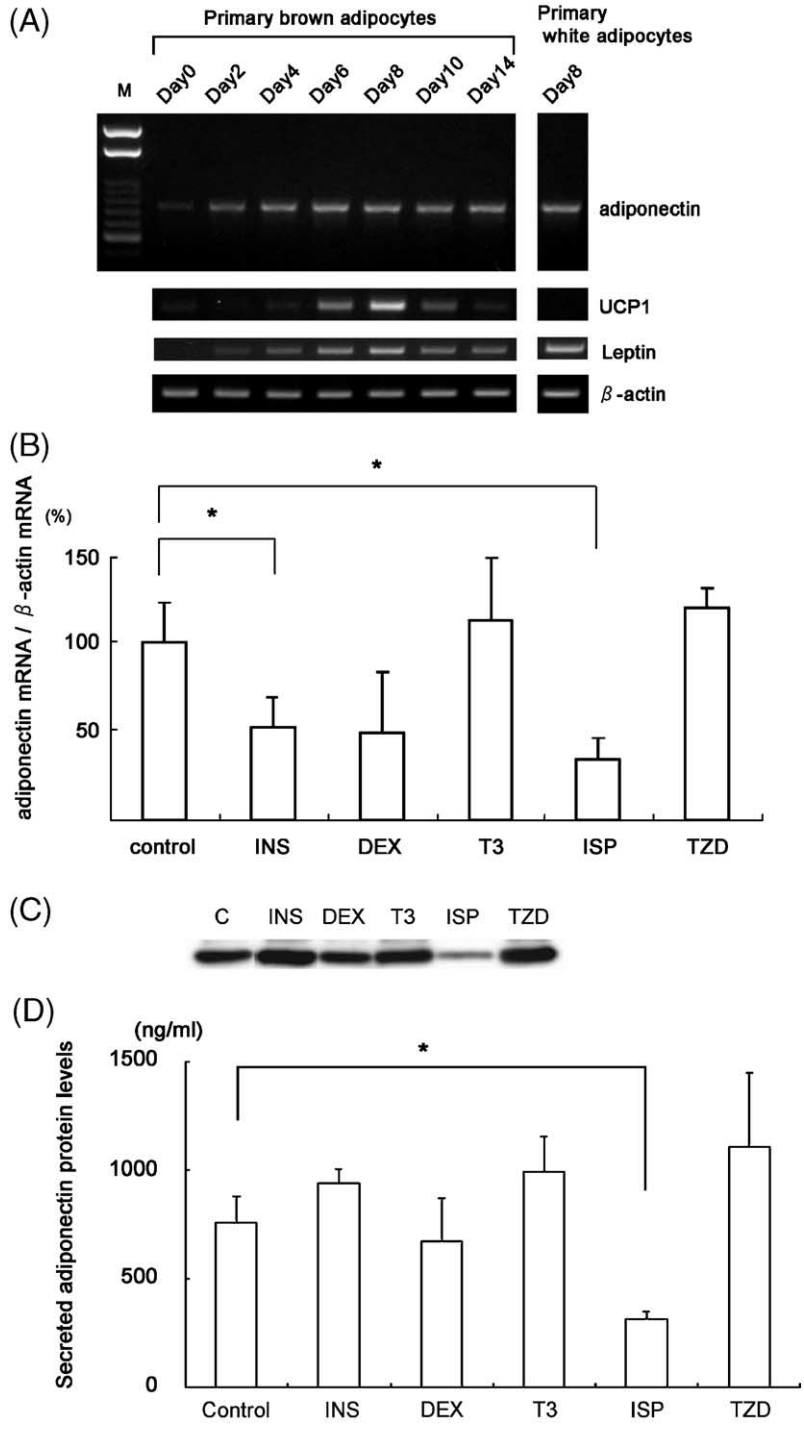


Fig. 6. Expression in primary brown and white adipocyte cultures. (A) RT-PCR analysis of adiponectin. Days 0–14 indicate the days after induction. M: 100-bp ladder marker. (B) Hormonal control of mouse adiponectin gene expression. Total RNA was extracted from differentiated brown adipocytes treated with 100 nM insulin (INS), 100 nM dexamethasone (DEX), 1 μM triiodothyronine (T3), 1 μM isoproterenol (ISP), or 100 nM troglitazone (TZD). Northern blot analysis with specific probes for mouse adiponectin and β-actin was used. mRNAs were quantified using the BAS2000 Phosphor Imaging System. The relative expression of adiponectin is indicated as a ratio relative to β-actin expression. Induced adiponectin expression is given as a ratio relative to its expression in untreated control cells (=100%). Results are given as means ± S.D. of at least three independent experiments. Asterisks indicate statistically significant results ($P < 0.05$). C and D: Hormonal control of mouse adiponectin protein secretion. Adiponectin protein was recovered from the culture medium of differentiated brown adipocytes treated for 48 h with the various reagents described in B. Immunoblot analysis of adiponectin secreted into the medium. Representative blots are shown in C. Concentration of adiponectin assayed using an ELISA kit (D). Results are given as means ± S.D. of three independent experiments. Asterisks indicate statistically significant results $P < 0.05$).

metabolism, with both paracrine and endocrine effects during the perinatal period. It has also been reported that cord plasma adiponectin levels correlate with birth weight and adiposity in human neonates [42,43]. Recombinant adiponectin enhanced the proliferation of osteoblasts [44]. Furthermore, adiponectin may be involved in fetal growth through bone homeostasis and/or adiposity.

3.5. Conclusion

We have reported the developmental expression of adiponectin and adiponectin receptors. In the fetus, adiponectin is synthesized in BATs and surrounding immature tissues, and secreted into the blood. The level of adiponectin in the blood peaks at birth. On the other hand, adiponectin receptors 1 and 2 are expressed at an earlier stage, and are widely expressed in various tissues. Using primary cultures of brown adipocytes, we found that adiponectin expression is regulated by various modulators, as it is regulated in WAT. Because WAT is not detectable in the perinatal period, adiponectin synthesized in BAT and immature adipose tissues might have an important role in endocrine function. Further studies are required to elucidate the role of adiponectin, especially in the perinatal period.

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