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ARTICLES

Adipogenic Potential of Human Adipose Derived Stromal Cells From Multiple Donors is Heterogeneous

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Abstract The current study was done to assess if heterogeneity existed in the degree of adipogenesis in stromal cells (preadipocytes) from multiple donors. In addition to conventional lipid-based methods, we have employed a novel signal amplification technology, known as branched DNA, to monitor expression of an adipocyte specific gene product aP2. The fatty acid binding protein aP2 increases during adipocyte differentiation and is induced by thiazolidinediones and other peroxisome proliferator activated receptor γ ligands. The current work examined the adipogenic induction of aP2 mRNA levels in human adipose tissue stromal cells derived from 12 patients (mean age ± SEM, 38.9 ± 3.1) with mild to moderate obesity (mean body mass index ± SEM, 27.8 ± 2.4). Based on branched DNA technology, a rapid and sensitive measure of specific RNAs, the relative aP2 level in adipocytes increased by 679 ± 93-fold (mean ± SEM, n = 12) compared to preadipocytes. Normalization of the aP2 mRNA levels to the housekeeping gene, glyceraldehyde phosphate dehydrogenase, did not significantly alter the fold induction in a subset of 4 patients (803.6 ± 197.5 vs 1118.5 ± 308.1). Independent adipocyte differentiation markers were compared between adipocytes and preadipocytes in parallel studies. Leptin secretion increased by up to three-orders of magnitude while measurements of neutral lipid accumulation by Oil Red O and Nile Red staining increased by 8.5-fold and 8.3-fold, respectively. These results indicate that preadipocytes isolated from multiple donors displayed varying degrees of differentiation in response to an optimal adipogenic stimulus in vitro. This work also demonstrates that branched DNA measurement of aP2 is a rapid and sensitive measure of adipogenesis in human stromal cells. The linear range of this assay extends up to three-orders of magnitude and correlates directly with independent measures of cellular differentiation. J. Cell. Biochem. 81:312–319, 2001.

Key words: adipose; stromal cells; heterogeneous; branched DNA; aP2

Mature adipocytes, the primary components of white adipose tissue are responsible for energy storage; this function is subject to hormonal control. In addition, mature adipocytes themselves secrete leptin, the *obese* gene product. Consequently adipose tissue is emerging as an endocrine organ capable of releasing its own hormonal product [Zhang et al., 1994; Pelleymounter et al., 1995].

To date, in vitro systems have been used to study adipocyte differentiation. These include both preadipose cell lines and primary cultures of adipose-derived stromal vascular precursor cells. Primary adipocytes have been successfully cultured from various species including human and offer several advantages over the preadipose cell lines. First, primary cells are diploid and thus reflect the in vivo situation better than aneuploid cell lines [Gregoire et al., 1998; Deslex et al., 1987]. Second, primary cells can be isolated at various stages of development and from different depots. The latter is especially important since significant physiological differences have been correlated with depot variation [Masuzaki et al., 1995; Lacasa et al., 1997a, 1997b; Montague et al., 1998].

It is possible that primary cultures will reveal some degree of heterogeneity among the stromal vascular preadipose cell populations. The purpose of the present study was to assess adipogenesis in stromal cells (preadipocytes) from multiple donors. In addition to conventional lipid-based methods, we have employed a novel signal amplification technol-

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ogy, known as branched DNA, to monitor expression of an adipocyte specific gene product aP2 [Spiegelman et al., 1983; Bernlohr et al., 1984, 1985; Hunt et al., 1986; Phillips et al., 1986].

aP2, also known as P442, is a 132 amino acid fatty acid binding protein with a molecular weight of 14.6 kDa [Spiegelman et al., 1983; Bernlohr et al., 1984, 1985; Hunt et al., 1986; Phillips et al., 1986]. Cyclic AMP [Yang et al., 1989], fatty acids [Amri et al., 1991], and ligands for peroxisome proliferator activated receptors all regulate aP2 gene at the transcriptional level [Ross et al., 1990; Graves et al., 1991, 1992a, b; Harris and Kletzien, 1994; Tontonoz et al., 1994; Kletzien et al., 1992]. The thiazolidinediones and other PPARγ ligands have proven to be potent anti-diabetic drugs [Spiegelman, 1998; Lehmann, 1995]. Consequently, methods to monitor intracellular aP2 mRNA levels have potential utility as screening strategies to identify novel anti-diabetic compounds and PPAR γ ligands.

The branched DNA (bDNA) technology employs signal amplification to detect and measure cellular mRNAs [Nolte, 1999]. This technique allows direct, quantitative detection of nucleic acid molecules without the necessity of amplifying the mRNA of interest. Unlike transfection methodologies which use artificial promoter-reporter constructs, bDNA examines the actions of the entire native promoter sequence within the cell's own nucleus following a physiological or pharmacological stimulus.

METHODS

Tissue Preparation

All subcutaneous adipose tissue specimens were obtained from liposuction surgeries with informed patient consent. Primary cultures were prepared as described in Halvorsen et al. (in press).

Proliferation Assay

Stromal cells from 12 different donors were cultured in micro titer plates (tissue culture grade, 96 wells, flat bottom well) at a seeding density of 3×10^4 cells/cm^2 in a 100 μl volume/well of either preadipocyte or adipocyte medium as previously described (Halvorsen, in press). The plates were incubated in a humidified atmosphere (37°C, 5% CO_2) for 7–10 days with feeding every third day. Cell proliferation was measured by either measuring the activity of mitochondrial dehydrogenases in the sample or by cell counting using a hemacytometer. For the former enzymatic assay 10 μl of Cell Proliferation Reagent WST-1 (Boehringer Mannheim, Indianapolis, IN) was added to each well with mixing at a final dilution of 1:10. The plates were then incubated in a humidified atmosphere (37°C, 5% CO_2) for 2 h after which they were shaken thoroughly for 1 min on a shaker. The optical absorbances of the samples were then read using a micro titer plate reader (Spectracount, Packard, IL) at a wavelength of 450 nm. In case of the latter assay, cells from 10 different donors were plated in 12-well plates at a seeding density of 6×10^3 cells/cm^2 in preadipocyte medium and fed every second day. Cells were harvested by trypsin/EDTA digestion and counted on a hemacytometer on days 0, 3, 6, 8, and 10 after plating.

Adipocyte Differentiation

Adipose tissue derived stromal cells from 12 different patients were cultured in micro titer plates (tissue culture grade, 96 wells, flat bottom well) (Coming, NY) at a plating density of 3×10^4 cells/cm^2 in either preadipocyte medium or adipocyte medium. Under the adipogenic conditions cells were differentiated for 3 days in differentiation medium (DMEM/ Ham's F-10 nutrient broth, 1:1, v/v; HEPES buffer (15 mM); Fetal Bovine Serum (3%); Biotin (33 μM), Pantothenate (17 μM), human insulin (100 nM), dexamethasone (0.5 μM), PPARγ agonist (1 μM) and antibiotics) after which they were maintained in adipocyte medium with feeding every 3 days.

bDNA Assay

The bDNA assays were performed with cells incubated under preadipocyte or adipogenic conditions for 1 week. Cells were incubated with lysis buffer provided in the Quantigene Expression kit (Bayer Corporation, Emeryville, CA) for 15 min at 37°C containing the appropriate oligonucleotide probes for aP2 or GAPDH [Burris et al., 1999]. The final detection was achieved by incubation with a chemiluminescent substrate dioxetane and measuring the light emission from its enzymatic degradation using a Quantiplex bDNA Luminometer (Chiron Diagnostics/Bayer). The probes for aP2 and GAPDH were designed

according to the Probe Designer Software (Bayer Corporation, Emeryville, CA) [Burris et al., 1999] and synthesized by Sigma-Genosys (Woodlands, TX).

Polymerase Chain Reaction

Total RNA was isolated from human adipose tissue using TriReagent (Molecular Research Center, Cincinnati OH) [Chomcynski and Sacchi, 1987]. Reverse transcriptase reactions were performed with 1 μg of total RNA using the GeneAmp RNA PCR Kit (Perkin Elmer, Branchburg, NJ). The resulting cDNA was serially diluted by factors of 10-fold (0.005–50 ng per reaction) and used in polymerase chain reactions performed using a 5 min 94°C start; a cycle of 30 sec at 94°C, 30 sec at 55°C, and 1 min at 72°C; and an 8 min extension at 72°C. Equivalent concentrations of the total RNA were used in the branched DNA reactions described above. Oligonucleotide primer sets were synthesized by Gibco-BRL (Gaithersburg, MD) specific for the human aP2 cDNA. Fragment size and number of amplification cycles are indicated in parentheses; the cycle numbers used were determined to be in the linear amplification range for the aP2 gene:

aP2 5′ GGCCAGGAATTTGACGAAGTC (251 bp)
aP2 3′ ACAGAATGTTGTAGAGTT-CAATGCGA (23 cycles)

Aliquots of the amplified PCR products were electrophoresed on a 2% agarose gel, stained with ethidium bromide and photographed. The PCR product was subcloned into the TA cloning vector (Invitrogen, San Diego, CA) and sequence confirmed by automated sequence analysis (performed by the Sequencing Core Facility at the Oklahoma Medical Research Foundation, Oklahoma City, OK).

Leptin ELISA Assay

The leptin ELISA assay was performed according to the manufacturer's instructions (R&D Systems, Minneapolis, MN) using 24-well plates seeded at a density of 3×10^4 cells/cm^2. Cultures were maintained under control or adipogenic conditions for two weeks and medium changed every third day. The leptin assays were performed using 72 h conditioned medium in the presence of serum. Values are expressed as pg of leptin per ml.

Fluorescent Methods: Nile Red Staining

The Nile Red staining method was performed according to a published protocol [Dorheim et al., 1993]. A stock solution (1000 ×) of Nile Red (Sigma-Aldrich, St. Louis, MO) was prepared in DMSO (1 mg/ml) and diluted 1:100 in phosphate buffered saline (PBS) immediately prior to use (10 × stock). Individual wells from a 24-well plate were washed three times with PBS and digested with trypsin/EDTA. The cells were suspended in PBS in a total volume of 400 μl, fixed by the addition of 40 μl of 5% paraformaldehyde, and stained with the addition of 44 μl of the 10 × Nile Red stock solution. The volume was adjusted to 1 ml with phosphate buffered saline and the gold fluorescence emission detected between 564 and 604 nm using a FACscan multiparameter flow cytometer (Becton Dickinson, San Jose, CA) (performed at the Duke Comprehensive Cancer Center Flow Cytometry Core Laboratory).

Histochemical Methods: Oil Red O Staining

The Oil Red O staining method is a modification of a published protocol [Green and Kehinde, 1975]. A stock solution of Oil Red O (0.5 g in 100 ml isopropanol) was prepared and passed through a 0.2 μm filter. To prepare the working solution, 6 ml of the stock solution was mixed with 4 ml of distilled water, left for 1 h at room temperature, and filtered through a 0.2 μm filter prior to use. Cells in 96-well plates were fixed with 10% formalin in PBS for 1 h at 4°C and stained with 30 μl of the Oil Red O working solution per well for 15 min at room temperature. The wells were rinsed three times with 150 μl of water. The dye retained by the cells was eluted by incubation with 100 μl of isopropanol. The OD_{540} was determined using a Packard Sprectracount plate reader. Blank wells (without cells) were stained with dye and rinsed in the same manner; these values were subtracted from the experimental (cell) data points to control for stain retention by the walls of the well.

RESULTS

We compared the degree of in vitro adipogenesis across a panel of human adipose tissue derived stromal cells (Table I). The donor ages ranged from 21 to 62 (38.9 ± 3.1, mean ± SEM) and their body mass indices ranged from 21.3 to 55.1 (27.8 ± 2.4, mean ± SEM). All primary cul-

TABLE I. Donor Information

Donor	Gender	Age(years)	Basal metabolic index (BMI)	Doubling time for cell proliferation (days)
1	F	56	21.6	
2	F	32	21.7	
3	M	35	23.8	8.1
4	F	37	34.6	4.8
5	F	28	23.6	2.6
6	F	39	55.1	2.91
7	M	37	32.5	1.65
8	F	42	25.2	3.56
9	F	62	31.7	3.33
10	F	34	23.1	1.76
11	F	29	22.5	2.32
12	F	39	25.9	1.38
13	F	21	26.9	
14	F	54	21.3	
Mean ± SEM		38.9 ± 3.1	27.8 ± 2.4	3.2

tures were derived from subcutaneous adipose tissue. The stromal cells were cultured for 1 week under preadipocyte (control) or adipogenic culture conditions prior to harvest. Over this time period, the cells under preadipocyte conditions exhibited a mean doubling time of 3.2 days based on hemacytometer counts (n = 10, Table I). In all donors, the preadipocyte proliferation rate exceeded that of the identical cells under adipogenic conditions so that, after 7 days in culture, the cell numbers in preadipocyte wells exceeded adipocyte wells by 1.46-fold (mean, n = 8) (data not shown).

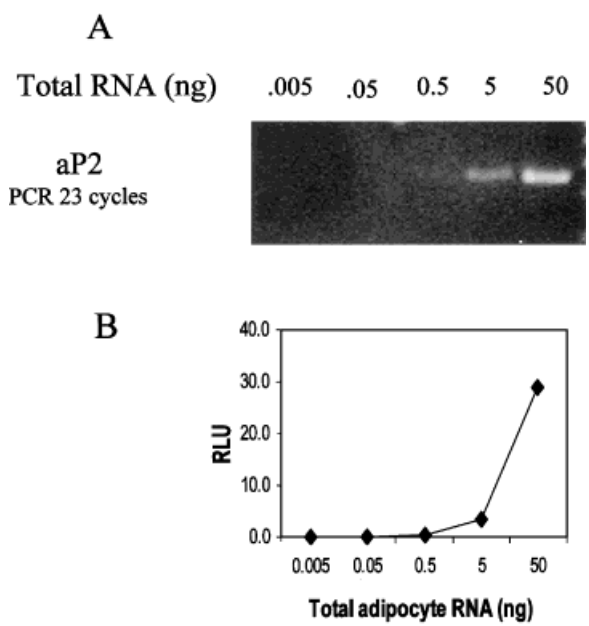

Fig. 1. bDNA assay compared to RT-PCR. **A:** Total RNA was isolated from adipocytes and RT-PCR performed using aP2 primers. The number of cycles used for PCR was 23. **B:** bDNA assay was performed using similar concentrations of purified adipocyte RNA (same donor) with aP2 probes.

aP2 mRNA Induction During Adipogenesis

Initial studies compared the sensitivity of aP2 mRNA detection by bDNA relative to that by polymerase chain reaction (Fig. 1). Each analysis examined serial dilutions of purified total RNA isolated from human cultured adipocytes. The bDNA measured the mRNA levels as relative luminescent units (RLU) while the PCR products were detected based on ethidium bromide staining. The polymerase chain reaction was conducted at a cycle number within the linear amplification range of the assay. The detection threshold for each method was approximately 5 ng of total RNA per reaction (Fig. 1).

The aP2 mRNA levels were determined by branched DNA (bDNA) assay under preadipocyte and adipocyte culture conditions in stromal cells derived from 12 individual donors (Fig. 2). The stromal cells under preadipocyte condition displayed a mean (± SEM) aP2 mRNA level of 0.29 ± 0.04 RLU (Fig. 2A). Under adipocyte conditions, this increased over two orders of magnitude to a mean (± SEM) of 202.5 ± 46.9 RLU (Fig. 2A). The individual donors exhibited some degree of heterogeneity in terms of their fold activation of aP2 mRNA (measured under adipogenic condition: preadipocyte condition), ranging from 372- to 1259-fold (Fig. 2B). Overall, the aP2 mRNA levels in adipocytes increased 679 ± 93-fold (mean ± SEM, n = 12) relative to the stromal cells (preadipocytes) after one week in culture.

As an additional control, the aP2 level in stromal and adipocyte cells was normalized

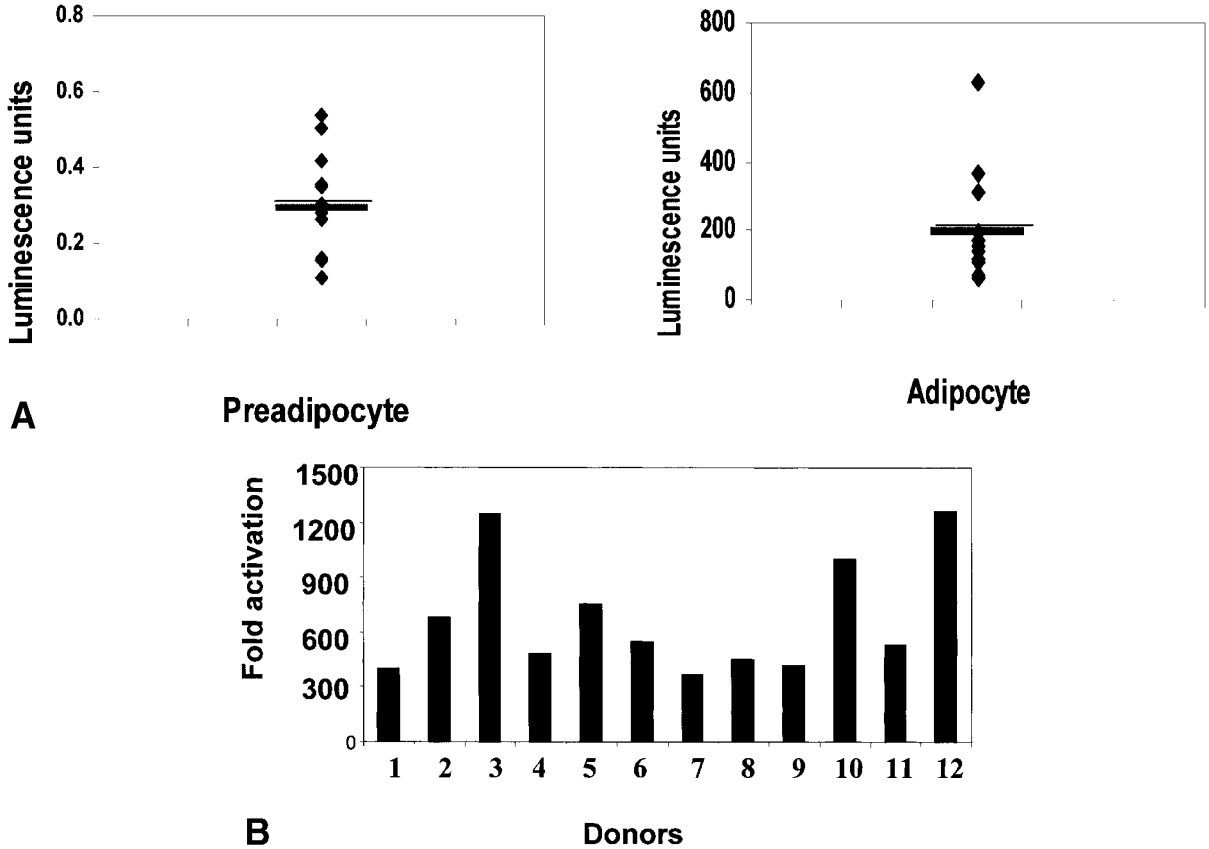

Fig. 2. **A:** bDNA assay: absolute values. The aP2 bDNA levels were determined using lysates from the stromal cells isolated from 12 different donors cultured under preadipocyte or adipocyte conditions. The assay on each donor was performed in quadriplicate and the mean aP2 bDNA values were plotted for either the preadipocyte or adipocyte condition. Note that there is a 1000 fold difference between the y-axis scale for luminescent signal for the preadipocyte and adipocyte conditions. **B:** bDNA assay: fold-activation. The average aP2 bDNA values under adipogenic conditions were divided by the average aP2 bDNA values under preadipocyte conditions for each of the 12 donors. The resulting "fold activation" was plotted for each of the individual donors.

relative to the *GAPDH* gene in a selected number of donors ($n = 4$). The fold activation calculated based on the GAPDH normalized data (1118.5 ± 308.1, mean $\pm$ SEM) was not significantly different ($P = 0.242$) from the fold activation calculated based on the raw bDNA data from these same donors (803.6 ± 197.5, mean $\pm$ SEM).

Leptin Induction During Adipogenesis

As an alternative measure of adipogenesis, leptin secretion was determined in 3-day conditioned medium from cells maintained 12 days in culture (Table II, $n = 8$ donors). Under preadipocyte conditions, leptin levels ranged from undetectable to 32 pg/ml; under adipocyte conditions, leptin levels increased to between 7755 and 10556 pg/ml (Table II). The mean overall leptin level was 9303 ± 297 pg/ml ($n = 8$).

Neutral Lipid Accumulation During Adipogenesis

As a second alternative measure of adipogenesis, the accumulation of neutral lipid was compared under preadipocyte and adipocyte culture conditions. The Nile Red fluorescent dye is hydrophobic and concentrates in the lipid droplets of mature adipocytes; this can be measured by flow cytometric methods (Fig. 3). After 2 weeks in culture, the percentage of Nile Red positive cells under adipocyte conditions was $54.2\% \pm 12.5\%$ (mean $\pm$ SEM, $n = 4$) as compared to $6.5\% \pm 1.1\%$ under preadipocyte conditions; this corresponds to an induction of 8.3-fold (Fig. 3). In comparable Oil Red O histochemical staining assays performed on cells after 4 weeks in culture, the relative amount of neutral lipid in adipocytes was 8.5 ± 2.4-fold

TABLE II. Leptin Secretion[a]

Donor	Preadipocytes	Adipocytes
3	ND	9325 ± 60
4	ND	9750 ± 660
5	ND	10556 ± 480
6	ND	9368 ± 98
8	32 ± 6	8974 ± 49
9	ND	9920 ± 198
10	16 ± 3	7755 ± 360
11	ND	8774 ± 960

[a]The leptin secretion level was determined by ELISA assay in 72 h conditioned medium from the stromal cells derived from 8 different donors cultured under either preadipocyte or adipocyte conditions for a 2-week period. Values are expressed as pg/ml of conditioned medium (mean ± SEM).

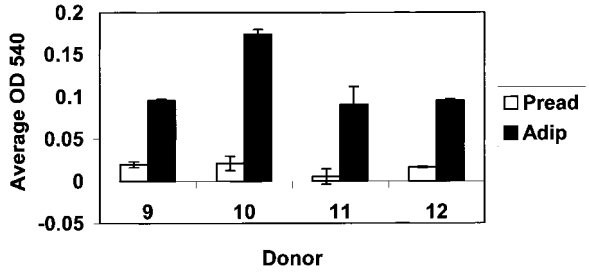

Fig. 4. Oil red O staining of stromal cells: Spectrophotometric analysis. Stromal cells derived from four individual donors were cultured for 4 weeks under preadipocyte or adipocyte conditions. The cells were harvested, fixed with formalin, and stained with the lipophilic dye, Oil Red O. The dye retained by the lipid vacuoles was measured by determination of the optical density at 540 nm and is shown on the y-axis (mean ± SD).

(mean ± SEM, n = 4) greater than that in stromal cells (preadipocytes) (Fig. 4).

DISCUSSION

This report demonstrates that preadipocytes isolated from multiple donors displayed varying degrees of differentiation in response to an optimal adipogenic stimulus in vitro. This heterogeneity is based on their ability to accumulate lipid and their ability to express the aP2 gene marker.

It is well documented that the fatty acid binding protein, aP2, is induced during adipocyte differentiation and it is routinely used as a lineage selective marker. Branched DNA detection of aP2 mRNA levels provides a reproducible and quantitative measure of adipocyte differentiation in vitro. Over a 7-day culture period, the assay displays a dynamic range of up to three orders of magnitude of aP2 expression in human adipose tissue-derived stromal cells under adipogenic conditions. Based on the fold activation of the gene between preadipocyte and adipocyte culture conditions, this assay can detect subtle differences among individual donors.

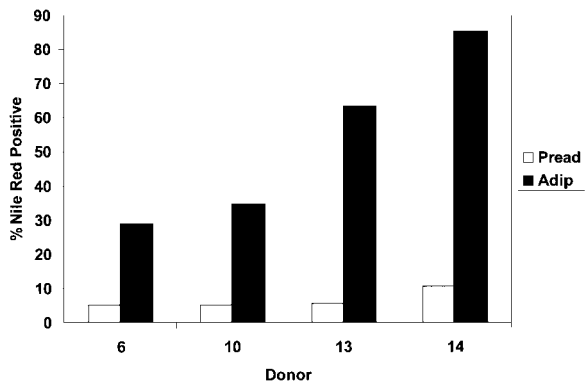

Fig. 3. Nile red staining of stromal cells: Flow Cytometry. Stromal cells derived from four individual donors were cultured for 2 weeks under preadipocyte or adipocyte conditions. The cells were harvested, fixed with paraformaldehyde and stained with the lipophilic fluorescent dye, nile red, prior to analysis by flow cytometry. The percent of cells staining positive for nile red under each culture condition are shown on the y-axis (mean ± SD).

Previous studies have utilized bDNA to quantify aP2 mRNA levels in differentiating human adipose derived stromal cells and macrophages [Burris et al., 1999; Pelton et al., 1999]. These investigators found that, following a 24 h induction period in the presence of PPARγ ligands, human adipose derived stromal cells increased their aP2 mRNA level up to 10-fold relative to undifferentiated cells [Burris et al., 1999]. Following a 7-day induction period, we observed that 1 μM BRL49653 (a PPARγ agonist) increased aP2 expression by 200-fold (data not shown). Thus, the current work confirms and extends the previous findings of others by demonstrating that the linear range of the aP2 bDNA assay extends upto three orders of magnitude [Burris et al., 1999; Pelton et al., 1999].

The bDNA assay compares favorably to other quantitative measures of adipocyte differentiation in vitro. Branched DNA detection of the aP2 mRNA exhibits a level of sensitivity similar to that displayed by the polymerase chain reaction. In contrast to traditional PCR assays, bDNA can be performed on simple cell lysates apart from purified total RNA, thereby reducing the required number of procedures [Mullis

and Faloona, 1987]. The adipogenic fold induction of the bDNA assay is similar to that displayed by the leptin ELISA. In the present study, the bDNA assay displayed a wider range among individual donors (>4 fold) as compared to the leptin ELISA (<2 fold). However it is possible that other factors such as stability and turnover of the leptin protein in culture medium relative to intracellular aP2 mRNA stability may contribute to this perceived difference in sensitivity [Bond et al., manuscript in preparation].

Flow cytometric and histochemical methods examining lipid accumulation revealed differences among individual donors; however, these assays exhibited only an 8-fold induction in adipocytes compared to preadipocytes. This is far less than the 679-fold induction displayed by the bDNA assay. This suggests that the aP2 bDNA assay offers several advantages for high throughput analysis of adipocyte differentiation in vitro. These include dynamic range, high sensitivity and detection of heterogeneity among donors.

The patient-to-patient variability may reflect differences in the genetic makeup of the donors. The heterogeneity in adipose derived stromal cell population that we observe in this study resembles that reported in other stromal cell systems [Phinney et al., 1999; Solchaga et al., 1999]. Phinney et al. [1999] had examined osteogenic differentiation in human marrow stromal cell cultures established from 22 different bone marrow donors. They found that the activity of the enzyme marker for osteogenesis, alkaline phosphatase varied among donors following an inductive stimulus. The level of induction ranged from 1 to 17 fold among the various donors. Solchaga et al. [1999] performed a similar analysis of rabbit bone marrow stromal cells from 241 individual preparations. They found that alkaline phosphatase activity ranged from 0.41 to 63.68. Thus the variation between individuals in the current study is comparable. Work in progress will elucidate whether other parallels exist between adipose tissue- and bone marrow- derived stromal cells.

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