Changes in integrin expression during adipocyte differentiation

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Summary

3T3-L1 preadipocytes require cAMP for maximal differentiation. Microarray analysis reveals that the integrins α5 and α6 are coordinately regulated by cAMP. α5 expression is gradually diminished during adipogenesis, whereas α6 is increased. Overexpression of α5 in preadipocytes results in enhanced proliferation and attenuated differentiation. Conversely, α6 overexpression is without effect. The GTPase Rac is normally inhibited during differentiation. However, overexpression of integrin α5 increases Rac activity. Constitutively active but not dominant-negative Rac inhibits differentiation when overexpressed in preadipocytes, implying its role downstream of α5 integrin in maintaining preadipocytes in an undifferentiated state. Moreover, α6 integrin is critically involved in clustering growth-arrested preadipocytes on basement membrane Matrigel. Perturbation of such clustering enhances Rho activity and promotes growth-arrested preadipocytes to reenter the cell cycle. These findings demonstrate a role for integrin α6 in connecting morphogenesis with signaling processes leading to terminal differentiation.

Introduction

The adipocyte functions both as a storage depot for fuel and an endocrine cell that senses hormonal and energy status, and in response secretes hormones, referred to as adipokines, to help maintain whole-body energy balance (Kershaw and Flier, 2004; Spiegelman and Flier, 2001). Adipocytes, osteoblasts, and chondrocytes descend from a common mesenchymal stem cell precursor (Gregoire et al., 1998; Grigoriadis et al., 1988; Kershaw and Flier, 2004; Pittenger et al., 1999). Adipogenesis begins in the prenatal period and continues throughout life. This multistep process is regulated by numerous hormones and is accompanied by dramatic changes in cell shape and gene expression (Gregoire et al., 1998; MacDougald and Lane, 1995; Rosen and Spiegelman, 2000; Rosen et al., 1979, 2000). The first step involves proliferation of mesenchymal stem cells followed by differentiation into preadipocytes. Fibroblast-like preadipocytes then migrate to and proliferate at the site of adipogenesis, where they undergo a second differentiation step to become round, spherical adipocytes (Ailhaud et al., 1992; Burdi et al., 1985; Cornelius et al., 1994).

While the signals that control the commitment of mesenchymal stem cells to an adipocyte fate remain largely unknown, downregulation of Rho activity appears to play a pivotal role in this process (McBeath et al., 2004). A recent study has indicated that, when human mesenchymal stem cells assume a round shape, adipogenesis is promoted via downmodulation of the RhoA-ROCK-tension pathway (McBeath et al., 2004). Indeed, earlier studies in mice with a targeted disruption of p190RhoGAP-B also showed that IGF-1-induced inhibition of Rho activity favors adipogenesis of embryonic fibroblasts over myogenesis (Sordella et al., 2003).

Preadipocytic cell lines such as 3T3-L1 are excellent in vitro models for studying hormonal regulation of adipocyte differentiation and metabolism (Gregoire et al., 1998; MacDougald and Lane, 1995; Rosen and Spiegelman, 2000). Postconfluent G_0 3T3-L1 preadipocytes undergo differentiation after continuous exposure to a regimen of adipogenic factors, including fetal bovine serum (FBS), dexamethasone (DEX), methylisobutylxanthine (MIX), and insulin (Green and Kehinde, 1975; Green and Meuth, 1974; MacDougald and Lane, 1995). Cells undergo differentiation after entering a period of mitotic clonal expansion, followed by growth arrest. The mitotic clonal expansion is believed necessary to unwind DNA, allowing transcription factors access to regulatory response elements present in adipose-specific genes. The expression of early adipose genes such as CCAAT/enhancer binding protein β (C/EBPβ) and C/EBPδ are initiated during this period of mitosis. Subsequently, C/EBPβ and C/EBPδ mediate the transcriptional activation of peroxisome proliferator-activated receptor γ (PPARγ) and C/EBPα. Through a combination of autocrine and paracrine mechanisms, these two master regulators of adipocyte differentiation then mediate the acquisition of the adipocyte phenotype (MacDougald and Lane, 1995; Rosen and Spiegelman, 2000; Rosen et al., 2000).

Among the components of the classic adipogenic cocktail, insulin (at a nonphysiologically high concentration) is known to act through the insulin-like growth factor 1 (IGF-1) receptor (Schmidt et al., 1990; Smith et al., 1988). Stimulation of the IGF-1 receptor by insulin leads to inhibition of Rho-ROCK pathway (Sordella et al., 2003) as well as activation of the MAP kinase pathway and subsequent mitotic clonal expansion (Tang et al., 2003). While glucocorticoid addition gives rise to an elevated cAMP concentration sufficient to induce differentiation of Ob1771 preadipocytes (Vassaux et al., 1992), this is not the case for certain other preadipocyte lines such as 3T3-L1. It has been commonly recognized that maximum differentiation of 3T3-L1 preadipocytes requires both DEX and MIX (MacDougald and Lane, 1995). However, the specific contribution of MIX and DEX to the process has not been completely elucidated.

Gene expression profiling has been extensively used as a means of identifying key regulatory factors involved in adipo-

cyte differentiation. We describe here a crucial role for integrins α5 and α6, the expression of which are differentially regulated during adipocyte differentiation. These studies implicate the importance of the extracellular matrix (ECM) receptors in the regulation of adipocyte differentiation and gene expression.

Results

Both DEX and MIX are required for 3T3-L1 adipocyte differentiation

Previous work has established DEX, MIX, and insulin as major inducers of adipocyte differentiation in vitro (Green and Kehinde, 1975; Green and Meuth, 1974; MacDougald and Lane, 1995). However, Qiu et al. found that DEX and MIX can induce significant 3T3-L1 adipocyte differentiation in the absence of insulin (Qiu et al., 2001). We reassessed the contribution of individual components to the differentiation process by treating cells with different combinations of DEX, MIX, and insulin in the presence of FBS. Adipocyte differentiation was evaluated by the expression of adipocyte marker proteins aP2, CAP and C/EBPα as determined by immunoblotting, and the accumulation of cytoplasmic triglyceride, as revealed by Oil Red O staining (Figure 1). Regardless of the presence of MIX and insulin, DEX was able to stimulate to a great extent the expression of aP2 and CAP, two genes downstream of PPARγ activation (Baumann et al., 2000; Thompson et al., 2004). The expression of C/EBPα, however, was upregulated only when MIX was included in the medium with DEX (Figure 1A). The combination of DEX and MIX also caused a slight decrease in the expression level of β-actin. Insulin, the sole factor known to induce mitotic clonal expansion (Qiu et al., 2001), was not required for expression of any of the marker proteins. Light microscopy and Oil Red O staining confirmed the results of immunoblotting. As shown in Figure 1B, DEX/MIX/insulin and DEX/MIX were the only two conditions that caused the fibroblast-like preadipocytes to differentiate into the round-shaped adipocytes. Interestingly, the adipocytes differentiated with DEX/MIX were significantly larger in size than the adipocytes differentiated in the presence of insulin plus DEX/MIX. Taken together, these results demonstrate that the presence of FBS, DEX, and MIX is necessary and sufficient to induce differentiation of 3T3-L1 preadipocytes.

Genes that are regulated by MIX

Although DEX alone was able to increase PPARγ expression and those of downstream genes, addition of insulin to the differentiation cocktail with DEX was not sufficient to induce differentiation. Thus, to determine the precise role of MIX in the preadipocyte differentiation, we performed gene microarrays. Total RNA was purified from postconfluent 3T3-L1 cells treated for 3 days with DEX/MIX/insulin (DI + M) or from cells treated with DEX/insulin containing no MIX (DI – M). Data derived from these two conditions are accessible at http://www.lifesciences.umich.edu/institute/labs/saltiel/saltiel_cellmetabolism.pdf. We designed a simple screen to define genes that are expressed at similar levels (within 2-fold of each other) among the three samples from the same condition and that are expressed at significantly different levels between the two conditions (DI + M versus DI – M) ($p < 0.01$; at least a 5-fold change). Of the ~22,000 genes and ESTs that are represented on the chip, 218 met these criteria. The gene names of 149 clones within these 218 clones could be identified. The rest were ESTs from uncharacterized genes.

Most genes exhibiting higher expression levels under the DI – M condition were housekeeping genes, while many of the genes whose expression level was higher under the DI + M condition represent those critical for adipogenesis and adipose-specific metabolism (see Table S1 in the Supplemental Data available with this article online). For example, EST clones representing various fat-specific secreted factors such as resistin, adiponectin, and adipsin were upregulated by at least 20-fold when the cells were cultured in DI + M compared to DI – M. The inclusion of MIX in the differentiation cocktail also elevated the expression of C/EBPα and PPARγ, two of the most important transcription factors for adipogenesis, by 36-fold and 16-fold, respectively. Interestingly, adaptor protein with PH and SH2 domains (APS), an insulin receptor substrate involved in Cbl tyrosine phosphorylation, was also found to be highly expressed under DI + M conditions. In fact, APS had the highest DI + M/DI – M fold change (29×) among all the genes encoding signaling proteins. Phosphodiesterase 4B was also elevated with a 14-fold increase.

Integrin α subunit expression changes during the differentiation of 3T3-L1 preadipocytes

Both integrin α5 and α6 subunits were differentially expressed in response to MIX in the differentiation cocktail (Table S2). Because of the important role of these proteins in adhesion and cell fate determination, we tested whether there was an expression switch of integrin α5 to integrin α6 mRNA and protein during the progression of differentiation of 3T3-L1 preadipocytes. Cells were differentiated using the standard cocktail containing DEX/MIX/insulin/FBS. The expression of integrin α5 and α6 proteins at different time points of differentiation was determined by RT-PCR and immunoblotting assays. As shown in Figure 2A, α5 was highly expressed in preadipocytes and stayed at a similar level during the first 2 days of adipogenesis. However, we were unable to detect α6 expression during this period. Expression of α5 integrin was significantly decreased after 3 days of differentiation, while there was a dramatic increase in the expression of α6 over this time period. As controls, mRNA level of Rac1 and protein level of β-actin remained constant throughout the differentiation.

To determine what components in the differentiation cocktail were responsible for the apparent expression switch of integrin α subunits, we treated postconfluent preadipocytes for 3 days with different combinations of DEX, MIX, and insulin. Immunoblotting analysis revealed that the integrin switch was only induced by DEX/MIX/insulin or DEX/MIX (Figure 2B). Coincidently, these were also the two conditions under which preadipocytes can undergo differentiation to adipocytes. Interestingly, inclusion of H-89, a specific inhibitor for cAMP-dependent protein kinase (PKA), did not affect either expression switch of α integrins or differentiation of preadipocytes, as revealed by aP2 immunoblotting (Figure 2C). Taken together, the results suggest that the switch of expression pattern of α integrins may be controlled by a cAMP-dependent but PKA-independent pathway that is specifically associated with the differentiation of 3T3-L1 preadipocytes.

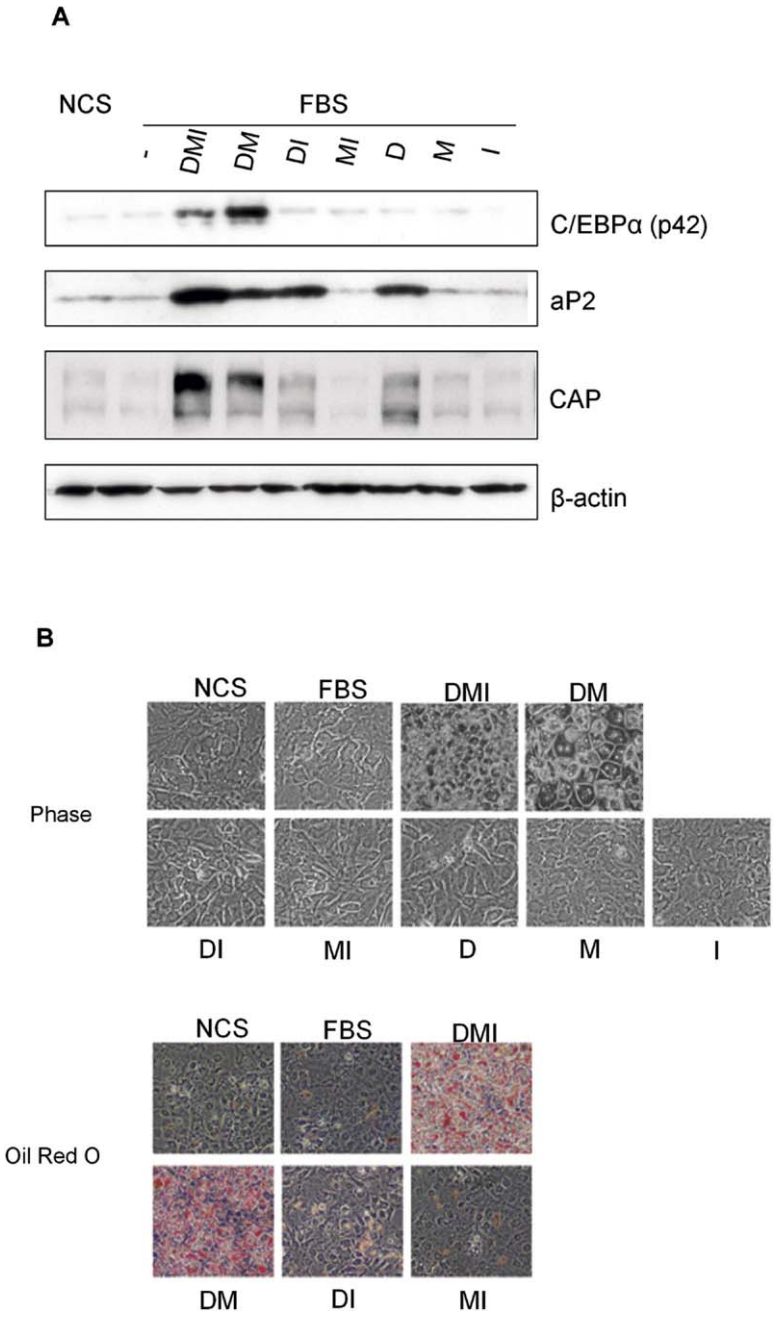


Figure 1. Differentiation of 3T3-L1 preadipocytes under different conditions

Preadipocytes were induced to differentiation with different combinations of DEX (D), MIX (M), and insulin (I) in the presence of FBS.

A) cell lysates were prepared on day 5 postdifferentiation induction. 20 μg of total protein were subjected to SDS-PAGE followed by immunoblot analysis for C/EBPα, aP2, CAP, and β-actin.

B) Phase-contrast images and Oil Red O staining of cells on day 5 of differentiation are shown.

To determine whether such an expression switch occurs in vivo, we isolated primary preadipocytes and adipocytes from mouse epididymal fat pad. Immunoblotting revealed aP2 expression in adipocytes but not in preadipocytes (Figure 2D). As a control, the expression level of flotillin-1 was comparable between these two populations. Consistent with the results obtained from 3T3-L1 cells, α5 integrin was expressed at a high level in preadipocytes but was undetectable in adipocytes. α6 integrin, on the other hand, was more abundant in adipocytes than in preadipocytes (Figure 2D).

To insure that the surface expression of integrin α subunits correlates with their total expression level, we analyzed both nonpermeablized preadipocytes and adipocytes by immunostaining using α5, α6, and β1 integrin antibodies specific for their extracellular domains. As shown in Figure 2E, integrin α5 was expressed on the cell surface at a high level in preadipocytes but at a considerably lower level in adipocytes. In contrast to α5, there was no appreciable surface expression of α6 integrin in preadipocytes, while a great amount of α6 was detected on the cell surface after differentiation. As control, β1 integrin was detected on the surface of both preadipocytes and adipocytes. Therefore, there is a switch in surface expression as well as total expression of α integrins during the differentiation process.

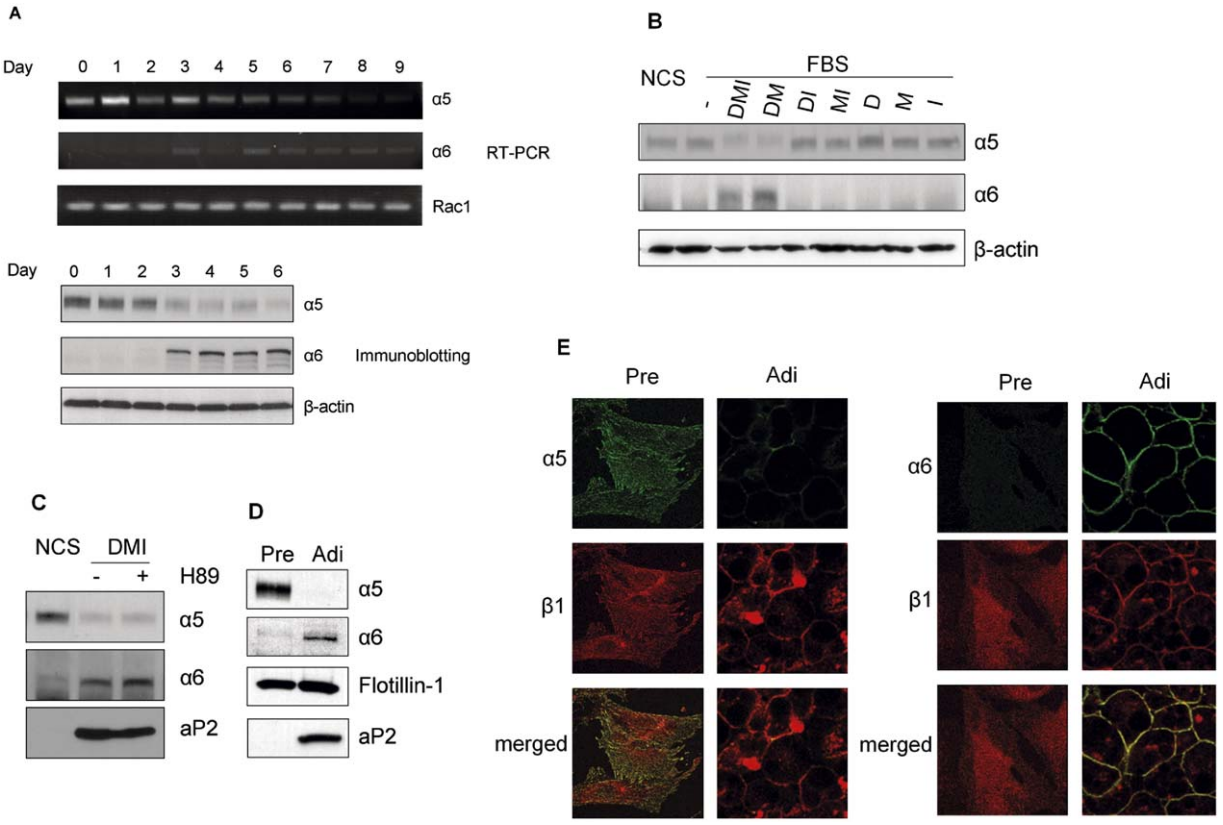


Figure 2. Expression switch of integrin α subunits during differentiation of preadipocytes

A) Total RNA or protein was extracted from cells at various days of differentiation. The levels of α5 and α6 integrins were analyzed by RT-PCR or immunoblotting.

B) Expression of α integrins was analyzed by immunoblotting in lysates from cells differentiated under different conditions for 5 days.

C) Cells were differentiated under DMI condition in the presence or absence of 5 μM H-89. Expression of α5, α6, and aP2 was revealed by immunoblotting of cell lysates.

D) Preadipocytes and adipocytes were isolated from mouse epididymal fat pad. Immunoblotting analysis was performed using antibodies against α integrins, flotillin-1, and aP2.

E) Preadipocytes and adipocytes were fixed and left unpermeablized. Integrin α5 and α6 was detected on the cell surface by immunostaining with FITC-conjugated antibodies that recognize their extracellular domains. β1 integrin was costained with an antibody against its extracellular domain followed by Alex594-conjugated secondary antibodies.

The α5 and α6 integrins act reciprocally to regulate proliferation and differentiation of preadipocytes

To address the role of integrins in adipogenesis, we attempted to stably express α5 and α6 subunits in 3T3-L1 preadipocytes and then differentiate these cells into adipocytes. The conventional methods for establishing stable cell lines have been inefficient for preadipocytes, largely because preadipocytes tend to lose differentiation potential throughout the numerous passages necessary for selection of drug-resistant clones. Thus, we resorted to a lentiviral-mediated system that has previously proven to be effective in final genetic modification of both dividing and nondividing cells. The lentiviral vector pHR-CMV-GFP, which contains the ubiquitously active cytomegalovirus (CMV) promoter to drive the expression of the GFP reporter gene, was used to evaluate gene transfer into 3T3-L1 preadipocytes. We exposed the replicating preadipocytes at low confluence to pHR-CMV-GFP at 5×10^7 particles per 10^5 cells. Seventy-two hours postinfection, over 95% of the cells were GFP positive (see Figure S1). No cytotoxicity was apparent, as the ability of the infected cells to differentiate into lipid droplet-gorged adipocytes remained intact. Next, we transduced preadipocytes with the lentiviral particles containing either α5 cDNA or α6 cDNA. The GFP virus served as a control for both integrins. Overexpression of integrin proteins in preadipocytes was demonstrated by immunoblotting. As shown in Figure 3A, an ~2-fold increase in the amount of α5 in the lysates from preadipocytes infected with α5 lentivirus was found when compared with that of endogenous protein in cells infected by GFP virus. A similar increase in the amount of α6 was observed when cells infected were differentiated to adipocytes (Figure 3D).

To investigate whether the integrin switch affects preadipocyte differentiation, we assayed the ability of the preadipocytes stably expressing α5 and α6 integrins to differentiate into adipocytes. By 5 days after the induction of differentiation, cells infected with either GFP virus or α6 virus were differentiated into adipocytes, as indicated by the appearance of a microscopic pattern of multilocular fat droplets. Oil Red O staining confirmed the accumulation of intracellular triglycerides in these cells. In contrast, cells overexpressing α5 integrin remained mostly fibroblastic with few fat droplets (Figure 3C). Immunoblotting showed that the expression of the adipocyte marker proteins C/EBPα and aP2 was decreased, though to a

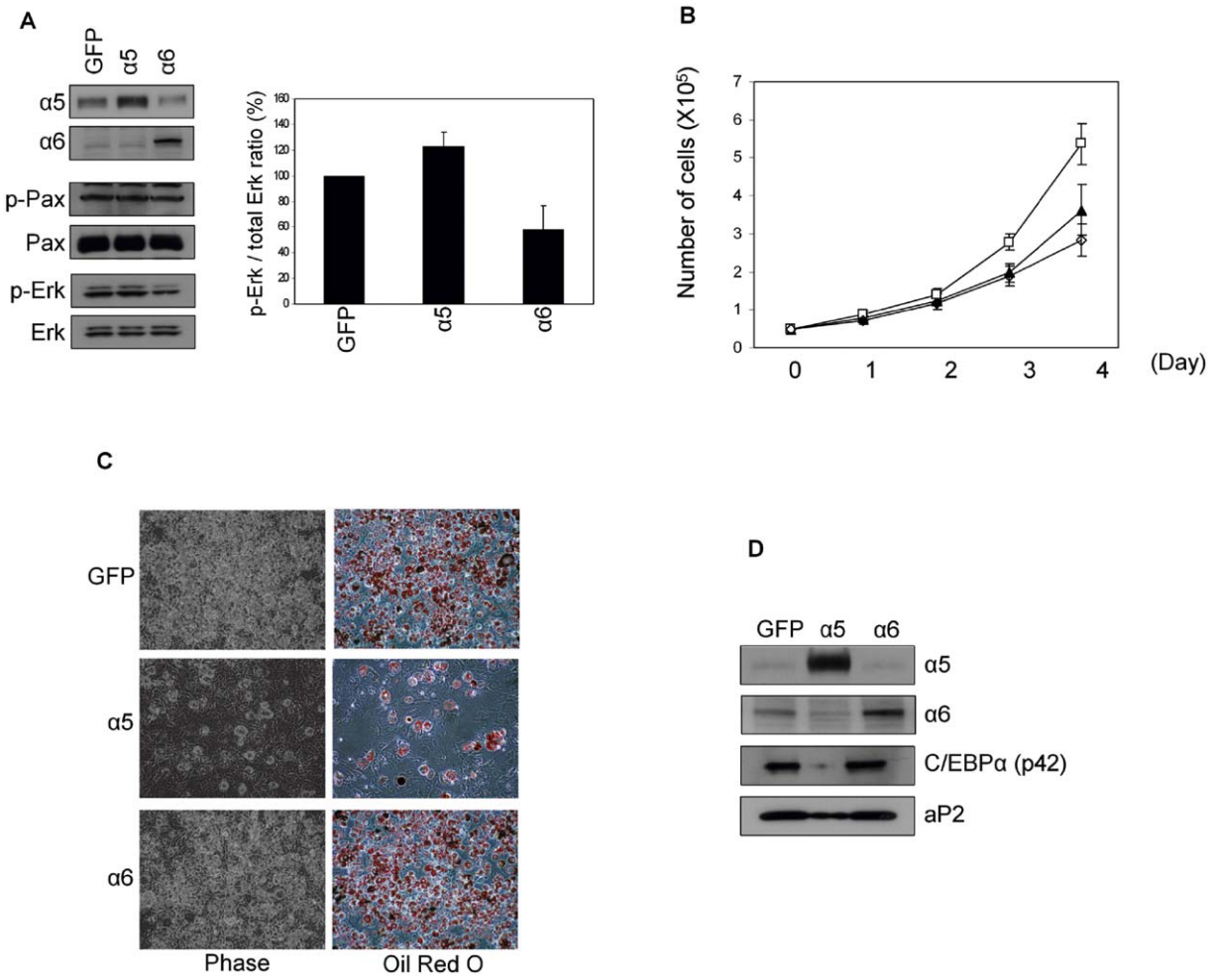


Figure 3. Proliferation and differentiation of 3T3-L1 cells overexpressing GFP, α5, and α6

A) Preadipocytes were transduced by lentiviral vectors expressing GFP, α5, or α6. Lysates were prepared from subconfluent preadipocytes and 20 μg of total protein was separated by SDS-PAGE. Integrins, pY118-Paxillin, total paxillin, phosphor-ERK, and total ERK were revealed by immunoblotting using specific antibodies. Quantification of ERK phosphorylation by densitometry analysis of two independent experiments is also shown (± SD).

B) Preadipocytes expressing GFP, α5, or α6 were plated at 0.5 × 10^5 in 60 mm dishes, and cell numbers were counted during different days of growth. The data reported are the means ±SD of results of three independent experiments. □, α5; ▲, GFP; ◇, α6.

C) Preadipocytes ectopically expressing GFP, α5, or α6 were induced to differentiation by DEX and MIX. The phase-contrast images and the Oil Red O staining of cells from day 7 postdifferentiation induction are shown.

D) Cell lysates were prepared day 7 postdifferentiation induction and analyzed by immunoblotting with antibodies against integrins, C/EBPα, and aP2.

different extent, in α5 cells compared with GFP or α6 cells (Figure 3D). This is consistent with the results of Oil Red O staining.

Because cell growth and differentiation are usually mutually exclusive, and preadipocytes undergo growth arrest before differentiation, we investigated whether ectopic integrin expression might affect the growth profile of 3T3-L1 preadipocytes. Figure 3B shows the growth curve of cells infected by α5 or α6 virus compared with the growth of GFP-expressing cells. The α5-expressing cells exhibited a significant increase in growth rate compared with GFP cells. All α5-expressing cells had a shorter doubling time (18 hr) and an increased growth rate compared with the GFP cells (doubling time, 23 hr). Conversely, the α6-expressing cell clones showed the opposite phenotype, with a slightly increased doubling time (25 hr) and a reduced growth rate. Therefore, the growth properties of α5- and α6-expressing cells were significantly different from the GFP-expressing cells.

A previous study using primary myoblasts has shown that ectopic α5 expression enhances activation of paxillin and MAP kinase, while ectopic α6 expression suppresses MAP kinase activation with a lesser effect on paxillin (Sastry et al., 1999). Since both paxillin and MAP kinase have been implicated in anchorage-dependent cell growth (Hagel et al., 2002; Howe et al., 2002; Turner, 2000a, 2000b), we examined whether ectopic integrin expression altered paxillin phosphorylation and MAP kinase activation in preadipocytes. MAP kinase activity was assessed by immunoblotting cell lysates with an antibody that specifically recognizes the phosphorylated active forms of the 42 and 44 kDa MAP kinase. As indicated in Figure 3A, α5 expression enhanced the level of active MAP kinase to a small degree compared with GFP expression. In contrast, the level of active MAP kinase in α6-expressing cells was decreased by ~55%. Paxillin phosphorylation was not significantly altered when either α5 or α6 was overexpressed.

Integrins regulate Rac activity in 3T3-L1 cells

As Rho GTPases transduce intracellular signals from growth factors as well as integrin receptors (Burridge and Wennerberg, 2004; Etienne-Manneville and Hall, 2002), we investigated whether ectopic expression of α5 and α6 integrins might affect the activity of RhoA and Rac. Lysates were prepared from postconfluent cells maintained in the regular growth media containing serum. GTP bound RhoA and Rac were isolated using the effector binding domain of rhotekin and Pak1, respectively. Active and total RhoA and Rac were normalized to total protein and analyzed by immunoblotting. As seen in Figure 4A, there was an appreciable amount of active Rac isolated from GFP cells. α5 expression increased Rac activity by nearly 3-fold, while α6 expression only had a modest effect. These results suggest that Rac may be involved in signaling downstream of the integrin switch. RhoA activity, on the other hand, was barely detectable in GFP cells from such a high-density culture. Expression of α5 or α6 integrins had no effect on RhoA activity (data not shown).

To assess the influence of cell density, we measured the activity of RhoA and Rac isolated from subconfluent and postconfluent preadipocytes. As was observed previously with mesenchymal stem cells (McBeath et al., 2004), increase in preadipocyte density dramatically diminished RhoA activity while slightly enhancing Rac activity (Figure 4B). As Rac positively regulates cyclin D1 expression (Coleman and Marshall, 2001; Mettouchi et al., 2001; Ridley, 2001), E2F stimulation, and pRb hyperphosphorylation (Gjoerup et al., 1998), we also evaluated whether Rho and Rac activity might be regulated during the differentiation of postconfluent 3T3-L1 cells. As shown in Figure 4B, Rac activity but not its expression level was downregulated upon differentiation. This change in Rac activity started 3 days after DMI induction, and the time course correlates well with the expression switch of integrin α subunits (Figure 4C). On the other hand, like in postconfluent preadipocytes, Rho activity remained hardly detectable in adipocytes (Figure 4B). Therefore, Rac activity appears to be regulated during the differentiation process, while RhoA activity is downstream of the change in preadipocyte density.

To determine the role of Rac in the adipogenic differentiation, we expressed either a constitutively active or a dominant-negative mutant of Rac (RacL61 or RacN17, respectively) in 3T3-L1 preadipocytes via lentiviral-mediated transduction. Both Rac mutants were fused at the N terminus with a single Myc-epitope tag. Immunoblotting with anti-Rac or Myc antibodies showed that both mutants were expressed at levels comparable to that of the endogenous proteins (Figure 4E). We then tested these cells for their ability to differentiate into mature adipocytes. After 5 days of differentiation induction, the phenotype of cells expressing either Rac mutant was compared with that of GPF-expressing cells. Oil Red O staining and immunoblotting of C/EBPα and aP2 showed that cells expressing RacN17 differentiated into adipocytes as well as those expressing GFP alone. In contrast, cells expressing RacL61 demonstrated fewer lipid droplets accompanied by a lower level of C/EBPα and aP2 expression (Figures 4D and 4E). Therefore, Rac negatively regulates the differentiation of 3T3-L1 preadipocytes, and the integrin switch appears to serve as a mechanism for shutting down Rac activity.

Morphological differentiation of growth-arrested preadipocytes depends on integrin α6

One of the key stages of de novo adipogenesis is the clustering of preadipocytes to form primitive fat tissue in which individual cells concomitantly adopt a round shape (Wasserman, 1965). To study the morphological behavior of preadipocytes, we replated 3T3-L1 cells 3 days after differentiation at a subconfluent density in various extracellular matrix environments. We found that cells migrated and aggregated to form a unique multicellular structure on top of the reconstituted basement membrane Matrigel. The cluster-like structure was formed at 6–8 hr after the cells were seeded, and it lasted 2–3 days (Figure 5A). This adipose tissue-like morphology was only formed on Matrigel, not on collagen I, indicating that a specific interaction between the integrin on 3T3-L1 cells and the ECM component in Matrigel is required for this morphogenetic process.

Matrigel is derived from Engelbreth-Holm-Swarm mouse sarcoma and is composed largely of basement membrane proteins including laminin, collagen IV, and proteoglycans. Since clustering of preadipocytes in vivo is likely mediated by adhesion and migration of preadipocytes on laminin, we included function-blocking antibodies in the culture media to test the involvement of integrin α6 in this process. As shown in Figure 5A, preincubation with integrin α6 mAb GoH3 inhibited the formation of the multicellular cluster structure of 3T3-L1 cells. Instead of clustering and adopting a round cell shape, cells dispersedly spread out and displayed a fibroblastic morphology. On the other hand, neither a nonspecific antibody nor a function-blocking mAb against integrin α5 had any effect, indicating that only laminin-integrin α6 engagement is required for this cellular morphogenesis.

We next examined the direct effects of integrin-blocking antibodies on preadipocyte spreading and migration. Cells from 3 days after differentiation attached and spread on Matrigel after 30 min of plating (Figure 5B). However, when plated in the presence of a function-blocking antibody against α6 integrin, cells attached but remained round. In contrast, α5-blocking antibody had no inhibitory effect on either cell attachment or spreading. Overall, less than 10% of the cells spread in the presence of α6 antibody, while close to 85% of the cells spread when no antibody or α5 antibody was included.

In order to examine the importance of α6 in preadipocyte migration, cells were seeded at a confluent density in a Matrigel-coated dish with its central area precovered by a coverslip. After 15 hr of incubation, the coverslip was removed and the cells were allowed to migrate as an inward growth into the resultant open space (Figure 5C). In the presence of either no antibody or α5-blocking antibody, cells were able to migrate into the open space and subsequently aggregate. In contrast, inclusion of α6-blocking antibody greatly reduced migration and aggregation. Therefore, integrin α6, presumably through its interaction with laminin, mediates attachment and migration of preadipocytes on Matrigel.

Integrin α6-mediated cell clustering regulates RhoA activity

Earlier studies have shown that downregulation of the RhoA-ROCK pathway is required for mesenchymal stem cells to undergo adipogenesis. As shown in Figure 4B, increase in preadipocyte density inhibited RhoA activity. Density-dependent cell shape change is believed to regulate cellular fate by modulat-

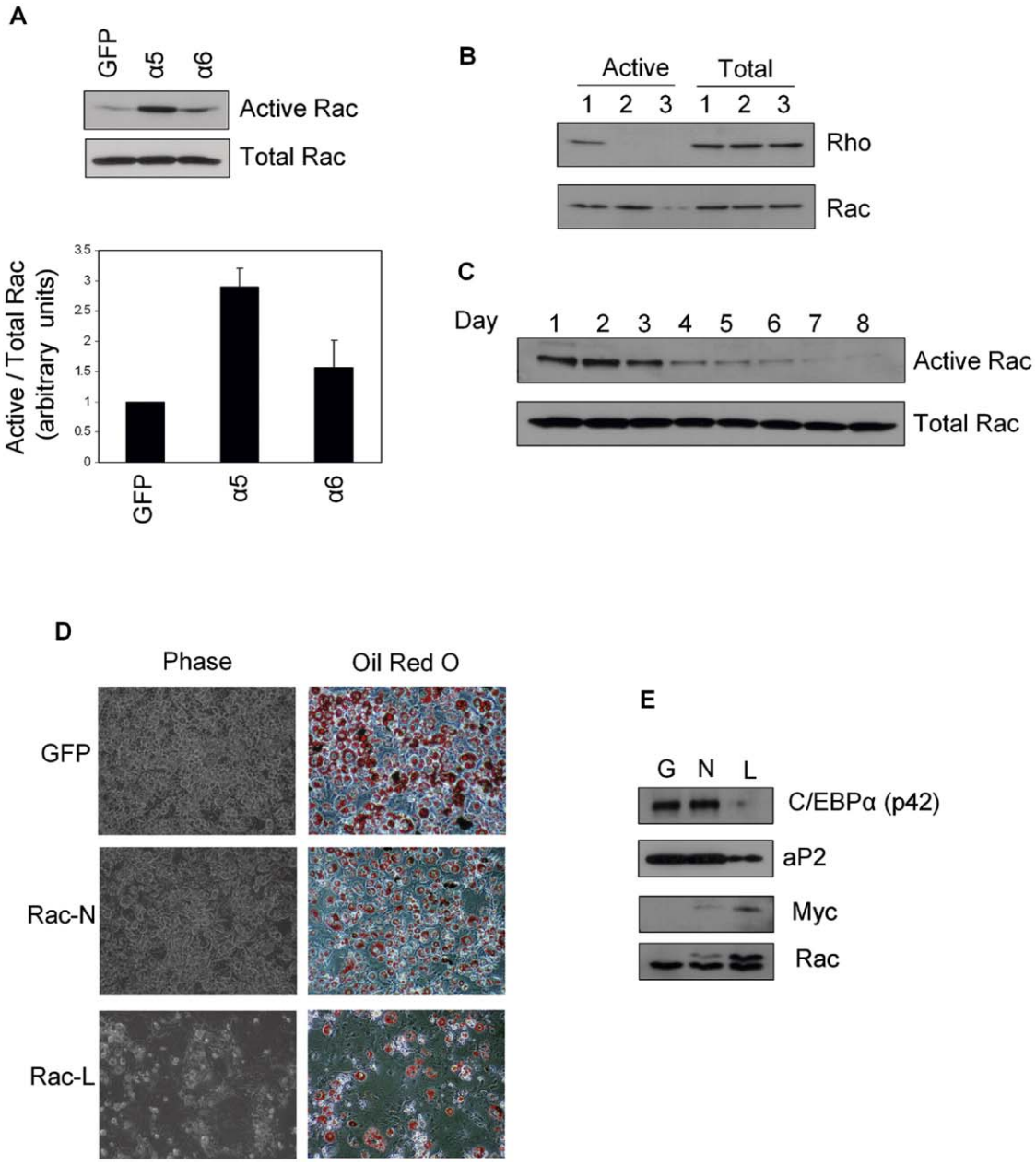


Figure 4. Integrin switch regulates Rac activity

A) Lysates were prepared from postconfluent preadipocytes ectopically expressing GFP, α5, or α6. Proteins pulled down by GST-PAK1 were separated by SDS-PAGE and then analyzed for bound Rac by immunoblotting. An aliquot of lysate was used for analyzing the total amounts of Rac. Quantification of active Rac by densitometry analysis of two independent experiments is also shown. Error bars represent SD.

B) Lysates were prepared from subconfluent preadipocytes (lane 1), postconfluent preadipocytes (lane 2), and adipocytes from day 7 postdifferentiation induction (lane 3). Rho and Rac proteins in their active forms were pulled down by GST-rhotekin and GST-PAK1, respectively, and were separated by SDS-PAGE followed by immunoblotting analysis. The amount of total Rho and Rac in the lysates was also revealed by immunoblotting.

C) Cells were collected at indicated times during differentiation, and Rac activity was measured by GST-PAK1 pulldown assays.

D) Constitutively active (RacL61) and dominant-negative (RacN17) mutants of Rac were expressed in preadipocytes via lentiviral mediated method. The phase-contrast images and Oil Red O staining of cells from day 7 postdifferentiation are shown.

E) Cell lysates were prepared and analyzed by immunoblotting with antibodies recognizing C/EBPα, aP2, Rac, and Myc.

ing RhoA activity (McBeath et al., 2004). We therefore investigated whether the cell density change resulting from integrin α6-mediated clustering could also negatively regulate RhoA activity in 3T3-L1 preadipocytes. We seeded cells from day 3 post DMI treatment at a subconfluent density on Matrigel in the presence of α5- or α6-blocking antibodies. After 8 hr of incubation, RhoA activity was analyzed by pull-down experiments using a GST-rhotekin protein. As shown in Figure 6A, integrin α6-blocking antibody significantly enhanced RhoA activity at all concentrations used when compared with no anti-

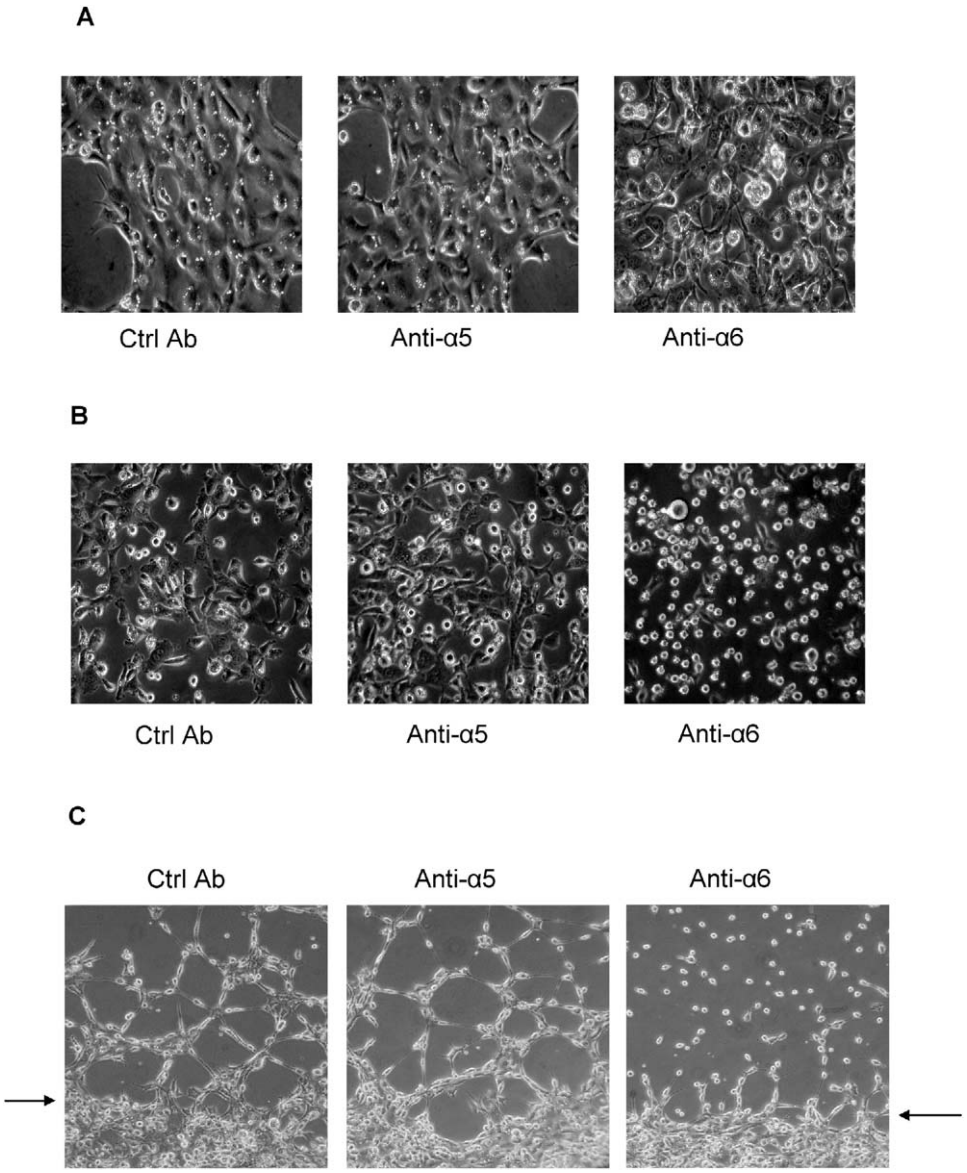


Figure 5. Clustering of growth-arrested preadipocytes on Matrigel depends on integrin α6

A) Preadipocytes from day 3 post DMI induction were replated at a subconfluent density on the surface of Matrigel for 8 hr and then photographed. Monoclonal antibodies against α integrins were added at 10 μg/ml at the beginning of the plating. Same concentration of transferrin receptor antibody was used as control.
B) Same as in (**A**), except that cells were photographed after 30 min of plating.
C) Preadipocytes from day 3 post DMI induction were allowed to migrate for 15 hr on Matrigel in the presence of different antibodies and then photographed. Arrows indicate the start point of migration.

body control. However, inclusion of α5-blocking antibody had no appreciable effect on RhoA activity. On the other hand, RhoA activity remained equally low when cells were plated at a confluent density, regardless of the type of blocking antibodies included. These findings suggest the possibility that, as was observed during high-density plating, integrin α6-mediated clustering of preadipocytes plated at low density downregulates RhoA activity.

Integrin α6-mediated cell clustering prevents growth-arrested preadipocytes from reentering the cell cycle

3T3-L1 preadipocytes are growth arrested at the G1 phase before terminal differentiation. Replating growth-arrested preadipocytes at a subconfluent density on a plastic surface or collagen 1 gel resulted in reentry of the cells into the cell cycle and subsequent proliferation (data not shown). To assess the effect of cell clustering on Matrigel on cell cycle reentry, we performed a colorimetric BrdU-incorporation ELISA to quantify cells undergoing proliferation. As shown in Figure 6B, cells treated with integrin α6-blocking antibody demonstrated significantly enhanced proliferation after 8 hr of seeding on Matrigel compared with untreated control cells (41% decrease). Integrin α5-blocking antibody did not affect mitotic activity, as proliferation did not differ between control and treated cells. Therefore, in addition to its morphogenetic effect, integrin α6

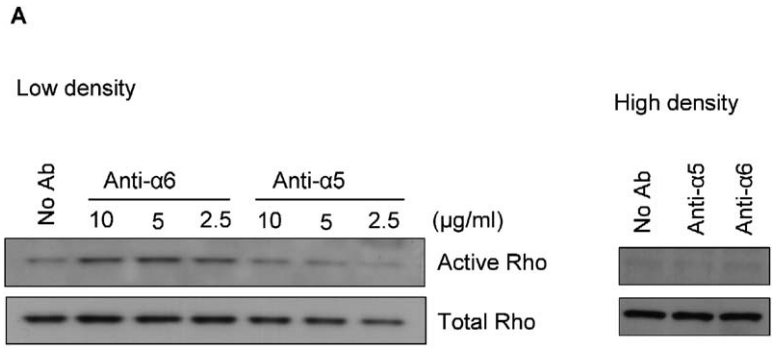
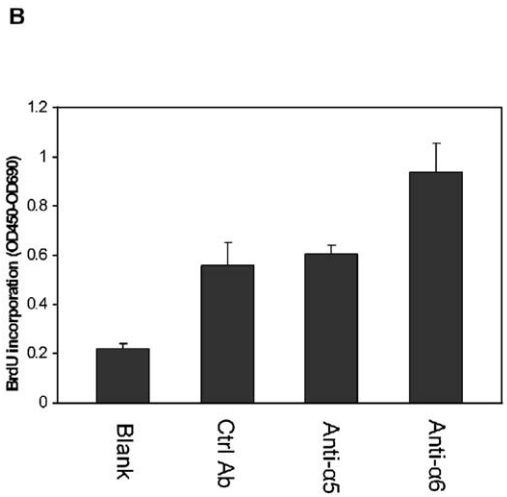


Figure 6. Clustering-induced downregulation of RhoA activity and cell cycle exit are dependent on cell density

A) Growth-arrested preadipocytes were plated at low or high density on Matrigel in the presence of different amounts of integrin antibodies. 8 hr after plating, cells were dispersed with MatriSperse Cell recovery solution, lysed, and incubated with GST-rhotekin. Proteins were separated by SDS-PAGE and then analyzed for bound RhoA molecules by immunoblotting. An aliquot of lysate was used for analyzing the total amounts of RhoA.

B) Preadipocytes from day 3 post DMI induction were replated at a subconfluent density on the surface of Matrigel for 8 hr followed by a 4 hr labeling period with BrdU. Then, cell proliferation was analyzed by Cell Proliferation ELISA BrdU (colorimetric). Error bars depict SD.



prevents growth-arrested preadipocytes from reentering cell cycle when plated at low density on Matrigel.

Discussion

Adipocyte differentiation is a multistep process in which mesenchymal stem cells first commit to becoming preadipocytes, and then preadipocytes differentiate into mature adipocytes (Ailhaud et al., 1992; Burdi et al., 1985; Cornelius et al., 1994). A hormonal regimen widely used to differentiate the model preadipocyte line 3T3-L1 in vitro usually includes cAMP phosphodiesterase inhibitors such as MIX. It is well established that cAMP enhances expression of both C/EBPα and C/EBPβ (Cao et al., 1991; Tang et al., 1999). It was also shown recently that cAMP initiates adipogenesis via the transcription factor cAMP response element binding factor (CREB) (Reusch et al., 2000). However, other findings have suggested an inhibitory role of α subunit of the stimulatory G protein (G_S) regarding differentiation of 3T3-L1 cells (Gettys et al., 1991; Wang et al., 1992). Thus, Gsα activity modulates adipogenesis independent of increased intracellular cAMP. Data presented here indicate that DEX and MIX are both required for upregulation of C/EBPα expression and eventual differentiation of 3T3-L1 preadipocytes, although DEX alone appears sufficient for the activation of PPARγ expression. A previous study by Qiu et al. has suggested that insulin is not necessary for inducing adipogenesis in 3T3-L1 cells (Qiu et al., 2001). However, our results do not rule out the role of insulin because of its likely presence in the FBS.

To uncover the specific gene expression programs that are controlled by cAMP, we conducted gene array analyses to evaluate multigenic expression profiles that were altered by inclusion versus exclusion of MIX in the prodifferentiative medium. Many of the genes whose expression was induced by MIX have previously been classified with regard to cell cycle regulation or/and adipogenic differentiation. Among these are C/EBPα and PPARγ, the two master regulators of adipogenesis. However, others have not been classified and should serve as basis for further studies on the identification and characterization of additional genes involved in adipocyte differentiation and metabolism.

The onset of adipogenesis is usually defined by extracellular matrix remodeling, characterized by the conversion from the fibronectin-rich stromal matrix to the laminin-rich basement membrane (Mandrup and Lane, 1997; Selvarajan et al., 2001; Smas and Sul, 1995). This is consistent with the idea that pre-

adipocytes lose their fibroblastic morphology as they turn into round adipocytes. Growth of preadipocytes on a fibronectin matrix inhibits adipocyte differentiation, and this effect is overcome by the addition of cytochalasin D, which disrupts actin filaments and promotes rounding-up of cells (Spiegelman and Ginty, 1983). The interplay between the changing ECM, cytoskeleton, and cell shape and gene expression programs during adipocyte differentiation is not yet understood. Here, we elaborate the roles of integrin α5 and α6, respective receptors for fibronectin and laminin, in proliferation and differentiation of 3T3-L1 preadipocytes. We report the presence of an expression switch from α5 to α6 at the growth arrest stage of differentiation, which is consistent with the ECM change during adipogenesis. Moreover, ectopic expression of α5 or α6 integrin produced contrasting phenotypes in proliferation/differentiation of preadipocytes.

Numerous studies have associated integrin α5 with anchorage-dependent proliferation of keratinocytes, epithelial, and muscle cells (Adams and Watt, 1990; Menko et al., 1998). Overexpression of integrin α5 in the integrin α5β1-negative intestinal epithelial cell line Caco-2 results in activation of EGFR and cell proliferation (Kuwada and Li, 2000). Moreover, ectopic α5 expression prevents myoblast cell cycle withdrawal via stimulation of MAP kinase and paxillin tyrosine phosphorylation (Sastry et al., 1999). We show here that although it promotes proliferation and inhibits differentiation of 3T3-L1 cells, α5 expression had only a minor effect on MAP kinase activity and no effect on paxillin phosphorylation in preadipocytes. The discrepancy between our results and previous findings may be caused by the difference in cell type and/or the difference in plating conditions. While cells in other studies were generally plated on a fibronectin-coated surface (Kuwada and Li, 2000; Sastry et al., 1999), α5-expressing preadipocytes used in our study were maintained on plastic surface in the presence of serum. The latter is also the condition under which 3T3-L1 cells are routinely grown and differentiated. In fact, activation of the EGFR and MAP kinase by EGF in α5-transfected Caco-2 cells was strictly dependent on plating of cells on fibronectin (Kuwada and Li, 2000). Therefore, in the absence of exogenous fibronectin, the effect of α5 expression in preadipocytes on cell spreading and activation of MAP kinase may not be significant when compared with that of α6 or GFP.

In a search for the mechanism underlying the enhanced proliferation and impaired differentiation of integrin α5-expressing cells, we reasoned that such a mechanism should be specific for integrin α5, since differentiation was achieved to the same extent in α6 cells as in control cells. Rho family GTPases are molecular switches transducing signals from integrin and growth factor receptors. Cell adhesion to fibronectin but not laminin is particularly efficient in activating the Rho family GTPase Rac, a process likely to be mediated by the guanine nucleotide exchange factor SOS (Mettouchi et al., 2001). In this study, we provide strong evidence to show that Rac might be one of the key proteins working downstream of integrin α5 to maintain preadipocytes in an undifferentiated state. First, Rac activity was stably enhanced by ectopic expression of α5 integrin in preadipocytes. Coincident with decreased expression of α5 integrin, there is a down-regulation of Rac activity during the differentiation of 3T3-L1 preadipocytes. Finally, overexpression of an active but not a negative mutant of Rac greatly decreased adipogenesis.

It is now well known that Rho GTPases regulate the actin cytoskeleton and cell adhesion, as well as activities such as cell cycle progression, differentiation, and gene transcription (Burridge and Wennerberg, 2004; Etienne-Manneville and Hall, 2002). Here, we demonstrate that downregulation of Rac activity occurs at the growth-arrest stage of adipogenic differentiation, whereas RhoA activity appears to be regulated by preadipocyte density rather than during the differentiation process per se. There are three explanations to account for the necessity to down-modulate Rac activity in adipogenesis. First, Rac may prolong proliferation of preadipocytes, which is consistent with the reported effects of Rac on cyclin D1(Coleman and Marshall, 2001; Mettouchi et al., 2001; Ridley, 2001), c-Myc (Chiariello et al., 2001), E2F stimulation, and pRb hyperphosphorylation (Gjoerup et al., 1998). Second, by promoting cell adhesion and spreading, Rac prevents the cell shape change and the establishment of the cortical actin structure necessary for adipocyte formation. Adhering cells are characterized by an elaborate network of stress fibers and focal adhesions, and thus are more prone to adopt a fibroblastic cell shape. In addition, cell adhesion and spreading in part reflect cytoskeleton tension (Huang et al., 1998; Huang and Ingber, 1999). In this regard, changes in cell shape and tension were shown previously to be determining factors for the adipogenic differentiation of mesenchymal stem cells (McBeath et al., 2004). Third, there is accumulating evidence for an important role of Rac-induced dynamics in nuclear processes, notably in chromatin remodeling (Rando et al., 2000). There is also strong evidence that cycles of actin polymerization and depolymerization can regulate gene transcription (Sotiropoulos et al., 1999). In summary, we thus postulate that Rac may act to modify the differentiation program of preadipocytes at multiple levels.

In contrast to the α5 subunit, the expression of α6 integrin is often linked to the differentiation of many cell types (Frade et al., 1996; Kadoya et al., 1995; Menko et al., 1998; Sastry et al., 1996; Selvarajan et al., 2001). During the differentiation of 3T3-L1 cells, expression of α6 integrin is correlated with the growth arrest of preadipocytes. As was observed in myoblasts, ectopic expression of α6 integrin in proliferating preadipocytes decreased the level of active MAP kinase and the rate of cell growth. This result suggests that α6 integrin also functions in favor of differentiation over proliferation of preadipocytes. Since overexpression of α6 integrin alone was not sufficient to replace MIX in inducing adipogenic differentiation (data not shown), it is likely that additional mechanisms must also be required.

In several prior studies, Matrigel has been used as a basement membrane model for de novo adipogenesis (Hausman et al., 1996; Kawaguchi et al., 1998; O'Connor et al., 2003; Tabata et al., 2000; Viravaidya and Shuler, 2002). Our results show that growth-arrested preadipocytes can form high-density cell clusters when plated at a subconfluent density on Matrigel, resembling a multicellular morphology adopted by cells in adipose tissue. Cell clustering has at least three phases: cell adhesion, cell migration, and cell-cell contact. Antibody inhibition assays further demonstrate that integrin α6 contributes to the clustering process by mediating adhesion and migration of preadipocytes on Matrigel, presumably through its interaction with laminin. Interestingly, integrin α6 together with tetraspanin/CD151 mediates the branching morphogenesis by endothelial cells on Matrigel in an established model of angiogen-

esis (Kazarov et al., 2002; Zhang et al., 2002). Therefore, proteins other than α6 integrin may also be involved in establishing the unique morphology for different cell types.

What are the biological roles of such preadipocyte clustering? It has been shown that downregulation of the RhoA-ROCK pathway is required for both adipose lineage commitment of mesenchymal stem cells and differentiation of precommitted preadipocytes (McBeath et al., 2004; Sordella et al., 2003). Among the many cues required for adipogenic differentiation in vitro, increased cell density appears to be critically important for downregulation of RhoA activity (Figure 4B) (McBeath et al., 2004). While cells can be easily grown to postconfluence in vitro, we propose that integrin α6-mediated cell clustering on basement membrane might be a mechanism for preadipocytes to achieve density in vivo. Indeed, we find that integrin α6-dependent clustering of subconfluent preadipocytes on Matrigel can negatively affect RhoA activity, as previously observed with cells plated at a confluent density. Such clustering may also assist in establishing contact inhibition and therefore prevents growth-arrested preadipocytes from reentering the cell cycle.

Experimental procedures

Antibodies and reagents

The C/EBPα (14AA), Myc (C-19), integrin α5 (H-104), and α6 (H-87) polyclonal antibodies were purchased from Santa Cruz Inc. The aP2 polyclonal antibody was kindly provided by Dr. David A. Bernlohr. The monoclonal blocking antibodies against α5 (5H10-27) and α6 (GoH3) were from BD Biosciences. Monoclonal β-actin antibody was obtained from SigmaAldrich. The Rac, Rho, and CAP antibodies were from Upstate Biotechnology, Inc. The paxillin, phosphor-paxillin, ERK, and phosphor-ERK antibodies were purchased from Cell Signaling. Matrigel Basement Membrane Matrix and Cell Recovery Solution were from BD Biosciences. Horseradish peroxidase-linked secondary antibodies were from Pierce Chemical Co. The Alexa Fluor secondary antibodies and phalloidin were from Molecular Probes. Polybrene were purchased from SigmaAldrich. The protease inhibitor mini tablets were obtained from Roche Diagnostics. The GST-PAK1 and GST-rhotekin agarose beads were from Cytoskeleton. The Cell Proliferation and ELISA Kit was from Roche Applied Science. Vectashield mounting medium was purchased from Vector Laboratories. Lipofectamine 2000 transfection reagent and reagents for tissue culture were obtained from Invitrogen.

Construction of lentiviral vectors containing α5, α6, and Rac cDNAs

To generate HIV-based lentiviral vectors, a typical three-plasmid expression system was used (Naldini et al., 1996) with modifications stated elsewhere (Wu et al., 2000). In brief, the original packaging construct pCMV-gag-pol was modified to contain the tetracycline (Tc)-response element (TRE, PCR amplified from pTRE-d2EGFP, clontech 6242-1) in replacement of the CMV promoter to obtain plasmid pTRE-gag-pol. In the presence of Tc-responsive transcriptional activator (tTA) expressed by pTet-Off plasmid (Clontech K1620-A), TRE drives expression of all viral elements required in *trans* except viral envelope and packaging sequence (ψ). The envelope expression plasmid contains a CMV-driven VSVG gene. The original transducing plasmids include *cis*-acting sequences of HIV required for packaging, reverse transcription, and integration, as well as various cDNA for enhanced green fluorescence protein (pHR-CMV-GFP), integrin α5 (pHR-CMV- α5), or integrin α6 (pHR-CMV- α6) that were subsequently used in lentivirus preparations.

The mouse α5 and α6 cDNAs were generated by RT-PCR. In brief, total cellular RNA was purified from 3T3-L1 preadipocytes, and adipocytes using the TRIzol reagent (Invitrogen). cDNA was synthesized with the SuperScript First-Strand Synthesis System from Invitrogen. Full-length α5 cDNA was obtained by one-step PCR (25 cycles) and was subcloned into BamHI and XhoI sites of pHRCTS-CMV-WPRE following digestion with *Bcl*I and *Xho*I. α6 cDNA was obtained by sequential ligation of two PCR fragments, flanked by *Bgl*II/*Xho*I and *Xho*I/*Sal*I, respectively, into pHRCTS-CMV-WPRE. Rac cDNAs fused at N terminus with Myc tag were derived from pRK5-Myc-Rac constructs by PCR and subcloned into pHRCTS-CMV-WPRE following *Bam*HI and *Xho*I digestion. For quantitative purpose, cDNA was synthesized as described above and used as a template for 15 cycles of PCR.

Cell culture

293T/17 cells (ATCC) were cultured in DMEM containing 10% fetal bovine serum (FBS). Mouse 3T3-L1 preadipocytes were maintained in DMEM supplemented with 10% calf serum, 100 U/mL penicillin G sodium, and 100 μg/mL streptomycin sulfate. Differentiation to adipocytes was induced by treatment of postconfluent cells with 10% FBS, 1 μg/mL insulin, 1 μM dexamethasone (DEX), and 0.5 mM isobutyl-1-methylzanthine (MIX). The differentiation medium was withdrawn 3 days later and replaced with medium supplemented with 10% FBS and 1 μg/mL insulin. After 2 days in insulin-containing medium, the cells were then cultured in DMEM containing 10% FBS.

Isolation of mouse primary preadipocytes and adipocytes

Isolated epididymal fat tissue was rinsed briefly in Krebs Ringer Buffer (KRB; 25 mM HEPES [pH 7.6], 120 mM NaCl, 5 mM KCl, 1 mM KH_2PO_4, 1 mM $MgSO_4$, 2.5 mM $CaCl_2$, and 2% BSA) and then minced in collagenase solution (1 mg/ml collagenase and 2 mM glucose in KRB). The mixture was then incubated in a shaking waterbath at 37°C for 30 min. The resulting cell suspension was filtered twice through a 100 μM cell strainer. The filtrate was allowed to settle for 3 min to separate into a top adipocyte layer, and an infranatant containing preadipocytes. The infranatant was drawn off with a needle and saved, while the adipocytes were further washed with KRB and allowed to settle as above. This process was repeated three to four times, until the infranatant was clear. The resulting adipocytes were then immediately lysed and analyzed by Western blotting.

To further purify the preadipocytes, infranatants were combined and centrifuged at 150 g. The pellet was resuspended in media and filtered through a 70 μM cell strainer. The filtrate was centrifuged again and resuspended in erythrocyte lysis buffer (154 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA) and incubated at room temperature for 10 min. The cell suspension was centrifuged at 100 g and then resuspended in KRB or lysed for Western blot analysis

Production of recombinant lentivirus and infection of 3T3-L1 preadipocytes

The lentiviral vectors (10 μg) were transfected into 293T cells cultured in a 15 cm dish together with the packaging mix (22 μg) by using Lipofectamine2000 from Invitrogen. The culture medium was refreshed after 15 hr and was collected at 60 hr posttransfection. The viruses were passed through 0.45 min filters and further concentrated by ultracentrifugation at 25,000 rpm for 90 min in a SW-28 rotor (Beckman). The viral pellets were resuspended in appropriate volumes of growth medium and then were used to infect 3T3-L1 preadipocytes. For this purpose, 3T3-L1 preadipocytes were usually seeded in a 6 cm dish overnight and were cultured subsequently in infectious media containing 8 μg/mL of polybrene for 15 hr.

Cell lysis and immunoblotting

3T3-L1 preadipocytes and adipocytes in 60 mm dishes were washed twice with ice-cold phosphate-buffered saline, and were lysed for 30 min at 4°C with buffer containing 50mM Tris-HCl (pH 8.0), 135mM NaCl, 1% Triton X-100, 1.0mM EDTA, 1.0mM sodium pyrophosphate, 1.0mM sodium orthovanadate, 10mM NaF, and protease inhibitors (1 mini tablet per 7 ml of buffer). The clarified lysates were mixed with 2× SDS sample buffer. Solubilized proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Individual proteins were detected with the specific antibodies and visualized by blotting with horseradish peroxidase-conjugated secondary antibodies.

Confocal fluorescence microscopy

Cells were routinely grown on glass cover slips in 6-well dishes. Following the fixation with 10% formalin for 20 min, cells were permeabilized with 0.5% Triton X-100 for 5 min, and were then blocked with 1% BSA and 1% ovalbumin for 1 hr. Primary and Alexa Fluor secondary antibodies were used at 2 μg/μl in blocking solution, and samples were mounted on glass slides

with Vectashield. Cells were imaged using confocal fluorescence microscope (Olympus IX SLA).

Rac and Rho activity assays

Rac and Rho activity assays were performed as previously described (Benard et al., 1999; Ren and Schwartz, 2000). In brief, 10^7 cells were grown in 10 cm dishes, washed in cold phosphate buffered saline, and lysed on ice in lysis buffer (50 mM Tris-HCl, [pH 7.4], 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10% glycerol, 5 mM $MgCl_2$, and protease inhibitors). Cleared lysates were incubated for 30 min at 4°C with glutathione S transferase (GST)-PAK1 or GST-rhotekin beads to precipitate GTP-bound Rac and Rho, respectively. Precipitated complexes were washed three times in lysis buffer and boiled in sample buffer. Total lysates and precipitates were analyzed on Western blot using monoclonal antibodies against Rac and RhoA.

Oil Red O staining

Intracellular triglyceride was stained by Oil Red O as described previously (Kasturi and Joshi, 1982). Cell monolayer was fixed in 10% buffered neutral formalin for 24 hr, rinsed with water followed by 70% ethanol, and stained with Oil Red O solution for 15 min. Excess stain was removed by washing with 70% ethanol until no more dye diffused out. The stained cells were washed finally with water before photographed under light microscope.

Measurement of cell proliferation using BrdU ELISA

The assay was performed using a colorimetric BrdU cell proliferation enzyme-linked immunosorbent assay kit (Roche). Aliquots of 10 ml BrdU labeling solution were added to each well 8–24 hr after seeding of the cells, and the cells were reincubated for 4 hr. After removal of the culture medium, the cells were fixed, and the DNA was denaturated in one step. Subsequently, the anti-BrdU–peroxidase conjugate was added and bound to the incorporated BrdU in newly synthesized cellular DNA. The immune complexes were detected by subsequent reaction with tetramethylbenzidine as substrate for 20 min. The reaction was stopped by addition of 25 ml 1 M H_2SO_4, and the reaction product was quantified by measuring the absorbance at 450 nm with reference to 690 nm using a Spectramax 250 plate reader (Molecular Devices Corporation).

Supplemental data

Supplemental Data include one figure and two tables and can be found with this article online at http://www.cellmetabolism.org/cgi/content/full/2/3/165/DC1/.

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