

Original Research Paper

Derivation of Adipocytes from Human Embryonic Stem Cells

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ABSTRACT

Human embryonic stem (hES) cells are undifferentiated and pluripotent cells that hold great therapeutic potential, but are hampered by our limited knowledge to promote specific cell differentiation. Here we provide the first report of the directed differentiation of hES cells into adipocytes. Embryoid bodies (EBs) derived from hES cells are shown to respond to factors that promote adipogenesis. Differentiated cells were observed that displayed the key features of adipocytes, i.e., expression of specific molecular markers, such as peroxisome proliferator-activated receptor γ 2 (PPAR γ 2), adipocyte fatty acid binding protein (aP2) and adiponectin, the secretion of leptin, and the accumulation of lipid droplets in cytoplasm. Taken together, our results demonstrate that adipocytes derived from hES cells in vitro can provide a novel model system to study human adipogenesis and obesity.

INTRODUCTION

HUMAN EMBRYONIC STEM (hES) CELLS can be propagated indefinitely in vitro, yet still retain potential for differentiation into a wide variety of cells representing progeny of three embryonic germ layers (1,2). Without a doubt, hES cells can provide many promising applications, including an unlimited source of cells for transplantation therapies to replace cells lost or impaired by disease and injury, a powerful in vitro model system for the studies of early human development, and a valuable tool for the drug development. Thus, intensified studies are under way in the field for establishing conditions to direct hES cell differentiation into certain therapeutically relevant cell types. To date, reports have described hES cell differentiation into sev-

eral cell types including neuronal (3), cardiomyogenic (4), hematopoietic (5), endothelial (6), and pancreatic cells (7).

It has been well recognized that adipose tissue is not only an energy storage organ but also an endocrine organ (8). Indeed, adipocytes link obesity, diabetes, and cardiovascular disease (9). Although key events and genes during adipogenesis have been identified largely through analysis of the differentiation process of immortalized mouse preadipocyte cell lines (10,11), there are significant differences between human and mouse adipocyte development. Thus far, the origins of the human adipocytes and the events and genes in control of human adipogenesis are yet to be defined fully.

As the first step to study molecular mechanisms of adipogenesis and to screen anti-obesity/diabetes drugs using

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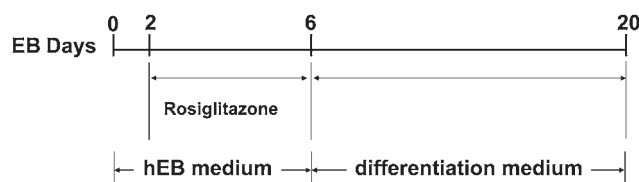


FIG. 1. Schematic diagram of the experimental protocol used for directing adipocyte differentiation from hES cells. EB: embryoid body.

human adipocytes, we demonstrate for the first time that hES cells can be differentiated into adipocytes.

MATERIALS AND METHODS

hES cell culture

Human ES cells used for this study were the WiCell H1 line (passages 32–50). The cells were cultured as previously described (12).

Embryoid body formation and induction of adipocyte differentiation

For embryoid body (EB) formation, hES colonies were digested by 200 IU/ml collagenase type IV (Invitrogen, Carlsbad, CA) and transferred to in ultralow attachment six-well plates (Corning Inc., Corning, NY) to allow their aggregation in suspension using EB formation medium consisting of 78% Dulbecco's modified Eagle medium (DMEM) with high glucose, 1% L-glutamine, 1% β -mercaptoethanol, 1% nonessential amino acid (NEAA), and 10% fetal bovine serum (FBS; Invitrogen). After 2 days,

the EB aggregates appeared denser, and the medium was changed with 20% of FBS. After 3–4 more days, EBs were transferred to six-well plates coated with 0.1% gelatin. To induce adipocyte differentiation from hES cells, the modified procedure (Fig. 1) was performed according to a previously described mouse protocol (13). Rosiglitazone (Cayman, Ann Arbor, MI), a peroxisome proliferator-activated receptor γ (PPAR γ) agonist was added to the EB growth medium. Medium was changed every day, and cells were treated for the indicated time periods.

Characterization of adipocytes

Oil Red O for lipid droplet staining was used as previously described (14). For the detection of leptin in culture medium, we used the human leptin enzyme-linked immunosorbent assay (ELISA) kit following the manufacturer's protocol (R&D System Inc, MN).

RT-PCR

The presence of Oct-4 and Nanog in the hES cells and adipocyte markers [PPAR γ 2, ADD1, adipocyte fatty acid binding protein (aP2) and adiponectin] in the directed differentiated hES cells were detected by RT-PCR using the primers shown in Table 1. 18S RNA was used as the internal control. Human adipose tissue RNA purchased from Clontech (Mountain View, CA) served as positive control for the study. The amplified products were separated on 1.2% agarose gels and detected by ethidium bromide staining.

Statistical analysis

All data, which were calculated as the percentage from three independent experiments, were expressed as the

TABLE 1. PRIMERS USED FOR RT-PCR

Gene	Primer sequence	Product size (bp)
Nanog	Forward, 5'-tgcctcacacggagactgtc-3'	354
	Reverse, 5'-tgctattctcggccagttg-3'	
Oct-4	Forward, 5'-gacaacaatgagaacct tcaggaga-3'	171
	Reverse, 5'-ttctggcgccggttacagaacca-3'	
PPAR γ 2	Forward, 5'-cagcaaacccctattccatg-3'	130
	Reverse, 5'-ctctgtgtcaacctgtca-3'	
ADD1	Forward, 5'-ggagccatggattgcacttc-3'	178
	Reverse, 5'-atcttcaatggagtgggtgcag-3'	
aP2	Forward, 5'-atgggatggaatcaacca-3'	104
	Reverse, 5'-gtggaagtgcgccttcat-3'	
Adiponectin	Forward, 5'-tccttacagaacacgccttca-3'	501
	Reverse, 5'-agggccacagaacgagag-3'	
18S RNA	Forward, 5'-ggaaggcaccaccaggagt-3'	317
	Reverse, 5'-tcagccccggacatctaag-3'	

mean \pm SD and were evaluated with chi-square test. A value of $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSIONS

Obesity is reaching epidemic proportions in United States (14). It poses a major public health problem by predisposing individuals to diabetes, hypertension, coronary heart disease, congestive heart failure, and stroke (15). Indeed, obesity contributes to approximately 300,000 deaths each year in the United States (16). However, the molecular determinants that mediate the process of obesity remain to be further defined. Although advances have been made to understand the adipogenesis using immortalized mouse preadipocyte cell lines (17), there is limited knowledge of human adipogenesis.

In this study, we investigated the adipocyte differentiation from hES cells as potential sources for generation

of human adipocytes and preadipocytes in vitro. The initial step in hES cell differentiation began when the cells were removed from the feeder layer and suspending into human EB medium containing serum. Approximately 80% of hES cell clumps started to organize into three-dimensional aggregates in suspension at day 2, and continued to grow into spherical EB-like structures at day 6. After plating onto the gelatin-coated wells, 95% of human EBs were attached and outgrown. To identify adipocytes in the hES cell differentiating system, we performed Oil Red O staining for triglycerides in human EB outgrowth aggregations. As shown in Fig. 2A, lipid droplets in cytoplasm appeared in some EB outgrowths. To confirm further that these cells are adipocytes, we detected the expression levels of adipocyte-specific genes. As expected, PPAR γ 2 and other adipocyte-specific genes (e.g., ADD1, aP2, adiponectin) were expressed in day-20 EB outgrowths (Fig. 2B). The human adipose tissue and undifferentiated hES cells were used as the positive and negative controls for the adipose markers, respec-

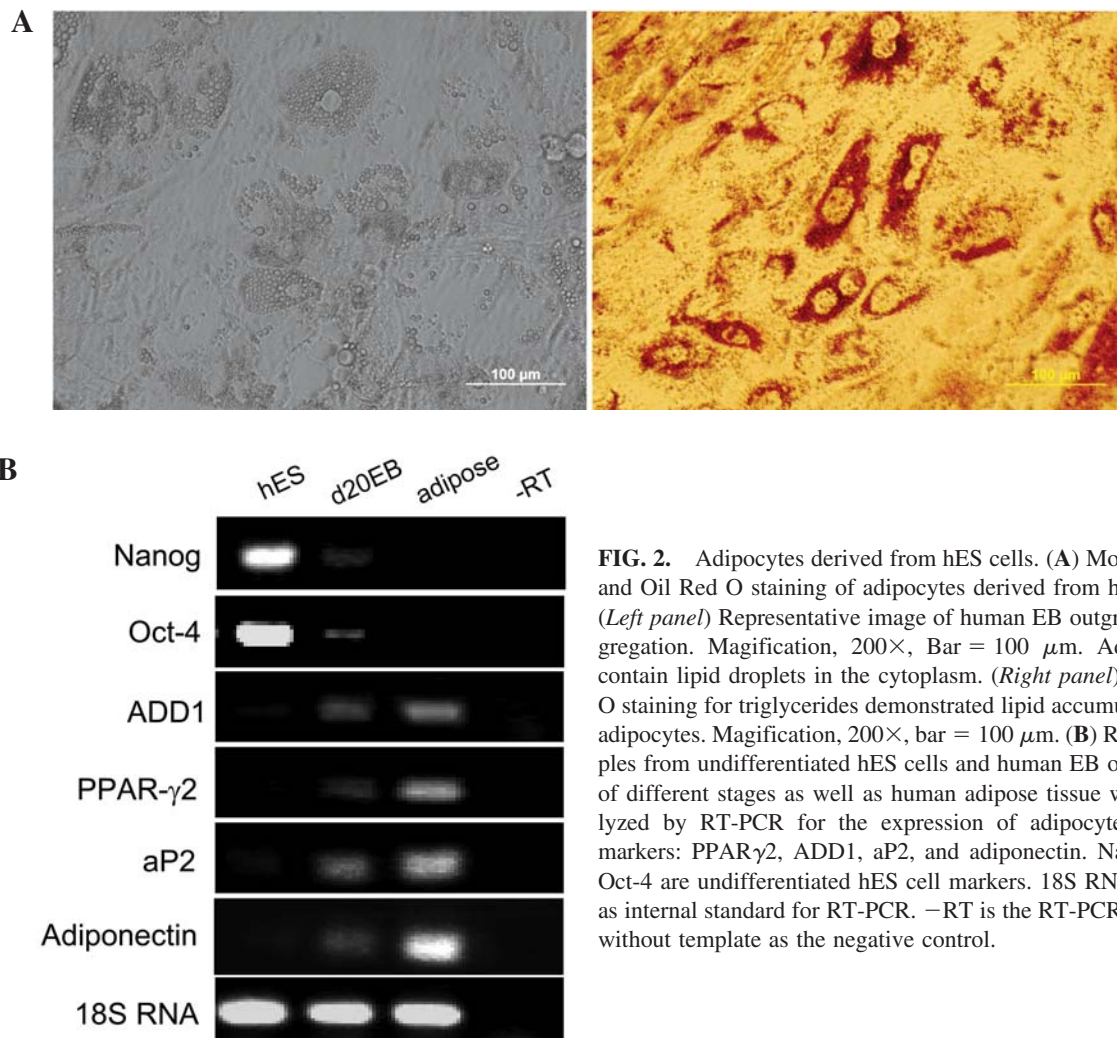


FIG. 2. Adipocytes derived from hES cells. **(A)** Morphology and Oil Red O staining of adipocytes derived from hES cells. *(Left panel)* Representative image of human EB outgrowth aggregation. Magnification, 200 \times , Bar = 100 μ m. Adipocytes contain lipid droplets in the cytoplasm. *(Right panel)* Oil Red O staining for triglycerides demonstrated lipid accumulation in adipocytes. Magnification, 200 \times , bar = 100 μ m. **(B)** RNA samples from undifferentiated hES cells and human EB outgrowth of different stages as well as human adipose tissue were analyzed by RT-PCR for the expression of adipocyte-specific markers: PPAR γ 2, ADD1, aP2, and adiponectin. Nanog and Oct-4 are undifferentiated hES cell markers. 18S RNA served as internal standard for RT-PCR. -RT is the RT-PCR reaction without template as the negative control.

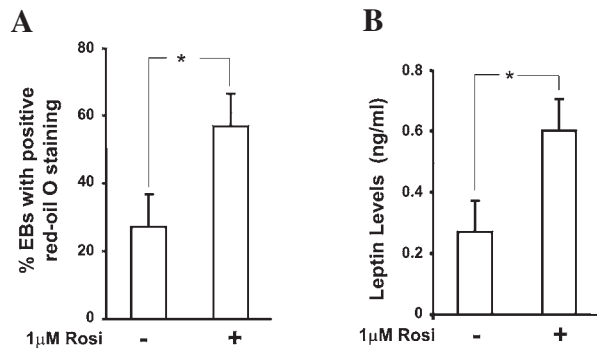


FIG. 3. Rosiglitazone enhances adipocyte differentiation from hES cells. (A) EBs were treated with Rosiglitazone as described in Fig. 1. The percentage of EB showing adipocyte colonies after 20 days in differentiation medium is shown. Data are expressed as means \pm SD ($n = 3$, $*p < 0.01$). (B) Leptin levels in the medium of EBs at day 20 were measured using an ELISA kit. Data are expressed as means \pm SD ($n = 3$, $*p < 0.01$).

tively. In addition, Nanog and Oct-4, markers of undifferentiated cells, were expressed in the ES cells and dramatically down-regulated in EBs (Fig. 2B). Taken together, our data demonstrate that adipocytes are existed in the spontaneous differentiated human EBs.

Emerging data suggest that PPAR γ is the master gene to control adipogenesis (18). PPAR γ functions as a heterodimer with retinoid X receptors (RXR), another member of nuclear receptor family, to bind to the PPAR-responsive element (PPRE) on its target genes (19). To explore the expression patterns of PPAR γ and related nuclear receptors in human ES cell differentiation, we performed quantitative real-time PCR using RNAs isolated from hES cells and EBs of day 2 to day 10. We found that PPAR γ and RXR γ were almost undetectable in hES cells, but steadily increased their expression in EBs from day 2 and reached maximal levels at day 6 (data not shown). These findings intrigue us to explore the directed differentiation of hES cell into adipocytes using rosiglitazone, a PPAR γ agonist. As outlined in Fig. 1, we treated the EBs with rosiglitazone (1 μ M) from day 2 to day 6. Interestingly, we documented that rosiglitazone significantly increased EB adipogenesis from 27.1% \pm 9.8% of no treatment to 57.0% \pm 9.6% (Fig. 3A, $n = 3$, $p < 0.01$), respectively. In addition, the level of leptin, an adipocyte-specific hormone, in the medium from rosiglitazone-treated EBs was significantly increased to 0.61 \pm 0.10 ng/ml versus 0.26 \pm 0.11 ng/ml ($n = 3$, $p < 0.01$) in the untreated medium (Fig. 3B). Taken together, this series of experiments established a method for directing adipocyte differentiation from hES cells.

In summary, our results documented for the first time that functional adipocytes could be differentiated from hES cells. Although adipogenic reagents were able to improve adipocyte differentiation from hES cells, the total

number of adipocytes was still lower in the present study. To improve the directed differentiation by genetic manipulation, our group has recently established stable and efficient genetic manipulation of hES cells using lentiviral vectors (12). Indeed, we are generating stable hES cell lines with temporal control of PPAR γ and CCAAT/enhancer binding protein γ (C/EBP α) expression, two master genes for adipogenesis. Using the combination of genetic and pharmacological approaches, we expect a better model system to be developed. By having an effective system to induce hES cells into adipocytes, we will provide a novel tool to study human adipogenesis and screen drugs for anti-obesity/diabetes.

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