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Expression of p107 and p130 during human adipose-derived stem cell adipogenesis

Ashley S. Ross, Rocky Tsang, Kris Shewmake, and Robert E. McGehee Jr.*

Department of Pediatrics, Division of Neonatology, University of Arkansas for Medical Sciences, Arkansas Children's Hospital, and Winthrop P. Rockefeller Cancer Institute, 4301 West Markham Street, Slot 512-5B, Little Rock, AR 72205, USA

Abstract

Within the first 24 h of hormonally stimulated adipocyte differentiation, murine 3T3-L1 preadipocytes undergo a mitotic expansion phase prior to terminal differentiation. During this time, the cell cycle regulatory proteins, p130 and p107 undergo dramatic differential expression and the transient increase in expression of p107 appears to be required for terminal differentiation. Recently, human adipose-derived human stem cells (hASC) of mesenchymal origin have been used as a model of human adipocyte differentiation and we sought to determine if differentiating hASC undergo clonal expansion and if the regulated expression of p130/p107 was similar to that observed during 3T3-L1 adipogenesis. Results indicate that differentiating hASC, unlike 3T3-L1 cells do not undergo clonal expansion and p130 expression gradually diminishes across differentiation. However, p107 expression is transiently increased during hASC differentiation in a manner analogous to 3T3-L1 cells suggesting a similar role for p107 in terminal differentiation in human adipocytes.

Keywords

3T3-L1 cells; hASC cells; Human adult stem cells; Adipocyte differentiation; p130; p107

The past 20 years have seen a dramatic increase in obesity in the United States. To date, more than 17% of American children and adolescents are considered overweight, and almost one third of adults are classified as obese [1]. This is particularly alarming given that obesity represents a significant risk factor for many diseases and health conditions including hypertension, dyslipidemia, type 2 diabetes, coronary heart disease, stroke, gallbladder disease, osteoarthritis, sleep apnea, and endometrial, breast, and colon cancers [1]. Due to the increasing epidemic of obesity and the health risks associated with the condition, much research has focused on identifying the underlying causes of obesity. However, in order to develop better control strategies, a better understanding of the molecular mechanisms that initiate differentiation of preadipocytes and stem cell into adipocytes in humans is still needed.

Significant portions of the current understanding of adipocyte differentiation have been obtained through the study of *in vitro* cellular models of adipocyte differentiation such as the murine 3T3-L1 preadipocyte cell line and the process of 3T3-L1 preadipocyte differentiation into mature adipocytes is well characterized [2,3]. Culturing 3T3-L1 cells in serum-supplemented medium results in growth to confluence, leading to contact inhibition and growth arrest [4]. Differentiation is induced by a 4 day hormonal treatment regimen consisting of insulin, dexamethasone, and 1-methyl-3-isobutylmethylxanthine [5]. Hormonal induction results in re-entry into the cell cycle, and the cells undergo one to two rounds of mitotic clonal

* Corresponding author. Fax: +1 501 603 1425. rem@uams.edu (R.E. McGehee Jr.).

expansion for about 24–36 h. They then permanently withdraw from the cell cycle and undergo terminal differentiation into mature adipocytes [6]. We have previously demonstrated that protein expression levels of two members of the retinoblastoma (Rb) tumor suppressor family, p130 and p107, are highly regulated within the first 24 h of adipocyte differentiation in 3T3-L1 cells and referred to as the p130:p107 switch [6]. These proteins regulate the transcriptional activity of the E2F proteins that control cell cycle progression [7]. In 3T3-L1 cells, p130 is constitutively expressed in quiescent day 0 preadipocytes while p107 levels are barely detectable. On day 1, following 24 h of hormonal stimulation, p130 levels fall dramatically and p107 levels rapidly increase. Activation of this switch in expression of p130:p107 is associated with mitotic clonal expansion and entry into the cell cycle in 3T3-L1 cells. By terminal differentiation on day 4, there is a resetting of the switch back to levels observed in quiescent day 0 cells [6]. Additional studies in which expression of these proteins were examined under conditions that uncoupled clonal expansion from differentiation revealed that p107 is associated with terminal adipocyte differentiation and not the clonal expansion phase, while p130 was more associated with clonal expansion and not terminal differentiation [8].

Several recent reports have demonstrated that human adipose-derived stem cells (hASC) isolated from human adipose tissue are multipotent and capable of undergoing *in vitro* differentiation into adipocytes as well as other mesenchymal lineages [9–11]. hASC therefore provide a unique cell model in which to investigate human adipogenesis. The molecular regulation of these cells have not been well characterized and here we sought to compare and contrast adipocyte differentiation in hASC to 3T3-L1 cells by examining; the time course of differentiation using two well established markers of adipocyte differentiation; the mitotic clonal expansion phase; and expression of the p130 and p107 protein levels.

Methods

Cell harvesting, cell culture, and adipocyte differentiation

All chemical were obtained from Sigma, St. Louis, MO unless otherwise noted. Lipoaspirate samples were obtained from donors undergoing elective liposuction. University of Arkansas for Medical Sciences Institutional Review Board approval and consents were obtained before tissue collection. 50–100 ml of adipose tissue (obtained via lipoaspirate) was placed in a sterile specimen cup and was brought immediately to the lab for hASC isolation as described by Gronthos [12]. Liposuction tissues were washed 3 times in twice the volume of lipoaspirate sample in Krebs–Ringer-bicarbonate solution (KRB) to remove contaminating blood in 50 ml conical tubes. KRB was aspirated and lipoaspirate was incubated with an equal volume of collagenase type I (1 g/L of KRB with 1% bovine serum albumin) for 2 h at 37 °C with intermittent aggressive agitation. Following centrifugation at 300 rpm for 5 min, the supernatant containing floating adipocytes were separated by aspiration. The resulting cell pellet was re-suspended in growth media consisting of Dulbecco's modified Eagle medium (DMEM)/Hams-F12 1:1, v/v (Gibco BRL, Gaithersburg, MD and Sigma, respectively), 10% fetal bovine serum (Harlan, Indianapolis, Indiana), 15 mM HEPES, 1% penicillin/amphotericin B and plated on tissue culture dishes at a density of approximately 3500 cells/cm². Following a minimum of two passages but, no more than six passages, cells were plated on 100 mm or 60 mm culture dishes at a density of ~35,000 cells/cm², allowed to reach confluence and treated on day 0 with differentiation medium consisting of growth media supplemented with 100 nM insulin, 1 μM dexamethasone, 0.033 mM biotin, 0.017 mM pantothenate, 0.25 mM isobutyl methylxanthine (IBMX), and 1 μM rosiglitazone for 3 days. On day 3, the media was changed to adipocyte maintenance medium consisting of growth media, 100 nM insulin, 1 μM dexamethasone, 0.033 mM biotin, 0.017 mM pantothenate. The cells were re-fed every 3 days with adipocyte medium [9,13]. Cells were stained with Oil Red O, harvested for RNA analysis, harvested for protein analysis, or counted via hemocytometer as previously described [8].

Oil Red O (ORO) staining for lipid detection

Adipocyte differentiation was detected using ORO stain as described previously [14]. Cells were washed in PBS and then fixed in 10% formalin. They were then washed in 50% isopropyl alcohol and stained for 10 min in a 60% (v/v) saturated ORO/H₂O solution. The stained cells were washed in 50% isopropyl alcohol and stained with Maer's hematoxylin. The plates were then preserved with a glycerin jelly and documented with whole plate and microscopic photographs.

Whole cell extracts and Western blots

Whole cells extracts were obtained using prior methods [14]. Extracts stored at -80 °C were quantitated using a Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA). Protein (50 µg) was aliquoted and run on 12% sodium dodecyl sulfate–polyacrylamide gels. Once resolved, the samples were transferred to nitrocellulose membranes (Micron Separations, Westboro, MA). All membranes were blocked for 1 h in PBS with 0.05% Tween 20 and a 5% non-fat powdered milk solution. Primary antibodies at a 1:200 dilution for p107 and p103 (Santa Cruz Biotechnology, Santa Cruz, CA) were incubated with the membranes for 1 h in PBS and Tween 20. This was followed by two 5 min washings in PBS and Tween 20. The membranes were then exposed to a secondary antibody at 1:2000 dilution for 30 min in a buffer containing 1:1 PBS/Tween 20:RIPA buffer (vol:vol). After the secondary antibody, the membranes were washed 3 times for 5 min in PBS and Tween 20. Immune complexes were visualized by chemiluminescence (CDP-Star, NEN Life Sciences, Boston, MA).

RNA isolation and real time RT-PCR

RNA was isolated from 100 mm plates of cells using TRI Reagent™ (Sigma, Technical Bulletin MB-205). After processing the RNA samples, all were run on 1% agarose, ethidium bromide containing gels and UV illuminated to identify the 28S and 18S ribosomal RNA. Formaldehyde-agarose gel and spectrophotometry were used to determine the quality and quantity of the isolated RNA, respectively. Random hexamer primers with TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA) were then used to reverse transcribe 1 µg of the total RNA. Amplification of the reverse transcribed RNA was conducted with 1× SYBR Green PCR Master Mix (Applied Biosystems) and 0.3 µM of gene-specific upstream and downstream primers during 55 cycles on a Rotor-Gene 3000 Real time Thermal Cycler (Sydney, Australia). The condition for each cycle composed of 20 s intervals of denaturation at 94 °C, annealing at 58 °C, and extension at 72 °C. The variability among the individual samples was normalized with amplified 18S ribosomal RNA expression. A standard curve was created with pooled RNA from the assayed samples, and the data for each sample was tabulated with respect to the 18S RNA expression.

Cell counting

Cells were grown as previously described [8] on 60 mm plates and stimulated to differentiate using the protocol outlined above. Cells were counted at the same time in daily intervals from day 0 to day 10 of differentiation. Each plate was washed in PBS prior to exposure to trypsin/EDTA 0.1%. Once cells were confirmed to be fully trypsinized by microscopy, cells were agitated and an aliquot removed and placed on a hemocytometer. The mean was determined from an $n = 5$ number of independent experiments and evaluated by Student t -test for comparison to number of cells present at day 0 of differentiation.

Results

Under appropriate hormonal stimulation as described in methods, hASC are capable of robust differentiation into adipocytes as assessed by Oil Red O staining of lipid accumulation on day

10 of differentiation (Fig. 1). Fig. 1A is representative 100 mm culture dish and Fig. 1B is a phase contrast photomicrograph (400×) of living cultured cells in which substantial lipid droplets are evident. Once differentiated, cells can be maintained in standard culture media for a number of days, however, the amount of lipid accumulation is not appreciably increased (data not shown).

When compared to 3T3-L1 cells that are typically fully differentiated by day 4, the hASC model requires a much more protracted time course and only at differentiation day 10 does Oil Red O accumulation begin to compare to differentiated 3T3-L1 adipocytes. Therefore, we sought to determine if expression of molecular markers of adipocyte differentiation were similarly delayed by examining expression of PPAR- γ and aP2 (also FABP4), two well characterized markers in a number of different adipogenic cell culture systems. Fig. 2 illustrates the data from the real time RT-PCR analyses for mRNA expression of aP2 and PPAR- γ from an average of two independent experiments. PPAR- γ mRNA expression levels begin increasing on day 8 followed by an increase in aP2 mRNA levels on day 9, with both reaching peak expression at day 10. The sequential expression profile of these two markers is similar to that observed in 3T3-L1 cells and consistent with the delayed lipid accumulation seen in Fig. 1A.

Another early event during 3T3-L1 differentiation is that they undergo re-entry into cell cycle and proceed through a requisite 1–2 rounds of mitotic clonal expansion [3]. To determine if hASC undergo a similar process, we examined mitotic clonal expansion phase by counting cells throughout the time course of differentiation. hASC plated on 60 mm plates were hormonally stimulated to differentiate as described in methods and cells counted daily from day 0 to day 10 post differentiation. Mean cell counts were obtained from five different experiments. Results shown in Fig. 3 reveal that hASCs do not undergo the clonal expansion phase. While there were 3 time points that were significantly different, there was no obvious doubling in cell number at any time throughout the course of differentiation.

During 3T3-L1 adipocyte differentiation, there is dramatic differential expression of p130 and p107 that is characterized by a rapid and transient increase in p107 mRNA and protein on day 1 [15]. By contrast, p130 protein levels are elevated in day 0 preadipocytes and transiently decreased via proteolytic degradation on day 1 of differentiation [16]. Following terminal differentiation, there is a reversion in expression back toward day 0 levels. Fig. 4A illustrates the pattern of p130 and p107 protein expression typically observed during 3T3-L1 differentiation. During the differentiation of hASC there is also a distinct and transient increase in expression of p107 protein levels (Fig. 4B). However, the rapid increase of expression occurs on day 4 of differentiation with levels falling back to pre-induction levels on day 7 in a pattern similar to that observed during 3T3-L1 adipogenesis. Expression of p130 during hASC differentiation is quite distinct from that observed in 3T3-L1 cells, in that p130 protein levels are evident at day 0 and they remain at that level or even slightly diminish across the time course of differentiation (Fig. 4B).

Discussion

We have shown that hASC are capable of differentiating into adipocytes as assessed morphologically by Oil Red O detectable lipid accumulation and biochemically by expressing mRNAs encoding PPAR- γ and aP2, two well described markers of adipocyte differentiation. Additionally, these events occur in a temporal profile as one would expect them to, in that there is expression of PPAR- γ , followed by aP2 and subsequent lipid accumulation [3]. The most obvious difference observed when comparing differentiation in hASC to 3T3-L1 cells is the time course required for terminal differentiation. In our hands, the hASC require a full 10 days to reach terminal differentiation, a result consistent with that previously reported [12]. This protracted time may well be explained by the fact that the 3T3-L1 cells are preadipocytes [3]

and already committed to an adipocyte lineage and hormonal treatment simply stimulates them to complete the process. In contrast, the hASC are clearly multipotential stem cells and can be capable under appropriate hormonal stimulation of differentiation into a variety of other mesenchymal and other cell lineages [17–19]. It is possible that much of the early time course in hASC adipogenesis is utilized in getting the cells to a preadipocyte stage for 2–3 days after which the time is quite similar to that observed in 3T3-L1 cells. Based on these observations, the hASC in addition to serving as a model of human adipocyte differentiation, may also allow investigations into some of the critical molecular events that occur between pre-committed adult stem cells and committed preadipocytes.

It also appears, that unlike hormonally stimulated 3T3-L1 cells, hASCs clearly do not undergo a mitotic clonal expansion stage as determined by examination of total cell number throughout the time course of adipocyte differentiation. In 3T3-L1 cells, the p130:p107 switch was originally thought to be involved in regulating the cell cycle re-entry leading to the clonal expansion phase [15]. However, it was subsequently reported using 3T3-L1 cells under pharmacological manipulation with the MEK inhibitors, PD98059, or U0126, that terminal adipocyte differentiation and clonal expansion could be uncoupled, resulting in complete adipocyte differentiation in the absence of clonal expansion [8]. These experiments also demonstrated that the transient increase in p107 protein expression was independent of clonal expansion, suggesting a role for p107 in terminal adipocyte differentiation, an observation consistent with another study that demonstrated antisense inhibition of p107 protein expression blocked differentiation in 3T3-L1 cells [16]. In the absence of clonal expansion with MEK inhibition, p130 protein expression was significantly altered such that instead of a transient decrease on day 1 of differentiation, there was a sustained gradual reduction in p130 levels across differentiation [8]. Interestingly, examination of the p130:p107 switch in differentiating hASC, reveal a pattern of expression for both of these proteins that is almost identical to that of the uncoupled 3T3-L1 cells treated with MEK inhibitors. Protein levels of p107 exhibit the same transient increase in expression as both normal and uncoupled 3T3-L1 cells, suggesting that p107 may also serve a role in human adipocyte differentiation.

These experiments provide preliminary insight into known regulators of adipogenesis in a human cell line that have been well established in murine models. hASCs represent a valuable resource for investigating obesity at a molecular level, and though beyond the scope of this work, additional investigations into the molecular regulation of hASC adipogenesis may provide better understanding of these events and allow the development of targets for interventions for an ever increasing obese population.

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Fig. 1. Morphological assessment on day 10 of hormonally stimulated adipocyte differentiation of human adipose-derived stem cells. (A) Oil Red O stained 100 mm cell culture plate. (B) Phase contrast photomicrograph illustrating lipid accumulation (100 $\times$).

Fig. 2.

Total RNA isolated from differentiating hASC were analyzed by RT-PCR. Primer sets and conditions used to generate the specific products for PPAR- γ (open squares) and aP2 (closed squares) are described in the Methods, and are expressed relative to endogenous 18S RNA.

Fig. 3. Protein extracts derived from the indicated day of hormonally stimulated differentiation in 3T3-L1 cells (A) or hASC (B) were analyzed by Western blot using an antibody recognizing either p130 or p107 as described in Methods. Expression of p107 protein levels exhibit a similar transient increase in expression in both models of adipogenesis, though occurring later in hASC.

Fig. 4.

Post confluent hASC were plated on 60-mm culture dishes and stimulated to undergo hormonally induced adipocyte differentiation. Cells were trypsinized and re-suspended in 2.5 ml of PBS and counted on a Spotlite hemocytometer as described in Methods. Results are presented as the mean number of cells/60 mm well $\pm$ SEM and statistical analysis of time points compared to day 0 were performed by Student's *t*-test ($n = 5$). * $P < 0.05$.