Genetic regulation of fatty acid synthetase expression in adipose tissue: overtranscription of the gene in genetically obese rats

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Abstract  We have investigated the molecular mechanism of the overactivity of fatty acid synthetase (FAS) in adipose tissue from the genetically obese Zucker rat. Purified FAS from lean and obese rat adipose tissues displayed kinetics constants, molecular weight, and immunological properties that were identical. Western blot analysis revealed that FAS overactivity in obese versus lean rat adipose tissue was paralleled by a proportionate increase in FAS mass, i.e., 4-fold increase in suckling normoinsulinemic 16-day-old pups and 25-fold in weaned hyperinsulinemic 30-day-old rats. The determination of absolute FAS mass disclosed that FAS was quantitatively a major protein in obese rat adipose tissue accounting for 13% of cytosolic proteins versus 2% in lean rat at 30 days of age. FAS hyperabundance could be ascribed to an increased relative rate of FAS synthesis that was 6-fold higher in obese than in lean rat adipose tissue. Northern blot analysis demonstrated that FAS mRNA levels in obese rats were increased 4- and 14-fold over those of lean rats at 16 and 30 days of age, respectively, in very close proportion to the 3- and 15-fold increases in FAS gene transcription rates revealed by nuclear run-on assays. Southern analysis of genomic DNA did not allow for detecting amplification or any major structural changes in the FAS gene.

It is concluded that FAS overactivity, shown here to be a life-long and general feature of all adipose tissue sites in the obese rat, arises primarily from FAS gene overtranscription.

Therefore, the unraveling of the biochemical defect in fatty rats might prove to be useful to a better understanding of human obesity where genetic factors also play a major role (2).

Lean and obese Zucker rats are morphologically identical until about 1 week of age when the first detectable signs of the obese phenotype, the enlargement of fat depots and fat cell sizes, occur (3). Any candidate for fat should therefore be expressed in obese rats at or before this stage of development. Recently we observed that adipose tissue fatty acid synthetase (FAS), an enzyme whose hyperactivity is a key feature of postweaning obese rats (4), met this criterion. By 7 days of age, i.e., well in advance of the onset of hyperphagia and hyperinsulinemia that develop during weaning (3, 4), FAS was already increased by 50% in preobese pup adipose tissue (5). Fatty acid synthetase is a multifunctional enzyme that plays a central role in de novo lipogenesis by catalyzing all the reactions in the conversion of acetyl CoA and malonyl CoA to long chain fatty acids. There has been ample evidence that fatty acid synthetase is the rate-limiting step for overall lipogenesis under a variety of physiopathological conditions (6, 7). Therefore, the overexpression of FAS activity in adipose tissue from mutant pups is likely to play a critical role in the development of the obese phenotype, suggesting FAS as a plausible candidate gene for the fatty mutation.

The obesity of the Zucker rat, first described by Zucker and Zucker (1), is inherited as an autosomal recessive mutation. It is thought to be initiated by a single gene defect (fa) the nature of which remains totally unknown. These rats develop a syndrome that closely resembles human obesity. Hyperphagia, hyperinsulinemia and normoglycemia, hypertriglyceridemia, hypertrophy and hyperplasia of fat cells as well as development of type 2 diabetes and renal complications are common features to both species.

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changes or increased FAS mass; 2) compare the rates of FAS synthesis and the levels of FAS mRNA and nascent mRNA transcripts in adipose tissues from lean and obese Zucker rats; and 3) examine whether major structural alterations in the FAS gene, as assessed by Southern analysis of genomic DNA, were present in the mutant rat.

MATERIALS AND METHODS

Animals

Obese (fa/fa) rats and their lean littersmates were bred in our laboratory from pairs originally provided by the Harriet G. Bird Memorial Hospital, Stow, MA. Known heterozygous Fa/fa lean females and obese fa/fa males were mated. From this mating, 50% of the litter is expected to be obese and 50% lean. Pups were routinely separated from their mothers at 28 days of age, and fed ad libitum on a standard laboratory diet (AO4, UAR, France). Rats at ages as stated in legends were killed by decapitation at 9-10 AM. The genotype of 16-day-old pups was identified as previously described (8). Adipose tissues were rapidly excised and processed according to the following experimental procedures.

Determination of FAS activity

Adipose tissues were homogenized in ice-cold 0.25 M sucrose, 1 mM dithiothreitol, 1 mM EDTA, 0.1 M phenylmethylsulfonylfluoride, pH 7.4, and cytosolic fractions were obtained by centrifugation (1 h, 10,000 g at 4°C). FAS activity (EC 2.3.1.85) was determined immediately by measuring malonyl CoA-dependent oxidation of NADPH at 35°C (9). One unit of enzyme activity represents 1 μmol of NADPH oxidized per min at 35°C. Protein concentration was determined by the method of Bradford (10) using bovine serum albumin as standard.

FAS purification

Adipose tissues from six to ten rats 6 weeks old, were pooled. Obese rats were maintained on a standard diet, but in lean rats, before they were killed, higher levels of FAS activity were induced by a nutritional treatment consisting of 48 h fasting followed by 3 days of refeeding on a high carbohydrate diet. Tissues were homogenized in 3 volumes of 50 mM potassium phosphate, pH 7.5, containing 0.2 mM sucrose, 20 mM NaF, 1 mM EDTA, 15 mM β-mercaptoethanol, 0.2 mM phenylmethylsulfonylfluoride, 10 mM benzamidine, 1 μg/ml pepstatin and leupeptin. FAS purification was performed as described by Ramakrishna and Benjamin (11), and resulted in a single band protein on SDS PAGE (Fig. 1A).

Preparation of FAS antibody

Antibody to rat adipose tissue FAS was raised in New Zealand rabbits by one intramuscular injection of 500 μg of purified FAS in complete Freund’s adjuvant, repeated twice at 2-week intervals but with incomplete adjuvant. Three weeks later, the immunoglobulins were purified from serum by ammonium sulfate precipitation and chromatography on DE 52 (Pharmacia). Preimmune serum was processed identically.

Western blot analysis of FAS

Adipose tissue cytosolic fractions were electrophoresed on a 7% SDS-polyacrylamide gel (12), electroblotted, and incubated with a 1/200 dilution of an anti-FAS antibody. The immune complex was detected by autoradiography after incubation with 125I-labeled protein A (Amersham). Bands corresponding to FAS were then excised from the membrane, counted, and total counts were corrected for background. Absolute quantities of FAS protein were determined by comparison with standard curves of purified FAS obtained on the same gel.

Immunoprecipitation and determination of relative rate of synthesis of FAS

Thirty-day-old lean and obese Zucker rats were killed 3 h after an intravenous injection of 100 μCi of [35S]methionine (1000 Ci/mmol). A labeling time of 3 h is short relative to the half-life of FAS (approximately 40 h (6)), and it can be assumed that the loss of labeling that occurs through degradation is negligible. This was verified in pilot experiments that showed that FAS labeling increased steadily from 2 to 3 h. Adipose tissue was excised and the cytosolic fraction was obtained as described above. [35S]Methionine incorporation into total proteins was determined after precipitation with 10% trichloroacetic acid followed by extensive washings. FAS was immunoprecipitated with an excess of anti-FAS IgG for 16 h at 4°C in 0.2 M potassium phosphate buffer containing Triton X-100 and deoxycholate (each at a final concentration of 0.5%). Antigen–antibody complex was isolated by centrifugation after addition of Pansorbin, washed extensively until no residual label could be detected in the
supernatant, and counted. Counts were corrected for non-specific trapping, estimated by running parallel samples using pre-immune IgG which did not precipitate any FAS-like protein on SDS PAGE (Fig. 1B). Results were expressed as relative rate of synthesis: radioactivity incorporated into FAS relative to the radioactive incorporated into total protein, in order to correct for possible differences in amino acid permeability and pool size between the two genotypes.

RNA preparation and Northern blot analysis

Total RNA from inguinal adipose tissue of lean and obese Zucker rats was isolated as described by Cathala et al. (13), analyzed by Northern blotting after electrophoresis on 1.5% agarose-0.66 M formaldehyde gels and transfer onto nylon membranes (HYbond N, Amersham). Hybridization with $^{32}$P-labeled cDNA insert (pFAS7, kind gift from A. G. Goodridge) encoding for rat liver obese Zucker rats was isolated as described by Cathala et al. (13), analyzed by Northern blotting after electrophoresis on 1.5% agarose-0.66 M formaldehyde gels and transfer onto nylon membranes (HYbond N, Amersham). Hybridization with $^{32}$P-labeled cDNA insert (pFAS7, kind gift from A. G. Goodridge) encoding for rat liver

Nuclear run-on transcription assay

Nuclei were isolated from adipose tissue of lean and obese Zucker rats aged 16 or 30 days. Tissues were homogenized (homogenizer 43, the Virtis Company, Gardiner, NY) in 5 ml/g of ice-cold lysis buffer containing 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl$_2$, 0.5% v/v NP40. The crude homogenate was filtered through a 20 µm Nylon mesh, and nuclei were pelleted twice by centrifugation (500 g, 5 min at 4°C). The nuclear pellet was layered onto a 5-ml cushion of glycerol storage buffer, checked under a light microscope, and stored at $-70^\circ$C in small aliquots.

The protocol used for nuclear run-on transcription experiments was as described by Zechner et al. (16), with no modification. Under these conditions, $^{32}$P-labeled CTP incorporation into labeled transcripts was linear with respect to time and amount of DNA.

Hybridizations, carried out for 3 days at $42^\circ$C, were performed in DNA excess with labeled nuclear RNA (1-2 $10^6$ dpm/ml) from lean or obese rats and an equal amount of unlabeled nuclear RNA from the opposite genotype. The addition of unlabeled RNA ensured that the hybridization conditions in both reactions were identical, thus allowing direct comparison of the signals for lean and obese rats. Quantification of autoradiograms was performed by densitometry and the results were corrected for non-specific hybridization (pBR322).

Southern blot analysis of genomic DNA

Genomic DNA from lean and obese Zucker rats was extracted, digested by restriction enzymes, and analyzed by Southern blotting as described (17), using pFAS7 probe.

RESULTS

The possibility that FAS hyperactivity in mutant rats was due to changes in structural properties of the enzyme was examined by comparing the electrophoretic mobility, the kinetic constants, and the immunological reactivity of the purified enzyme from lean and obese rats. Fig. 1A shows that, after purification, adipose tissue FAS migrated as a single band of approximately 220,000 daltons molecular mass whatever the rat genotype. Purified enzymes had similar specific activities (average of three preparations 1650 and 1620 nmol of NADPH oxidized/min per mg protein at 35°C in lean and obese rats, respectively). The apparent $K_m$ values of the purified enzymes for acetyl CoA and malonyl CoA were not significantly different between the two groups of rats ($K_m$ for acetyl CoA: 26.6 ± 6 µM vs. 22.4 ± 6.4 and $K_m$ for malonyl CoA: 49.8 ± 8.2 µM vs. 44.7 ± 4.6 in lean vs. obese rats, three determinations; $K_m$ for NADPH was not determined). In addition, the immunoreactivity of FAS as assessed by immunotritration was not different between the two groups of rats (data not shown).

The amount of FAS was quantified in adipose tissue from lean and obese rats by Western blot analysis. Determinations of absolute enzyme mass were made possible by the availability of highly purified FAS and monospecific antibody enabling us to construct standard curves as shown in Fig. 2. These studies were carried out in inguinal adipose tissues (the only site to be developed at early ages) from suckling (16 days old) and post-weaning (30 days old) rats, in order to assess the genotype effect independently of the frank hyperinsulinemia that develops in the mutants at weaning. Obese pups 16 days old displayed subcutaneous adipose tissues two times heavier than those of their lean littermates. FAS activity and mass were both increased 4-fold over lean rat values (Table 1). In contrast, the increase in insulin concentration in obese versus lean pups did not reach statistical significance, suggesting that FAS expression and insulin levels were independent variables. This conclusion was further supported by plotting FAS activity against insulin level using the individual data from Table 1 plus those from an additional litter containing four lean and five obese pups whose FAS activity was: 8.7 µU/mg protein versus 41.6, $P < 0.001$, and insulinemia: 17.1 µU/ml versus 32.9, NS in lean versus obese, respectively. The grouping of these 18 individual data disclosed that there was no relationship between FAS activity and insulin concentration in suckling Zucker pups ($r = 0.18$). After weaning, obese rat adipose tissues displayed a 27-fold increase in FAS activity together with a 25-fold increase in FAS mass over lean rat
adipose tissues. It is noteworthy that the changes in FAS activity and mass on a per cell basis would be identical to those observed on a tissue basis, since the number of fat cells per tissue is the same in lean and obese rats at these early ages (18). Furthermore, this study shows that FAS is a major protein in adipose tissue from post-weaning obese rats amounting to more than 13% of total cytosolic proteins versus only 2% in lean rat adipose tissue.

As previously reported (4), a dramatic 20-fold elevation in FAS activity occurs in inguinal adipose tissue of lean rats between suckling and weaning (Table 1). The present data demonstrate that this is achieved through a proportionate increase in protein enzyme, documenting for the first time the molecular basis of the large “weaning” effect on FAS activity in adipose tissue. Table 1 also shows that the “weaning” effect is considerably amplified in obese Zucker rats where enzyme activity and mass are increased 140-fold and 120-fold, respectively, from 16 to 30 days of age.

In order to clarify the mechanism involved in the overabundance of FAS protein in adipose tissue from obese as compared to lean rats, the relative rates of FAS synthesis were examined in 30-day-old animals using the pulse-labeling method. Table 2 shows that the labeling of FAS in obese rats is increased 14-fold over that of lean rats whereas the labeling of total soluble proteins is only twice higher, providing clear evidence of a selective stimulation of FAS synthesis in the mutant rat. When expressed as relative rates, FAS synthesis was found to be 6-fold higher in obese than in lean rats, which interestingly is the same fold increase as that observed for the specific enzyme activity (see Table 1). Table 2 also reveals that FAS synthesis is a rather minor process in lean rat adipose tissue, accounting for less than 3% of soluble protein synthesis. In contrast, FAS synthesis in obese rat adipose tissue represents 17% of the synthesis of total soluble proteins, a finding in excellent agreement with the above conclusion that FAS is a major cytosolic protein in adipose tissue from post-weaning obese rats.

**TABLE 1.** FAS activity and mass in inguinal adipose tissue from lean and obese Zucker rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>16 Days Old</th>
<th>30 Days Old</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lean (3)</td>
<td>Obese (6)</td>
</tr>
<tr>
<td>Tissue weight (mg)</td>
<td>109 ± 4.2*</td>
<td>251 ± 4.6</td>
</tr>
<tr>
<td>FAS activity (mU/mg protein)</td>
<td>3.5 ± 1.15*</td>
<td>7.8 ± 0.86</td>
</tr>
<tr>
<td>FAS activity (mU/tissue)</td>
<td>6.3 ± 1.67*</td>
<td>23.5 ± 2.12</td>
</tr>
<tr>
<td>FAS mass (µg/tissue)</td>
<td>4.4 ± 0.95*</td>
<td>17.9 ± 2.8</td>
</tr>
<tr>
<td>µg FAS ×100/µg cytosolic prot</td>
<td>0.23 ± 0.02*</td>
<td>0.65 ± 0.05</td>
</tr>
<tr>
<td>Insulinemia (µU/ml)</td>
<td>34.5 ± 6.2</td>
<td>44.8 ± 4.5</td>
</tr>
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Values are given as means ± SEM with the number of independent determinations in parentheses. Statistical differences between the two genotypes were assessed by Student's t test (*, P < 0.001); nd, not determined.

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TABLE 2. Relative rates of FAS synthesis in adipose tissue of lean and obese Zucker rats (30 days old)

<table>
<thead>
<tr>
<th></th>
<th>Lean (4)</th>
<th>Obese (5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dpm into total proteins</td>
<td>102734 ± 21245</td>
<td>229544 ± 20890</td>
</tr>
<tr>
<td>dpm into FAS</td>
<td>2754 ± 257</td>
<td>38394 ± 5908</td>
</tr>
<tr>
<td>dpm FAS/100 dp protein</td>
<td>2.96 ± 0.54</td>
<td>16.70 ± 1.83</td>
</tr>
</tbody>
</table>

Values are means ± SEM with the number of animals studied in parentheses. In this experiment, the ratio of specific FAS activity (U/mg protein) in obese to lean rats was 6.

In order to document further the mechanism of the genotype effect on FAS expression, a Northern analysis of total RNA from lean and obese rat adipose tissues was performed at both 16 and 30 days of age. As it has been previously reported (19, 20), we observed that two mRNA species were present in rat adipose tissue (Fig. 3). There was no genotype effect on the pattern of migration of FAS mRNAs but the signals from obese rat adipose tissues were dramatically stronger than those from lean rats. The stimulatory genotype effect was specific for FAS mRNA as no change was detectable in adipsin mRNA levels (21).

The results depicted in Fig. 4A show that FAS mRNA levels in obese rats were increased about 4- and 14-fold over those of lean rats at 16 and 30 days of age, respectively.

The dramatic increase in FAS mRNA in mutant rat adipose tissue could be achieved through a genotype effect on mRNA stability or/and FAS gene transcription. In order to answer this question we carried out transcription run-on analysis in isolated adipose tissue nuclei from lean and obese rats. As Fig. 4B demonstrates, the increase in FAS transcription rates was commensurate with the increase in mRNA levels in suckling as well as in weaning animals. Moreover, FAS gene transcription was specifically enhanced in obese rats as compared to lean rats since there was no genotype effect on PEPCK gene transcription (Fig. 5).

The possibility that FAS gene overtranscription in mutant rats might result from large gene modifications was

![Fig. 3. FAS mRNA content in adipose tissue of lean (L) and obese (O) Zucker rats. Adipose tissues were pooled from four to six pups at 16 days of age and used individually at 30 days of age for RNA extraction. Northern blot analysis of total RNA (60 μg) was performed as described in Materials and Methods, using pFAS7 encoding for FAS and pAD20 encoding for adipsin. The autoradiogram shown is representative of four independent experiments.](image)

![Fig. 4. Effect of the fatty genotype on FAS mRNA levels (arbitrary densitometric units/60 μg total RNA) in adipose tissue (A) and FAS gene transcription rates in nuclei (B). Adipose tissue RNA and nuclei were isolated as described. The results are expressed as fold-increase over the values in lean rats. Values are means ± SEM of four independent experiments.](image)
Fig. 5. Nuclear run-on transcription assay in lean and obese Zucker rats. Signals were obtained after hybridization of recombinant cDNA encoding for FAS (pFAS7), PEPCK (pPCK10), and nonrecombinant control vector (pBR322) with labeled transcripts from lean (upper dots) or obese (lower dots) rats. Results are representative of four independent experiments.

Fig. 6. Southern blot analysis of genomic DNA of lean (L) and obese (O) Zucker rats, digested with four restriction enzymes. Blots were hybridized with pFAS7 cDNA. The sizes (in kb) of DNA markers are indicated on the right vertical axis.

DISCUSSION

The hypothesis that FAS might be the site of the primary genetic lesion of the fatty mutation prompted us to delineate the molecular mechanism(s) underlying FAS overactivity in the mutant rat. Our data show that FAS enzyme molecules in adipose tissue of fa/fa rats exhibit catalytic properties, immunoreactivity, and electrophoretic mobility indistinguishable from the FAS molecules present in adipose tissue of lean littermates. These observations strongly suggest that FAS overactivity in adipose tissue of obese rats is mediated by an increase in enzyme mass rather than by structural changes. The finding of an overabundance of FAS proportionate to the increase in enzyme activity establishes that the fatty mutation exerts a control on FAS activity through an effect on FAS content. One possibility is that the rate of FAS synthesis is increased in obese rat adipose tissue. This is supported by our observation that the extent of changes in labeled methionine incorporation into FAS appears to account for the extent of changes in FAS mass. Consistent with increased FAS synthesis rates, we found large increases in FAS mRNA accumulation in obese rat adipose tissue. Investigating this alteration further, we were able to show that FAS gene transcription rates, as assessed in the nuclear run-on assay, were increased in close proportion to FAS mRNA changes, in suckling as well as in weaning pups. This provides strong evidence that the transcriptional level was the primary defective step responsible for FAS overexpression in this genetic obesity. A limitation of our study is the lack of direct assessment of the role of post-transcriptional regulatory mechanisms such as FAS mRNA or protein stability. However, from our data it can be inferred that the role, if any, played by those mechanisms is of minor importance in this model.

This study, which provides the first information on the absolute FAS mass in rat adipose tissue under different physiopathological conditions, reveals that FAS becomes one major cytosolic protein in genetic obesity. The present data disclose also that FAS overexpression, shown previously to emerge in the neonate preobese pup (5), is a lifelong and general feature of all adipose tissues sites. The
pathogenetic role of this alteration in the development and maintenance of the obese phenotype may be easily understood. FAS overactivity will act as a metabolic drive, channeling dietary substrates into adipose tissue fat stores; this would happen whatever the food intake level of the rats, in good keeping with the well-established observation that hyperphagia is not a necessary precondition for the development of Zucker rat obesity (3, 22, 23).

The shunting of nutrients into adipose tissue would entail two physiological consequences, a compensatory hyperphagia and a secondary hyperinsulinemia.

The main finding of this study, that the fatty mutation activates the transcription of FAS gene in adipose tissue, raises new questions as to the underlying mechanism(s). By Southern blot analysis we have excluded gene amplification and large rearrangements of the gene. However, this does not preclude the possibility of specific cis-acting regulatory sequences in the obese rat FAS gene. These sequences would drive tissue-specific expression of the gene, since liver in obese suckling pups expresses normal FAS activity level (4). Alternatively, rather than being the site of the mutation, the FAS gene could be the target of the mutated gene. It can be speculated that the  fa  gene either produces an activator or suppresses an inhibitor acting in  trans  on the FAS gene. The elucidation of these issues, under current investigation in our laboratory, should provide valuable insights into the nature of this genetic disorder.

The mediation of the genotype effect on FAS transcription through hormonal changes, as yet unidentified, cannot be ruled out at the present time. The 5' flanking region of the FAS gene in both goose (24) and rat (25) has been shown to contain putative response elements for sev-

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**Fig. 7.** Tissue weight (B) and FAS activity (A) of three adipose tissue sites in lean and obese Zucker rats from 7 to 83 weeks of age. One unit of enzyme activity represents 1 μmol of NADPH oxidized per min at 35°C.
eral hormones. The presence of a triiodothyronine regulatory element has been demonstrated (24) that explains the well-documented T3 positive effect on FAS gene transcription (26, 27). However, the observation that serum T3 levels are not increased, but rather decreased, in obese as compared to lean Zucker rats (28), does not support a role for this hormone in the effect described here. Insulin, though its blood level may be slightly increased in suckling obese pups, is not a good candidate either. We provide evidence here that the expression of adipose tissue FAS activity and blood insulin level are independent variables in suckling pups. This establishes the insulin-independence of the fatty genotype effect on FAS transcription in these pups. However, the implication of this hormone in the dramatic amplification of the genotype effect observed after weaning (and the well-documented development of a frank hyperinsulinemia) remains a possibility. It has been reported that insulin in vivo exerted a transcriptional activation of the FAS gene in mouse liver (29). In contrast, in chick embryo hepatocytes in culture, insulin was ineffective on FAS gene transcription when added alone, but amplified the transcription caused by T3 (26). Nothing is known about the regulation of FAS gene transcription in rat adipose tissue, and to our knowledge this is the first report of a transcriptional control of the FAS gene in this tissue. Very recently, FAS mRNA levels in adipose tissue have been shown to depend upon the fat content of the diet, but the level of this dietary control was not documented (30).

In conclusion, in suckling as well as in weaning genetically obese rats, adipose tissue FAS overactivity is due to an overabundance of structurally normal FAS molecules that results from an oversynthesis of the enzyme mediated by an accumulation of FAS mRNA. The responsible defective step is an overtranscription of FAS gene, the mechanism(s) of which remain(s) to be elucidated. These findings should be beneficial to ongoing research on genetic obesity and provide basis for future investigation.

We wish to thank Drs. A. G. Goodridge, R. W. Hanson, and H. Green for their gifts of cDNA probes encoding for FAS, PECK, and adipin, respectively.

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