Agouti Expression in Human Adipose Tissue

Functional Consequences and Increased Expression in Type 2 Diabetes

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It is well recognized that the agouti/melanocortin system is an important regulator of body weight homeostasis. Given that agouti is expressed in human adipose tissue and that the ectopic expression of agouti in adipose tissue results in moderately obese mice, the link between agouti expression in human adipose tissue and obesity/type 2 diabetes was investigated. Although there was no apparent relationship between agouti mRNA levels and BMI, agouti mRNA levels were significantly elevated in subjects with type 2 diabetes. The regulation of agouti in cultured human adipocytes revealed that insulin did not regulate agouti mRNA, whereas dexamethasone treatment potently increased the levels of agouti mRNA. Experiments with cultured human preadipocytes and with cells obtained from transgenic mice that overexpress agouti demonstrated that melanocortin receptor (MCR) signaling in adipose tissue can regulate both preadipocyte proliferation and differentiation. Taken together, these results reveal that agouti can regulate adipogenesis at several levels and suggest that there are functional consequences of elevated agouti levels in human adipose tissue. The influence of MCR signaling on adipogenesis combined with the well-established role of MCR signaling in the hypothalamus suggest that adipogenesis is coordinately regulated with food intake and energy expenditure. ***Diabetes*** **52:2914–2922, 2003**

Over the past decade, it has become apparent that the agouti/melanocortin system is a critical component of several biological pathways, including body weight homeostasis. Many genetic and pharmacological studies have shown that agouti and agouti-related protein (AGRP) compete with pro-opiomelanocortin (POMC)-derived peptides for binding sites on melanocortin receptors (MCRs) to regulate food intake and energy expenditure. Altered expression levels of agouti (1,2), AGRP (3,4), MC3-R (5) and MC4-R (6), and POMC (7) have been shown to disrupt the control of body weight in mice. However, to date, there have been no published observations implicating agouti, per se, in human obesity or type 2 diabetes.

The mouse and human agouti proteins are structurally similar, but the expression pattern of the mouse and human agouti genes is very different. The transcription of the wild-type mouse agouti is temporally regulated, being expressed solely in the skin during part of the hair growth cycle (1). Human agouti is expressed in diverse tissues: adipose tissue, testis, heart, liver, kidney, ovary, and skin (8,9). The divergence in expression patterns between mice and humans strongly suggests that agouti may have functions other than pigmentation in humans. The presence of agouti, and MCRs, in human adipose tissue raises questions as to their normal function in adipose tissue and whether dysregulation of agouti in adipose tissue might be associated with an obese, insulin-resistant, or diabetic phenotype.

In the original human agouti cloning study by Kwon et al. (8), an RT-PCR analysis of RNA obtained from subcutaneous fat from three normal females revealed agouti expression in two of the three fat samples and suggested that agouti might be differentially regulated in humans. More recently, agouti mRNA was found to be negatively correlated with BMI in men but not in women (10). In our recent studies, we engineered transgenic mice to overexpress agouti in adipose tissue in order to study the potential biological role of agouti/melanocortin signaling in human adipose tissue (11). The aP2-agouti transgenic mice become significantly heavier than nontransgenic littermates by 8–10 weeks of age and have almost a twofold increase in fat mass compared with nontransgenic mice (12). Moreover, there were no detectable changes in food intake in the aP2-agouti mice, suggesting changes in energy utilization and/or nutrient partitioning (13).

In the present study, expression levels of human agouti and MCRs were studied in human mesenchymal stem cells as they were differentiated into lipid-storing adipocytes. Having established the presence of both agouti and MCRs in human adipose tissue, agouti expression levels were measured in patients with a wide range in BMI and in patients with type 2 diabetes. Additionally, experiments with transgenic mice that express agouti in adipose tissue

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11β-HSD-1, 11β hydroxysteroid dehydrogenase type 1; AGRP, agouti-related protein; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; IBMX, isobutyl-methylxanthine; MCR, melanocortin receptor; αMSH, α melanocyte-stimulating hormone; POMC, pro-opiomelanocortin; PPAR, peroxisome proliferator–activated receptor.

and isolated preadipocytes were used to determine whether there was a possible functional role of the agouti/melanocortin system in adipose tissue. Several interesting findings have emerged from these experiments. The data demonstrate that human agouti expression is upregulated by glucocorticoids and that agouti and the cortisol-producing enzyme 11β hydroxysteroid dehydrogenase type 1 (11β-HSD-1) are upregulated in patients with type 2 diabetes. Third, we find that the agouti/melanocortin system is a potent and direct regulator of adipogenesis.

RESEARCH DESIGN AND METHODS

Human mesenchymal cell differentiation. Cells (BioWhittaker) were plated at a density of 2–5 × 10^3/cm^2 and grown at 37°C in an atmosphere of 95% air and 5% CO_2 as previously described (14). Cells were grown to 100% confluence in the growth medium supplemented with 5% fetal bovine serum (FBS). Five-day postconfluent cells were incubated in adipogenesis induction medium (AIM-M199; 1 mmol/l dexamethasone, 0.2 mmol/l indomethacin, 170 nmol/l insulin, 0.5 mmol/l isobutyl-methylxanthine [IBMX], 10% FBS, 0.05 units/ml penicillin, and 0.05 μg/ml streptomycin) for 3 days. Next, the cells were incubated for 1 day in adipogenesis maintenance medium (AMM-M199; 170 nmol/l insulin, 10% FBS, 0.05 units/ml penicillin, and 0.05 μg/ml streptomycin) and then switched to AIM for 3 days. After a third exposure to AIM, cells were maintained in AMM for up to 21 days after the initiation of differentiation.

Isolation and differentiation of mouse preadipocytes. Epididymal and inguinal fat pads, collected from 19- to 21-day-old FVB, β-actin promoter-agouti (BAP-agouti), and aP2-agouti mice, were minced and digested with collagenase class I (2 mg/ml; Worthington Biochemical) in a shaking bath for 1 h at 37°C. Dissociated cells were filtered through a 100 μmol/l cell strainer (Becton Dickinson Labware) and separated by centrifugation at 360*g* for 5 min. Pelleted preadipocyte cells were resuspended for 1 min. in erythrocyte lysis buffer (Sigma) to remove erythrocyte contamination and centrifuged at 360*g* for 5 min. Cells were plated in 35-mm Petri dishes (p = 0) in DMEM/F12 medium (Invitrogen Life Technologies) supplemented with 15% of FBS (Invitrogen Life Technologies) or in complete medium. Subconfluent primary cultures were trypsynized and replated (p = 1) at 2 × 10^4/cm^2 in a 24-well culture plate and kept in complete medium. At confluency, cells received differentiation medium (DMEM/F12 with 5% FBS supplemented with 1.7 μmol/l insulin, 1 μmol/l dexamethasone, and 0.5 mmol/l IBMX for 5 days. In the experiment in which RNA was collected from the adipocytes, the medium was replaced with DMEM/F12 with 5% FBS for 2 additional days in the presence of 2 μmol/l rosiglitazone and 10 nmol/l insulin. To measure lipid content in the adipocytes, cells were fixed in 10% solution of formaldehyde in aqueous phosphate buffer for ≥1 h, washed with 60% isopropanol, and stained with Oil Red-O solution (in 60% isopropanol) for 10 min. The cells were then repeatedly washed with water, photographed, and destained in 100% isopropanol for 15 min. The optical density of the isopropanol solution was measured at 500 nm.

Human biopsies. All human studies were performed after institutional review board approval and written informed consent were obtained. After local anesthesia, adipose tissue was collected by needle biopsy of the superficial subcutaneous adipose tissue lateral to the umbilicus.

Mice. Transgenic mice were maintained on the FVB/N background at the Pennington Biomedical Research Center as previously described (11). All mice were fed a diet containing 11% fat by weight (Mouse Diet 5015; Purina Mills). Food and water were provided ad libitum. All data are from mice that are hemizygous for the transgene or their nontransgenic littermates.

RNA isolation and RT-PCR analysis. Total RNA was isolated by the Trizol method (Life Technologies) and applied to Rneasy columns (Qiagen). The concentration of RNA was determined using spectrophotometry, and the quality was verified by visualizing on a gel. For nonquanitative RT-PCR, 1 μg total RNA was used for the cDNA synthesis and 1:40 of that reaction was used as a PCR template.

The following primer sets were used. Human (forward, reverse primer): agouti (TGTCTCTATTGTGGCGCTGAA, GGAAGAAGCGGCACTG); MC1-R (GCAGCAGCTGGACAATGTCA, ATGAAGAGCGTGCTGAAGACG); MC2-R (ATCACCTTCACGTCGCTGTT, CGTTATTCCCATGGATTCTA); MC3-R (CCAAAAGAAGTATCTGGAG, AGTTCATGCCGTTGCAGCC); MC4-R (GCTACGAGCAACTTTTTCTC, GTACTGGAGAGCATAGAAG); MC5-R (ACCTGCACTCCCCCATGTAC, CAGGCACTGGACATGCTCAC); peroxisome proliferator–activated receptor (PPAR)γ (TGCCAAAAGCATTCCTGGTT, CGCTGTCATCTAATTCCAGTGC); adipsin (CAGGGTCACCCAAGCAACA, CAGGGTCACCCAAGCAACA); cyclophilin (GGAGATGGCACAGGAGGAAA, CGTAGTGCTTCAGTTTGAAGTTCTCA). Mouse (forward, reverse primer): MC1-R (CACTCAGGAGCCCCAGAAGA, CACCAGACTCACCAGCCCTAG); MC2-R (GCGCCGCACCATCATC, GGGAGCGGGCAAGTAAGAAC); MC3-R (CTCTGGGCATCGTCAGTCTGA, GGATAAACTGGTCCTCCAAGGTC); MC4-R (GCGGGTCGGGATCATCA, GGTACCCTGGCGGATGGT); MC5-R (AGGTGTTCCTGACCCTGGGT, CAGGCATTGGACATGCTCAC); PPARγ (AGAGTCTGCTGATCTGCGAGC, CGGATCGAAACTGGCACC); aP2 (GTGATGCCTTTGTGGGAACC, CGCCCAGTTTGAAGGAAATCT); cyclophilin (TCCATCGTGTCATCAAGGACTT, CTCATCTGGGAAGCGCTCA).

The taqman probe primer system (Applied Biosystems) was used for quantitative RT-PCR. Primer/probe sets are as follows. Agouti: forward CAACTCCT-CTGTGAACCTACTGGAT, probe 6-FAM-CCCTTCTGTCTCTATTGTGgcGCTGAAC-BHQ (lower case letters denote intro/exon boundry), reverse TGCCGATCTGTTTGGATTTCT; 11β-HSD-1: forward GGCTTATCATCTGGCGAAGATG, probe 6-FAM-GGATACCACCTTCTGTAGAGTTTCTTTT-BHQ, reverse ACCTCGCTGTCACCACCACATGGG. RT-PCR was carried out in one tube using the ABI PRISM 7700 SDS instrument (Applied Biosystems) using 50 ng total RNA as the template. The master mix included 300 nmol/l primers, 100 nmol/l probe, reverse transcriptase MuLV, RNase inhibitor, and AmpliTaq GOLD (Applied Biosystems). The Ct value for every sample was measured in duplicate, and mRNA levels were normalized to cyclophilin mRNA levels (forward GGAGATGGCACAGGAGGAAA, probe 6-FAM-CATCTACGGTGAGCGCTTCCCCG-BHQ, reverse CGTAGTGCTTCAGTTTGAAGTTCTCA). The agouti and MCR PCR products were cloned into a T/A cloning vector and sequenced.

Cell proliferation assay. Human preadipocytes were purchased from Zen Bio. The donors were five healthy women between 20 and 60 years of age. The average BMI was 26.6 kg/m^2, and none were morbidly obese. Preadipocytes were isolated by centrifugal force after collagenase treatment. We received undifferentiated preadipocytes at passage 2 and plated equal numbers of cells in a 96-well plate. The cells were cultured in DMEM/F-10 (1:1 [vol/vol]) with 10% FBS until ~50% confluence. The human cells were then treated with the various combinations of α melanocyte-stimulating hormone (αMSH) and murine agouti (93aa-132aa; Phoenix Pharmaceuticals) overnight. The next morning, fresh media containing BrdU was added. Three hours later, BrdU uptake was measured by enzyme-linked immunosorbent assay (ELISA) (Amersham Pharmacia). For mouse preadipocyte proliferation assays, preadipocytes were isolated from 19- to 21-day-old mice by collagenase digestion and centrifugation. The preadipocytes were grown to confluence, trypsinized, and split equally into 96-well plates. The mouse cells were then treated with the various combinations of αMSH and agouti overnight. The next morning, fresh media containing BrdU was added. Three hours later, BrdU uptake was measured by ELISA (Amersham Pharmacia).

Statistical analyses. Unpaired *t* tests were used to compare diabetic and nondiabetic individuals (JMP version 4.0; SAS Institute, Cary, NC). Agouti and 11β-HSD-1 mRNA expression were compared across sex and diabetes status using a two-way ANOVA. Correlation between agouti and BMI was performed using Pearson correlation. Statistical significance was set a priori at 0.05.

RESULTS

The MCR status in FVB/N mouse adipose tissue and each of the cell lines used in these experiments were tested because different mouse strains may express different MCR, receptor status may be affected by culture conditions, and cell lines can change over time. To our knowledge, there is only one publication on the expression of MCRs in human adipose tissue, and this study indicates that all MCRs are expressed in human subcutaneous fat (15). In the present study, RNA was isolated from human mesenchymal stem cells and 3T3-L1 cells as they differentiated into adipocytes, as described in RESEARCH DESIGN AND METHODS. RNA was also obtained from intact mouse inguinal and human subcutaneous fat and from mouse adipose tissue after it was fractionated into stromal vascular and adipocyte fractions. The presence of all known MCR was then examined by RT-PCR. As shown in Fig. 1*A*, both MC1-R and MC2-R were detected in the human mesenchymal stem cells before and after differentiation and in human adipose tissue. MC1-R was found predominately in the preadipocyte fraction and MC2-R predominately in the adipocyte fraction. The other MCRs were not detected in

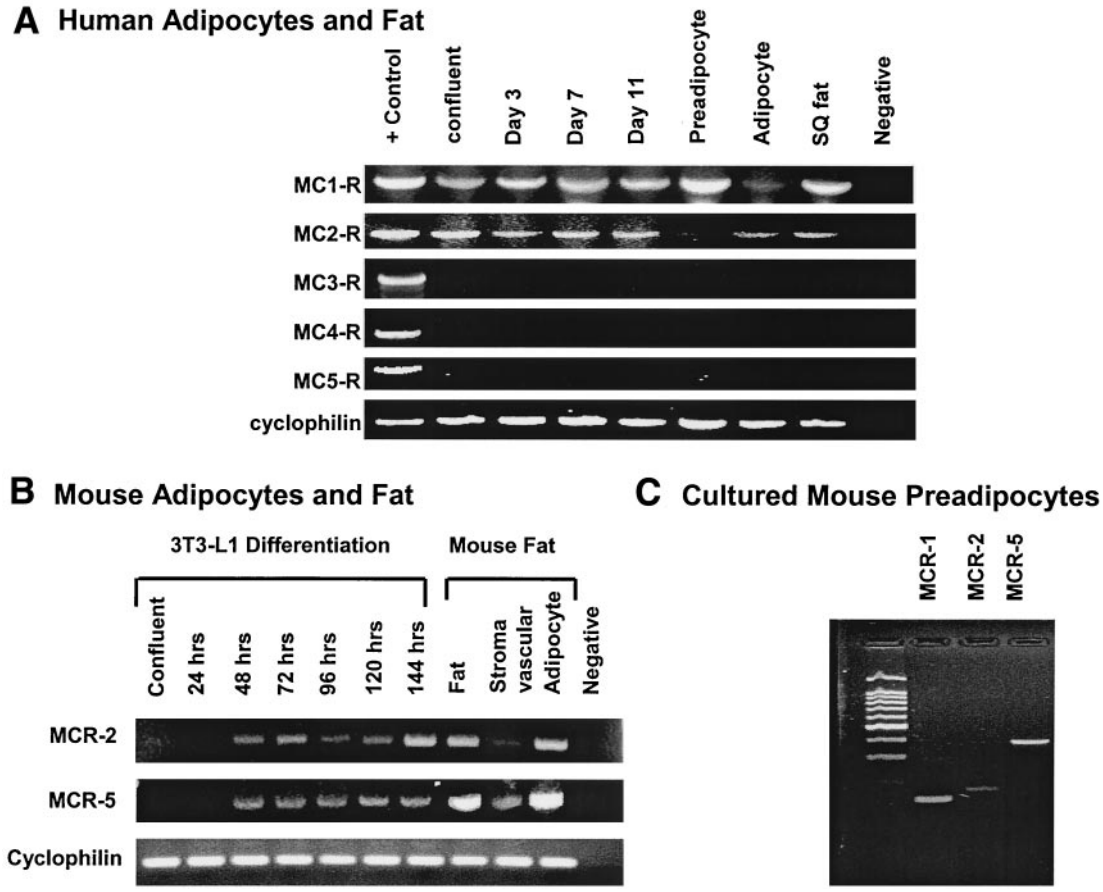

FIG. 1. RT-PCR analysis of MCRs in mouse and human adipose tissue and adipocytes. *A*: RNA was isolated from human mesenchymal stem cells as they were differentiated into adipocytes, as described in RESEARCH DESIGN AND METHODS (*lanes 2-5*), from preadipocytes that were isolated from human subcutaneous fat and grown to confluence (*lane 6*) and after they were differentiated into adipocytes (*lane 7*) and from whole human subcutaneous fat (*lane 8*). *B*: RNA was isolated from 3T3-L1 mouse adipocytes as they were differentiated into adipocytes, from whole mouse fat pads, and from mouse fat that had been separated into the stromal vascular and adipocyte fractions by collagenase digestion and centrifugation. *C*: RNA was isolated from mouse preadipocytes isolated from combined white fat depots and grown to confluence. For all samples, first-strand cDNA was synthesized from 2 μg total RNA and 1/40th of the reaction was used as template for 35 cycles of PCR.

human cells or fat. A critical problem with RT-PCR detection of MCR mRNA is that the MCR genes are intronless. Therefore only a small amount of genomic DNA contamination in the RNA preparation will give a false-positive. It is unclear if the human RNA purchased for the previous study (15) was DNAse treated for the RT-PCRs. Hence, it is possible that the commercial RNA may have been contaminated with genomic DNA.

RT-PCR analysis detected both MC2-R and MC5-R in murine 3T3-L1 cells (Fig. 1*B*) as they differentiated into adipocytes, an observation which is consistent with a previous study on the MCR subtype expression in 3T3-L1 adipocytes (16). None of the other MCR subtypes were detectable in 3T3-L1 cells. MC2-R and MC5-R were detectable in FVB/N mouse adipose tissue, and they were found primarily in the adipocyte fraction. MC1-R, MC2-R, and MC5-R were found in mouse preadipocyte culture (Fig. 1*C*).

To examine the agouti expression pattern during human adipocyte differentiation, human mesenchymal stem cells were cultured to 95% confluence and induced to differentiate as described in RESEARCH DESIGN AND METHODS. We observed that the expression of agouti mRNA increased through each cycle of exposure to the differentiation media and declined when the cells were returned to basal media (Fig. 2*A* and *B*). The decline in agouti expression that occurred when cells were returned to the basal media suggested that individual components of the differentiation media were capable of regulating agouti expression. We observed that the addition of dexamethasone to either confluent mesenchymal stem cells or differentiated adipocytes increased agouti expression to the same level observed with the complete differentiation cocktail (Fig. 2*C*). These in vitro results indicate that glucocorticoids upregulate agouti expression in human adipocytes.

Since transgenic mice that express agouti in adipose tissue become obese (12), agouti mRNA was quantitated in the subcutaneous fat from humans with a broad range of BMIs. There was no correlation between agouti expression levels and BMI in either men or women (Fig. 3*A*). However, agouti mRNA was significantly greater in women than in men (Fig. 3*B*). Agouti mRNA was also measured in individuals with type 2 diabetes with body fat similar to moderately obese people (Table 1 and Fig. 3*A*). There were significantly higher levels of agouti expression in diabetic subjects than in nondiabetic individuals (Table 1 and Fig. 3*B*). The data in Fig. 3*A* demonstrate that type 2 diabetic subjects have higher agouti levels than nondiabetic subjects across a range of BMIs. Again there was a

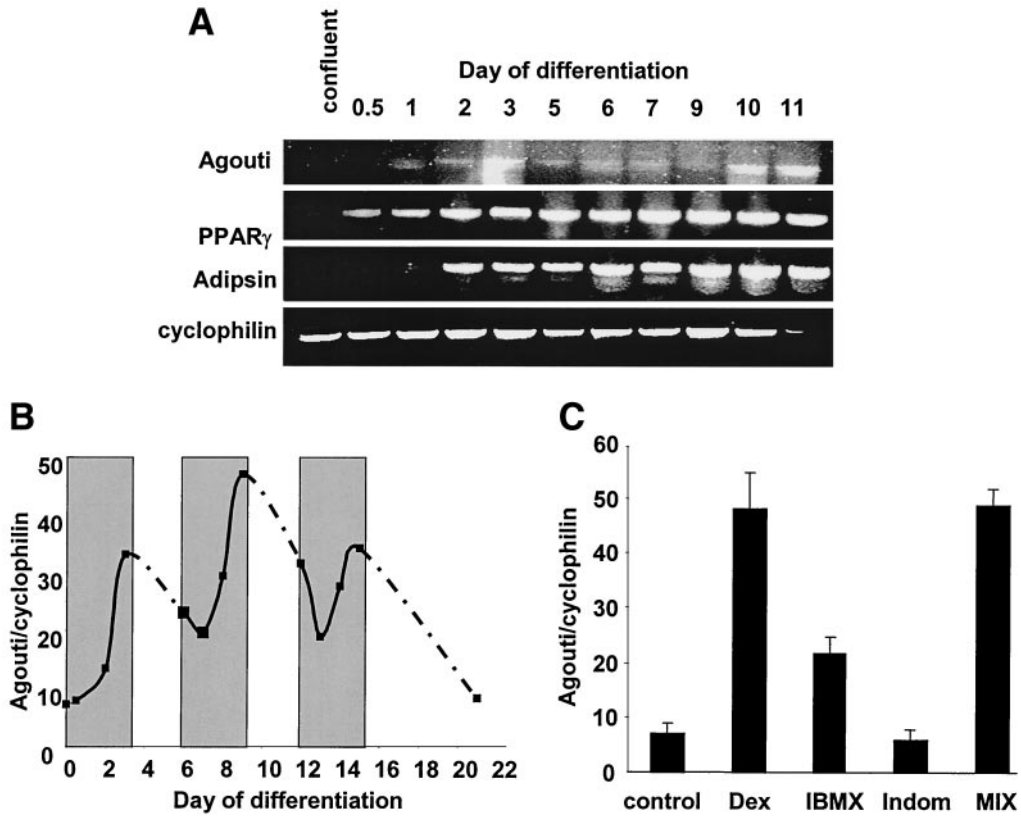

FIG. 2. Quantitation of agouti mRNA in human fat and adipocytes. *A*: RT-PCR analysis of agouti during the differentiation of human mesenchymal stem cells into adipocytes. PPARγ and adipsin were measured as markers of differentiation. *B*: Quanitative RT-PCR of agouti during human mesenchymal stem cell differentiation into adipocytes. Gray blocks show the times when cells were fed with adipocyte differentiation media (FBS + insulin + dexamethasone [Dex] + IBMX). At all other times, the cells were fed with FBS and insulin. *C*: Regulation of agouti by dexamethasone and IBMX in differentiated human adipocytes. Indom, indomethacin.

sex effect, with diabetic women having the highest overall agouti expression levels (Fig. 3*B*).

Cell culture experiments with either confluent preadipocytes or differentiated adipocytes demonstrated that glucocorticoids potently increase agouti expression. While elevated glucocorticoid levels may play a significant role in the development of obesity and type 2 diabetes (17), there are no definitive reports linking elevated circulating glucocorticoid levels to the obese/diabetic state. Additionally, we found no relationship between cortisol secretion (as measured by salivary cortisol) and agouti mRNA (data not shown). However, the enzyme 11β-HSD-1 can regulate intracellular glucocorticoid concentrations by regenerating active glucocorticoid (cortisol in humans, corticosterone in mice) from inactive cortisone and 11-dehydrocorticosterone. We observed that 11β-HSD-1 mRNA levels were significantly higher in the same cohort of diabetic subjects that had elevated agouti expression levels (Fig. 3*C*), demonstrating another correlation between glucocorticoids and agouti.

Having observed the elevated agouti levels in diabetic humans, transgenic mice and cell culture experiments were used to determine whether there were functional consequences of agouti expression in adipose tissue. Microarray data from 3-week-old aP2-agouti transgenic mice suggested a relationship between agouti expression and preadipocyte hyperplasia (data not shown). Therefore, we measured the DNA content from the epididymal fat pads of aP2-agouti transgenic mice to determine cell density. Both total DNA and DNA per milligram fat were significantly higher in the transgenic mice at 4 weeks of age, indicating that more preadipocytes were present in the fat pads of aP2-agouti mice (Fig. 4*A*). The increased amount of DNA in the fat of aP2-agouti mice led us to examine the effects of agouti and αMSH on preadipocyte proliferation in vitro. We observed that αMSH inhibited proliferation in both mouse and human preadipocytes (Fig. 4*B* and *C*). Our data also indicate that agouti has a slight stimulatory effect on proliferation, but it is clear that agouti blocked the inhibitory effects of αMSH. Interestingly, AGRP was not able to block the inhibitory effects of αMSH on proliferation (data not shown). Forskolin was also a potent inhibitor of proliferation, but agouti was not able to block the inhibitory effects of forskolin (data not shown). These data, taken together with the increased DNA content at 4 weeks of age in the aP2-agouti mice, suggest that αMSH and agouti regulate adipocyte proliferation in a reciprocal fashion.

Several studies have shown that PPARγ is an essential transcription factor for differentiation and maturation of adipocytes (18–24). Additionally, ectopic expression of PPARγ in nonprecursor fibroblast cells promotes lipid accumulation and confers the characteristics of mature adipocytes (25). Recent studies have also suggested that the STAT (signal transducers and activators of transcription) family of transcription factors may also be important in fat cells (26–29). We have previously shown that both agouti treatment of 3T3-L1 adipocytes and agouti expres-

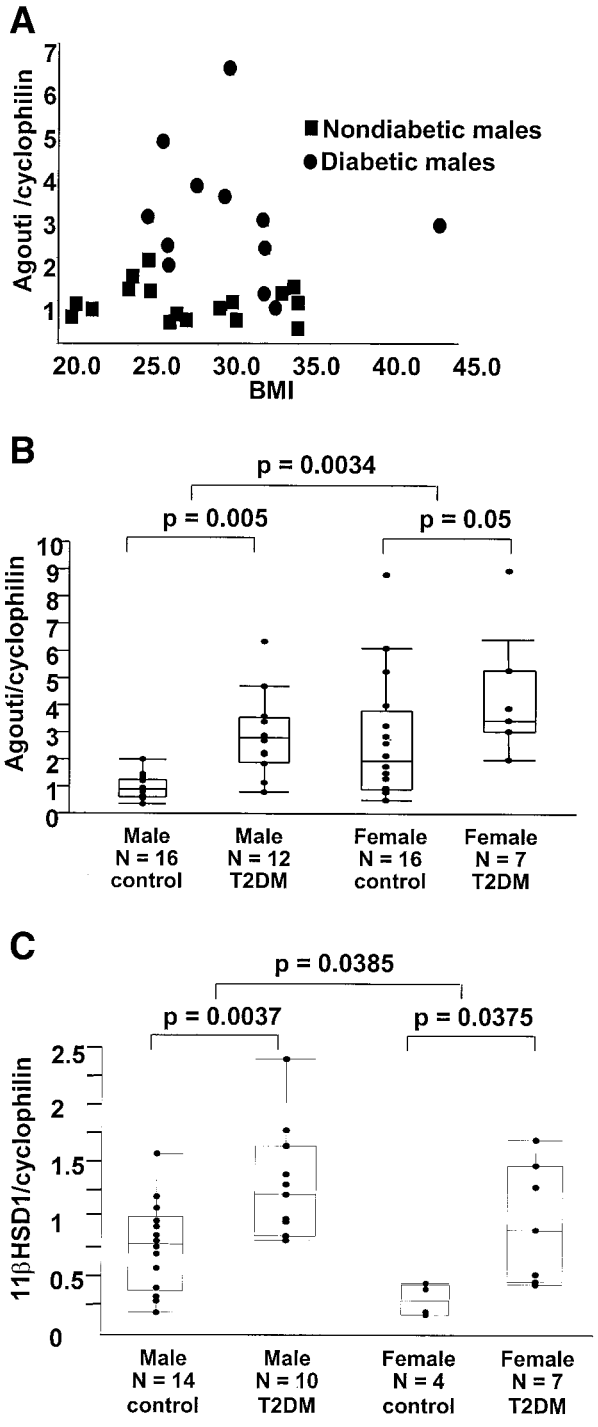

FIG. 3. Expression of agouti and 11β-HSD-1 mRNA in human subcutaneous adipose tissue. Agouti and 11β-HSD-1 mRNAs were measured as described in RESEARCH DESIGN AND METHODS and normalized to cyclophilin mRNA. *A*: There was no relationship between BMI and agouti mRNA, but agouti (*B*) and 11β-HSD-1 (*C*) mRNA are increased in diabetes. T2DM, type 2 diabetes.

sion in mouse adipose tissue increases STAT1, STAT3, and PPARγ protein levels (13). Since PPARγ is a key regulator of adipocyte differentiation and agouti induces PPARγ, we initiated experiments to address the ability of agouti to influence adipocyte differentiation. The levels of differentiation were measured by lipid accumulation in nontransgenic and BAP-agouti transgenic mice (30). Cells from the

TABLE 1
Variables of the study population

Variable	Nondiabetic control	Diabetic	*P* value
Sex (F/M)	8/17	7/12	NA
Race (black/white)	7/17	5/14	NA
BMI (kg/m^2)	29.12 ± 4.40	32.55 ± 5.23	0.01
Body fat (%)	30.10 ± 8.70	32.76 ± 8.99	NS
Waist (cm)	94.96 ± 12.30	108.05 ± 12.26	<0.001
Insulin (μU/ml)	11.70 ± 7.12	17.14 ± 9.42	0.01
Glucose (mg/dl)	95.40 ± 10.40	182.32 ± 48.55	<0.001
Triglyceride (mg/dl)	139.68 ± 63.34	230.16 ± 206.23	0.01
HbA_{1c} (diabetic subjects only)	N/A	7.71 ± 1.85	NA
Agouti/cyclophilin mRNA	1.56 ± 1.42	3.44 ± 1.90	<0.001
11β-HSD-1/ cyclophilin mRNA	0.61 ± 0.11	1.15 ± 0.10	<0.001

Data are means ± SD.

BAP-agouti mice were included in this study because the B-actin promoter will drive agouti expression in the preadipocytes. However, preadipocytes isolated from the aP2-agouti mice should not express agouti because the aP2 promoter is not active until late in adipocyte differentiation. Preadipocytes were isolated from 19- to 21-day-old mice by collagenase digestion and centrifugation. The preadipocytes were plated and grown to confluence and induced to differentiate as described in RESEARCH DESIGN AND METHODS. After 5 days of differentiation, the cells were stained with Oil Red-O to measure lipid accumulation. Only ~20% of the cells from wild-type mice had significant lipid accumulation (Fig. 5*A*). However, both the addition of 10 nmol/l COOH-terminal agouti peptide to wild-type preadipocytes (Fig. 5*B*) and the ectopic expression of agouti (Fig. 5*C*) caused a significant increase Oil Red-O staining. After photography, the Oil Red-O was extracted from the adipocytes and quantified (Fig. 5*D*), further demonstrating that agouti increases adipocyte differentiation. In a separate set of experiments, RNA was extracted from the confluent preadipocytes and daily after addition of the differentiation cocktail. PPARγ mRNA is already at high levels in the confluent preadipocytes isolated for the BAP-agouti transgenic mice. Additionally, aP2 mRNA appears earlier, and the band is more intense in the preadipocytes isolated from the transgenic mice. The data presented in Fig. 5 demonstrate that agouti treatment of preadipocytes from nontransgenic mice increases adipogenesis in vitro and that endogenous expression of agouti increases adipogenesis in vitro and in vivo.

DISCUSSION

The hypothalamic melanocortin system is an important regulator of body weight and appetite. The aim of these studies was to understand the role of the melanocortin system in adipose tissue. These studies reveal for the first time that expression of agouti mRNA is increased in diabetes, but not obesity, and that glucocorticoids upregulate agouti mRNA in vitro. They also reveal that there is increased expression of the cortisol-activating enzyme 11β-HSD-1 in diabetes. We also provide compelling data

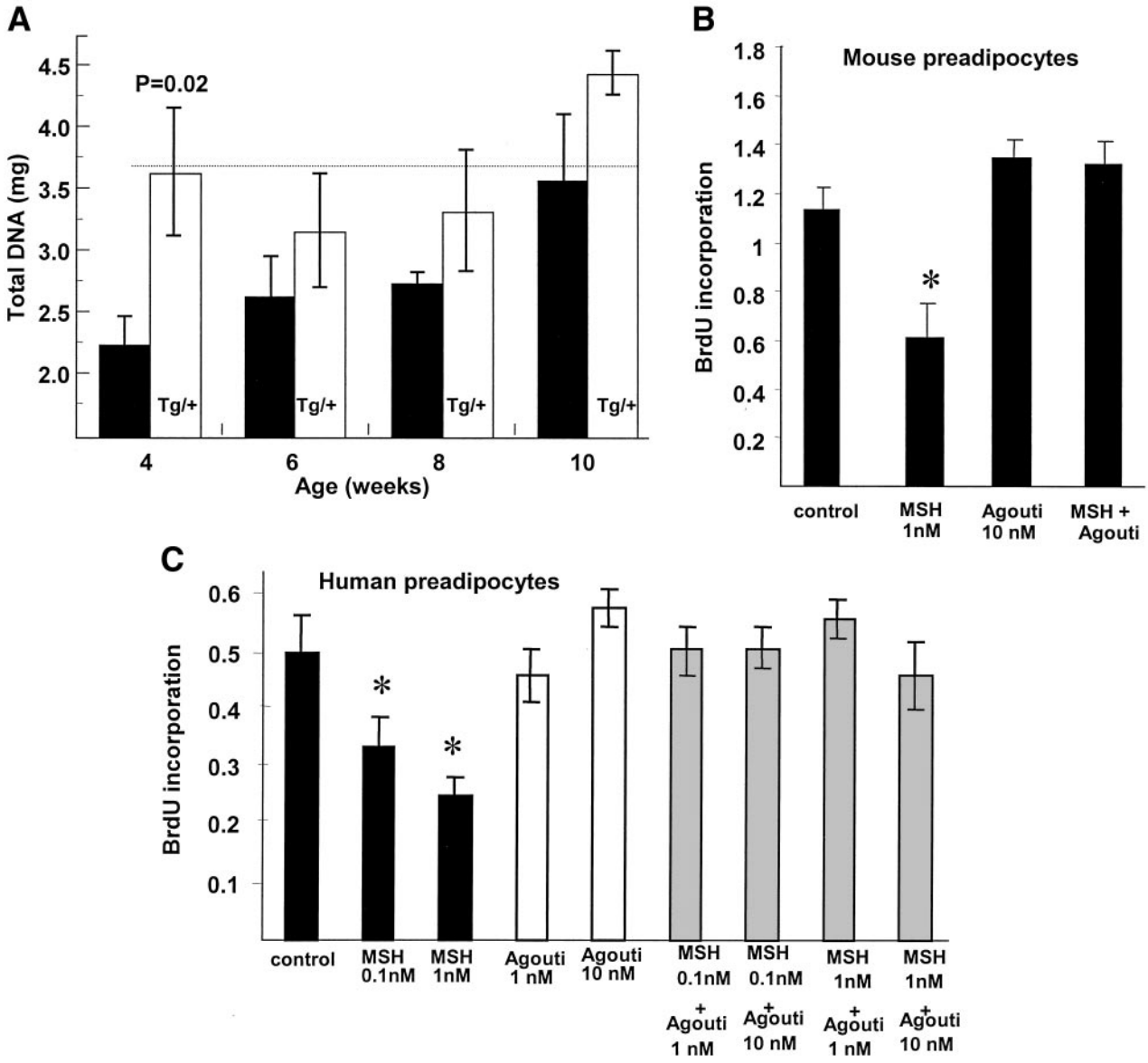

FIG. 4. Effects of agouti preadipocyte proliferation. *A*: As an estimate of cell density, DNA content was measured in aP2-agouti and nontransgenic mice at 4, 6, 8, and 10 weeks of age (n = 5–7 mice per data point). Data are presented as mean ± SE. *B* and *C*: Preadipocytes from mice and humans were isolated by centrifugal force after collagenase treatment. They were grown to confluence, trypsinized, and split equally into 96-well plates. The cells were then treated with the various combinations of αMSH and agouti overnight. The next morning, fresh media containing BrdU was added. Three hours later, BrdU uptake was measured by ELISA. Data are means ± SE from three separate experiments. *P < 0.05 vs. control values.

on the functional consequences of local agouti production; αMSH inhibits proliferation of human and murine preadipocytes, and agouti blocks this effect. Furthermore, agouti enhances differentiation of murine and human preadipocytes in vitro. Combined with previous data demonstrating that agouti upregulates PPARγ protein levels, this suggests that agouti is a paracrine factor in adipose tissue that can regulate both proliferation and differentiation of preadipocytes.

In addition to the effects of agouti on adipogenesis, there is considerable precedence for the regulation of mature adipocyte metabolism by agouti and POMC-derived peptides. The mRNA levels for fatty acid synthase and stearoyl-CoA desaturase, two key enzymes in de novo fatty acid synthesis and desaturation, respectively, were dramatically increased in obese (A^{vy}) mice relative to lean (a/a) controls (31). Additionally, treatment of fully differentiated 3T3-L1 adipocytes with recombinant agouti protein increased fatty acid synthase and stearoyl-CoA desaturase mRNA levels (31). A potential mechanism for the increased fatty acid synthase and stearoyl-CoA desaturase levels is that they are transcriptional targets of PPARγ. In opposition to the adipogenic effects of agouti, ACTH, αMSH, and β-LPH are potent lipolytic hormones, but considerable interspecies variability exists in the lipolytic response of adipocytes to melanocortins (32). The negative effects of melanocortins on adipogenesis (inhibition preadipocyte proliferation and increased lipolysis), combined with the positive effects of agouti on adipogenesis (increased PPARγ and differentiation), strongly suggest a coordinate control of the adipogenesis.

Agouti appears to antagonize αMSH and ACTH stimula-

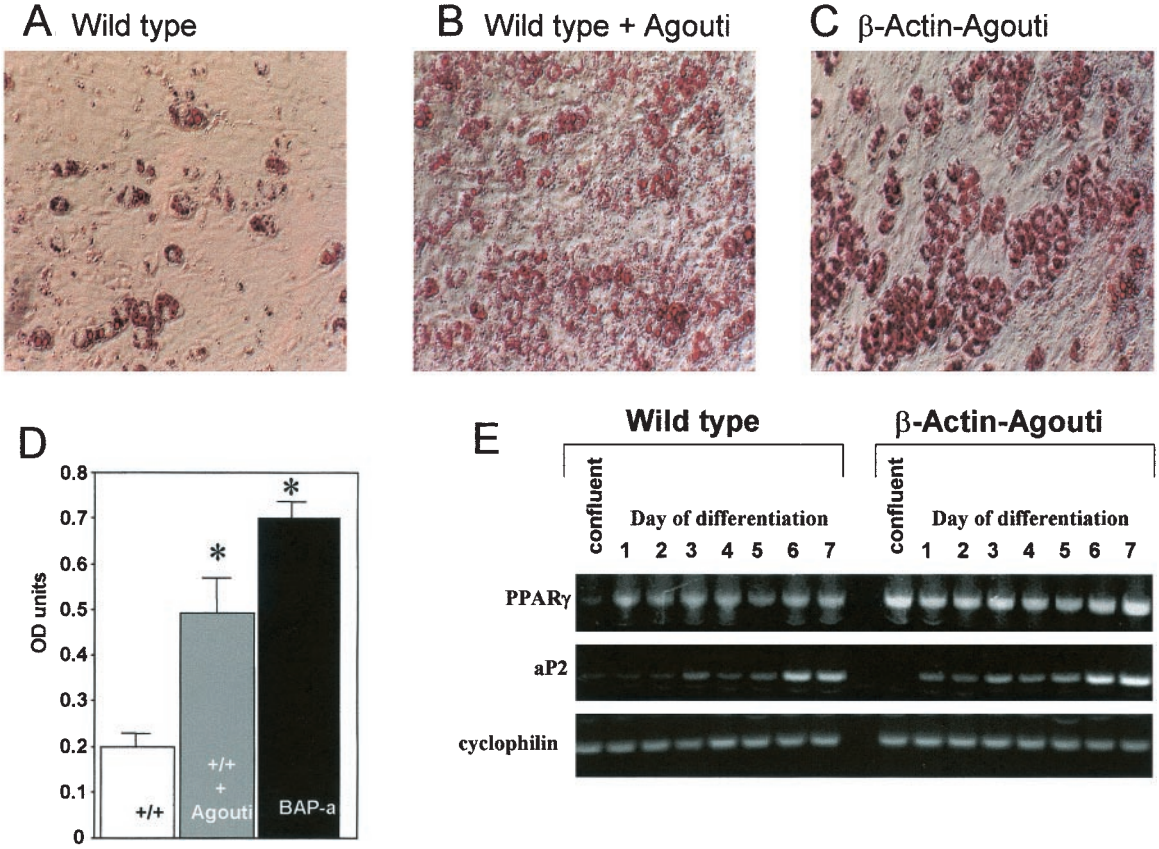

FIG. 5. Effects of agouti on adipocyte differentiation The preadipocytes from nontransgenic mice and BAP-agouti (BAP-a) transgenic were plated and grown to confluence in DMEM/F12 with 15% FBS. At confluence, the cells were trypinized and split equally into 12-well plates After reaching confluence the cells were induced to differentiate by changing the medium to DMEM containing 10% FBS, 0.5 mmol/l 3-isobutyl-1-methylxanthine, 1 μmol/l dexamethasone, and 1.7 μmol/l insulin. After 48 h, this medium was replaced with DMEM/F12 supplemented with 15% FBS, and cells were maintained in this medium for 2 more days until fixed and stained with Oil Red-O. *A*: Adipocytes from nontransgenic mice. *B*: Adipocytes from nontransgenic mice treated with 10 nmol/l agouti from the start of differentiation. *C*: Adipocytes from BAP-agouti mice. *D*: After photography the Oil Red-O was extracted from the adipocytes with isopropanol and quantitated via spectrophotometry. Data are presented as means ± SE and are from two independent experiments with four wells per treatment. *E*: In a separate experiment RNA was isolated from preadipocytes at confluency and after initiation of differentiation. RT-PCR was used to detect mRNA for PPARγ and aP2 as markers of differentiation. OD, optical density.

tion of cAMP at all five of the MCRs (33,34). αMSH stimulates cAMP levels at all the MCRs except MC2-R, and ACTH stimulates cAMP levels at all the MCRs (33). Previous studies demonstrated that mouse adipocytes express high-affinity binding sites for melanocortin peptides (35) and that MC1-R, MC2-R, and MC5-R are expressed in adipocytes and adipose tissue (16,36). In these studies, high expression levels of MC1-R and low levels of MC2-R were observed in the human preadipocytes (Fig. 1), and based on the inactivity of αMSH at MC2-R, it is logical to suggest that MC1-R is responsible for the antiproliferative effects of αMSH in preadipocytes.

It should be noted that the inhibitory effects of αMSH cellular proliferation are not restricted to preadipocytes. One of the hallmark phenotypes of the yellow obese syndrome is neoplasia and an increased incidence of tumors (reviewed by Wolff et al. [37] and Yen et al. [38]). Additionally, treatment of melanocytes with αMSH promotes terminal differentiation, resulting in an inhibition of proliferation, and agouti reverses αMSH effects and inhibits pigmentation and promotes proliferation (39–41).

We have previously shown that agouti increases PPARγ expression in fully differentiated 3T3-L1 adipocytes and in mice that ectopically express agouti in adipose tissue (12,13). The present study demonstrates that agouti stimulates adipocyte differentiation. Once again, the mechanism appears to be mediated through PPARγ, since PPARγ mRNA levels are as high in the confluent undifferentiated preadipocytes from agouti-expressing mice, as compared with the fully differentiated adipocytes.

Perhaps the most clinically relevant findings are the upregulation of human agouti by glucocorticoids and the elevated levels of agouti and 11β-HSD-1 mRNA in subcutaneous fat from patients with type 2 diabetes. Taken together, these data suggest a model where by the hyperglycemia of diabetes activates 11β-HSD-1 transcription and increased local production of cortisol, which then increases agouti mRNA. An alternate explanation is that a common factor on induces both agouti and 11β-HSD-1 transcription. Given that dexamethasone potently upregulates agouti in vitro, we hypothesize that the paracrine model depicted in Fig. 6 is a more probable explanation and warrants further investigation. 11β-HSD-1 causes visceral obesity when overexpressed in transgenic mice (42), but this effect is unlikely to be due to the upregulation of agouti, as murine adipose tissue does not express agouti. On the other hand, activity of 11β-HSD-1 in human adipose tissue is increased in visceral adipose tissue and has been

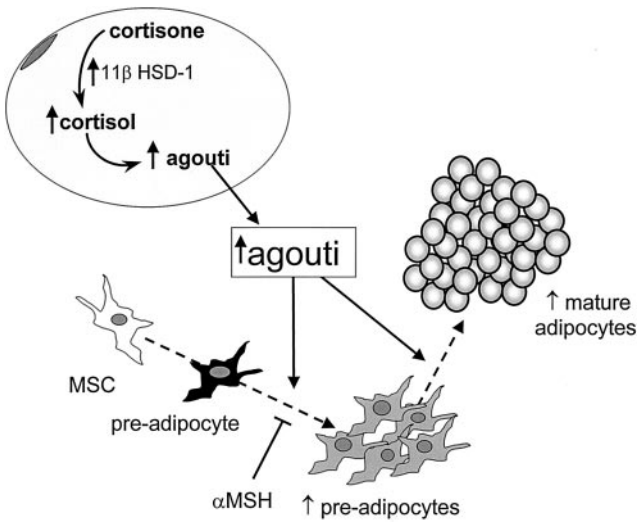

FIG. 6. Agouti regulation of adipogenesis: a working model. Increased cortisol, either through the local production of cortisol by 11β-HSD-1 or increased activity of the hypothalamic-ituitary-adrenal axis, leads to increased agouti transcription. Agouti increases both proliferation (by blocking the effects of αMSH) and differentiation (through the upregulation of PPARγ protein).

proposed as a cause of central adiposity (43). Our data suggest that agouti might play a role in central obesity as a downstream target of glucocorticoids. Cortisol might be produced locally via 11β-HSD-1 or the result of an overactive hypothalamic-pituitary-adrenal axis (44,45). Either way, increased exposure of adipose tissue to cortisol might serves as a stimulus for adipose tissue proliferation and differentiation, and these data suggest that the autocrine/paracrine production of agouti is a putative mediator of this effect.

When combined with previous studies in transgenic mice overexpressing agouti, the functional implications of increased agouti protein in adipose tissue are clear: agouti blocks the antiproliferative effects of αMSH and promotes preadipocyte differentiation (probably through the upregulation of PPARγ). Thus, increased agouti is potentially important as a paracrine factor in the regulation of adiposity, either during development or later in life. Given that type 2 diabetes may be influenced by impaired adipogenesis and "spillover" of lipid into skeletal muscle and liver (46), the upregulation of agouti mRNA in type 2 diabetes might be an attempt to proliferate and differentiate new adipocytes in order to store excess lipid in adipose tissue.

In conclusion, mRNA levels were significantly elevated in subjects with type 2 diabetes. The regulation of agouti in cultured human adipocytes revealed that insulin did not regulate agouti mRNA, whereas dexamethasone treatment potently increased the levels of agouti mRNA. This finding, as recapitulated in vivo in humans, where increased expression of the cortisol-activating enzyme 11β-HSD-1 was upregulated in concert with agouti in type 2 diabetes. Experiments with cultured human preadipocytes and with cells obtained from transgenic mice that overexpress agouti demonstrated that MCR signaling in adipose tissue regulates both preadipoycte proliferation and differentiation. Taken together, these results reveal that agouti can regulate adipogenesis at several levels and suggest that there are functional consequences of elevated agouti levels in human adipose tissue. The influence of MCR signaling on adipogenesis combined with the well-established role of MCR signaling in the hypothalamus suggest that adipogenesis is coordinately regulated with food intake and energy expenditure.

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