Biochemical and Biophysical Research Communications **275,** 115–120 (2000)
doi:10.1006/bbrc.2000.3201, available online at http://www.idealibrary.com on IDEAL®

# Retinoblastoma Protein Phosphorylation via PI 3-Kinase and mTOR Pathway Regulates Adipocyte Differentiation

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Received July 5, 2000

**In the early phase of adipocyte differentiation, transient increase of DNA synthesis, called clonal expansion, and transient hyperphosphorylation of retinoblastoma protein (Rb) are observed. We investigated the role of these phenomena in insulin-induced adipocyte differentiation of 3T3-L1 cells. Insulin-induced clonal expansion, Rb phosphorylation and adipocyte differentiation were all inhibited by the PI 3-kinase inhibitors and rapamycin, but not the MEK inhibitor, whereas the MEK inhibitor, but not PI 3-kinase inhibitors or rapamycin, decreased c-fos induction. We conclude that insulin induces hyperphosphorylation of Rb via PI 3-kinase and mTOR dependent pathway, which promotes clonal expansion and adipocyte differentiation of 3T3-L1 cells.** 

*Key Words:* **clonal expansion; adipocyte differentiation; retinoblastoma protein; mTOR; PI 3-kinase; 3T3-L1 adipocyte; insulin.**

Adipocyte differentiation of 3T3-L1 cells is induced by various drugs and hormones, such as dexamethazone (Dex), isobutyl methylxanthine (IBMX), thiazolidine derivatives, thyroid hormone and insulin (1, 2). Before the initiation of differentiation, 3T3-L1 cells have to be rendered confluent, withdraw from the cell cycle and arrest in the G1 phase (3). In the early phase of the differentiation process, the cells transiently reenter the cell cycle, resulting in a transient increase in DNA synthesis and several rounds of cell division. This phase of differentiation, called mitotic clonal expansion, was reported to be essential to complete the differentiation process (4, 5).

Abbreviations used: PI 3-kinase, phosphatidyl inositol 3-kinase; mTOR, mammalian target of rapamycin; Rb, retinoblastoma protein; IBMX, isobutyl methylxanthine.

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Insulin initiates intracellular signals by binding to its receptor, thereby activating intrinsic tyrosine kinase activity of the receptor and stimulating tyrosine phosphorylation of intracellular substrate proteins, which serve as adapter proteins to recruit various SH2 containing signaling molecules. Two major elements of insulin signaling pathway consist of PI 3-kinase and ras-MAP kinase pathways, which lead to pleiotropic biological effects including adipocyte differentiation. Several studies have examined the mechanism of insulin-induced adipocyte differentiation. For example, Tomiyama *et al.* showed that wortmannin, an inhibitor of PI 3-kinase, inhibits differentiation of 3T3-L1 cells (6). The importance of PI 3-kinase pathway was also demonstrated by a study in which expression of dominant negative mutants of PI 3-kinase inhibited adipocyte differentiation (7). Akt, a serine/threonine kinase downstream of PI 3-kinase, was also reported to be important in adipocyte differentiation, since the expression of a constitutively active form of Akt caused spontaneous differentiation of 3T3-L1 cells in the absence of insulin (8, 9). Yeh *et al.* reported that treatment of 3T3-L1 cells with rapamycin, an inhibitor of mTOR signaling pathway, inhibited adipocyte differentiation and reduced the extent of clonal expansion as assessed by the increase of cell number (10).

Retinoblastoma protein (Rb) is a nuclear phosphoprotein that negatively regulates growth by inhibiting cell cycle at G1 phase. Rb exerts its growth-inhibitory effects in part by binding to and inhibiting critical regulatory proteins including members of E2F family of transcription factors. The activation of E2F is necessary for the G1-S transition and the expression of various growth-related genes (11, 12). Rb is hyperphosphorylated by cyclin-Cdk complexes and becomes inactive, thereby releasing E2F. Recently, Chen *et al.* demonstrated that Rb is necessary for adipocyte differentiation (13). Rb is transiently hyperphosphorylated in the early phase of adipocyte differentiation (3) and the time course of Rb phosphorylation resembles that



0006-291X/00 $35.00

of clonal expansion. These observations raise a possibility that clonal expansion is induced by the inactivation of Rb. However, the mechanism and the role of Rb phosphorylation in clonal expansion and insulin-induced adipocyte differentiation are not well understood.

In this study, we provide an evidence that insulin induces hyperphosphorylation of Rb via PI 3-kinase and mTOR dependent pathway, which appears to be essential for clonal expansion and adipocyte differentiation.

## MATERIALS AND METHODS

*Materials.* A rabbit polyclonal anti-c-fos antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A mouse anti-Rb antibody was from PharMingen (San Diego, CA). Bromodeoxyuridine (BrdU), an anti-BrdU antibody and Chemiluminescence reagents were obtained from Amersham Corp. (Arlington Heights, IL). Rhodamine-conjugated anti-rabbit IgG and Fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG antibodies were obtained from Jackson Laboratories (West Grove, NY). PD98059 was from New England Biolabs, Inc. (Beverly, MA). LY294003, wortmannin and rapamycin were purchased from Sigma (St. Louis, MO). Electrophoresis reagents were from Bio-Rad (Hercules, CA). All other reagents were from standard suppliers.

*Cell culture condition.* 3T3-L1 cells were purchased from American Type Culture Collection (ATCC) (Rockville, MD) and maintained in Dulbecco's modified Eagle's medium (DMEM; high glucose) supplemented with 10% DCS (donor calf serum) in a 10% $CO_2$ environment prior to differentiation protocol. Differentiation into adipocytes were carried out as described previously (14) with some modifications. Briefly, confluent monolayers of 3T3-L1 cells were incubated in differentiation medium, i.e., DMEM (high glucose) containing various combinations of 500 $\mu$M 3-isobutyl-1-methylxanthine (IBMX), 0.4 $\mu$g/ml dexamethazone, and 5 $\mu$g/ml insulin for 2 days (serum was not changed during this period), followed by incubation in DMEM with freshly added 10% FCS and 5 $\mu$g/ml insulin for 2 days. Then, the medium was changed to DMEM with 10% FCS every other day until the cells were used for experiments.

*Oil Red O staining.* Nine days after the initiation of differentiation, the rate of adipocyte differentiation was evaluated by lipid staining with Oil Red O. 3T3-L1 cells on a coverslip were washed with PBS three times and fixed for 1 h in 10% formaldehyde. Oil Red O stock solution (0.5% Oil Red O in isopropanol) was diluted with equal volume of distilled water and filtered, then added to the fixed cells for 15 min. After washing with distilled water twice, the cells were mounted and observed under microscope.

*BrdU incorporation assay.* Twenty hours after the initiation of differentiation, 3T3-L1 cells on a coverslip were incubated with BrdU for 6 h at 37°C. The cells were fixed with 3.7% formaldehyde in PBS for 20 min at 22°C. The fixed cells were permeabilized and blocked in a buffer containing 0.5% Nonidet P-40 and 5% BSA in PBS for 15 min at 22°C. Then, the cells were incubated with rat polyclonal anti-BrdU antibody in a buffer containing 10 mM $MgCl_2$ and deoxyribonuclease I for 1 h at 22°C. After rinsing twice with PBS, the cells were incubated with rhodamine-labeled donkey anti-rat IgG antibody for 1 h at 22°C. The coverslips were mounted and the cells were analyzed with a Microphot-FXA fluorescence microscope (Nikon, Tokyo, Japan).

*c-fos induction assay.* Ninety min after the initiation of differentiation, 3T3-L1 cells on a coverslip were fixed with 3.7% formaldehyde in PBS for 15 min at 22°C. The fixed cells were permeabilized in a buffer containing 0.3% Triton X-100 in PBS and blocked in a buffer containing 0.3% Triton X-100 and 2% BSA in PBS for 30 min at 22°C. Then, the cells were incubated with rabbit polyclonal anti-c-fos antibody for 1 h at 37°C. After rinsing twice with PBS, the cells were incubated with FITC-labeled donkey anti-rat IgG antibody for 1 h at 37°C. After washing with PBS twice and distilled water once, the coverslips were mounted and the cells were analyzed with a Microphot-FXA fluorescence microscope (Nikon, Tokyo, Japan).

*Western blotting.* Before or after the initiating of adipocyte differentiation, the cells were lysed in the solubilizing buffer containing 30 mM Tris, 150 mM NaCl, 10 mM EDTA, 0.5% sodium deoxycholate, 1% Nonidet-P40, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 $\mu$M aprotinin, 0.5 $\mu$g/ml leupeptin, 1 mM $Na_3VO_4$, 160 mM NaF, pH 7.4, and incubated for 15 min on ice. The cell lysates were centrifuged at 4°C for 10 min to remove the insoluble materials and the supernatants were boiled with Laemmli sample buffer. The cell lysates were electrophoresed on 7.5% SDS–PAGE and transferred onto polyvinylidene difluoride membrane in the Trans-Blot cell apparatus (Bio-Rad). The membranes were immunoblotted with anti-Rb antibody, and subsequently with horseradish peroxidase-conjugated anti-mouse antibody. The proteins were visualized with chemiluminescence reagents according to the manufacturer's protocol (Amersham).

## RESULTS

### *Adipocyte Differentiation Is Inhibited by the PI 3-Kinase Inhibitors and Rapamycin, but Not the MEK Inhibitor*

To evaluate which component of insulin signaling pathway is important for the insulin-induced adipocyte differentiation, we first examined the effects of the pharmacological inhibitors for insulin signaling molecules, i.e., wortmannin and LY294003, (PI 3-kinase inhibitors), rapamycin, (an inhibitor of mTOR-p70 S6 kinase pathway) and PD98059, (a MEK I inhibitor) on adipocyte differentiation rate as assessed by Oil Red O staining of the cells (Fig. 1). Treatment of the cells with either insulin alone or IBMX + Dex alone stimulated differentiation of only a small percentage of the cells. (Fig. 1). However, when IBMX + Dex was present, addition of insulin markedly increased the rate of adipocyte conversion to more than 95% (Fig. 1). PI 3-kinase inhibitors or rapamycin inhibited the insulin-stimulated adipocyte differentiation in a dose-dependent manner, whereas the MEK inhibitor had no effect.

### *Clonal Expansion Is Inhibited by the PI 3-Kinase Inhibitors and Rapamycin, but Not the MEK Inhibitor*

To explore the relationship between the clonal expansion and adipocyte differentiation stimulated by insulin, we examined DNA synthesis as assessed by BrdU incorporation of the cells by immunostaining at 24 h after the induction of differentiation (Fig. 2). Only a few cells (3%) exhibited BrdU incorporation in the absence of adipogenic stimuli. Treatment with insulin alone stimulated BrdU incorporation of 21% of the cells, whereas treatment with IBMX + Dex alone re-

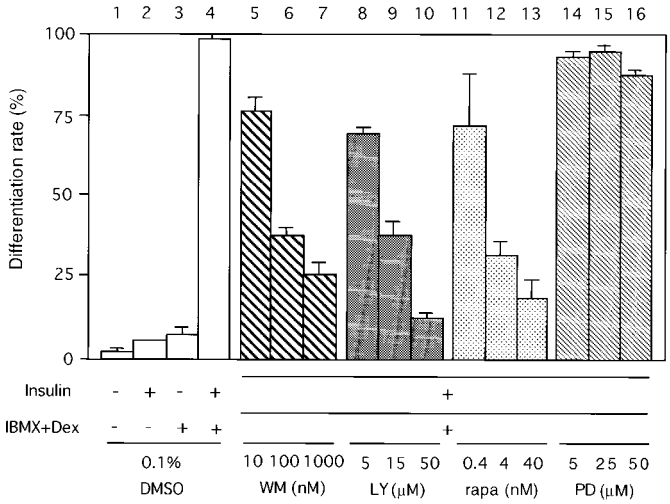


**FIG. 1.** Adipocyte differentiation is inhibited by the PI 3-kinase inhibitors and rapamycin, but not the MEK inhibitor. Adipocyte differentiation of 3T3-L1 cell was enhanced by the indicated combination of insulin and IBMX + dexamethazone. When differentiation was enhanced by insulin + IBMX + dexamethazone, the effects of pretreatment with wortmannin (lanes 5–7), LY294003 (lanes 8–10), rapamycin (lanes 11–13), or PD98059 (lanes 14–16) were examined. These chemical inhibitors were added to the cells 15 min before the initiation of differentiation. The rate of adipocyte differentiation was morphologically evaluated on day 9 by staining the lipid droplets in the cells with Oil Red O and counting the number of the cells which clearly accumulated lipid droplets. The results were shown as mean ± SE of three independent experiments.

sulted in BrdU incorporation of only 6% of the cells. When IBMX + Dex was present, addition of insulin significantly increased the number of the cells that incorporated BrdU (57%) (Fig. 2). BrdU incorporation was inhibited by both of the PI 3-kinase inhibitors (wortmannin and LY294003) in a dose-dependent manner. Rapamycin also inhibited BrdU incorporation in a dose-dependent manner. However, the inhibitory effect of rapamycin at the maximal concentration was partial (46.3%). On the other hand, the MEK inhibitor did not significantly affect BrdU incorporation.

### *c-fos Induction Is Inhibited by the MEK Inhibitor, but Not the PI 3-Kinase Inhibitors or Rapamycin*

Insulin is known to induce c-fos protein in the immediately early phase of its mitogenic process after the activation of MEK/MAPK (15, 16). We examined whether adipocyte differentiation and the clonal expansion are also related with the induction of c-fos protein. The induction of c-fos protein was evaluated by immunostaining with anti-c-fos antibody at 90 min after the induction of differentiation (Fig. 3). In the absence of adipogenic stimuli, only 11% of the cells exhibited staining of c-fos in the nuclei. Treatment with insulin alone resulted in positive c-fos staining in 19% of the cells. IBMX + Dex alone induced c-fos protein more efficiently than insulin, resulting in 41% of the cells positive for c-fos. When, differentiation was induced by insulin + IBMX + Dex, neither PI 3-kinase inhibitors nor rapamycin affected c-fos induction, whereas the MEK inhibitor inhibited it in a dose-dependent manner.

### *Phosphorylation of Rb Is Inhibited by the PI 3-Kinase Inhibitors and Rapamycin, but Not the MEK Inhibitor*

Retinoblastoma protein (Rb) is a tumor suppresser protein, which inhibits cell proliferation by controlling progression through the restriction point within the G1 phase to S phase of the cell cycle. Several studies have recently reported that Rb is required for adipocyte differentiation (13). Therefore to evaluate the role of Rb in the insulin-stimulated adipogenesis, we examined the effect of inhibitors for insulin signaling molecules on the phosphorylation status of Rb at 24 h after induction of differentiation (Fig. 4). Prior to the initiation of differentiation, most of Rb was hypophosphorylated as seen as a single band. At 24 h after initiation of differentiation with insulin + IBMX + Dex, hyperphosphorylated form of Rb appeared as a mobility shift on the gel. When the cells were treated with IBMX + Dex alone, Rb remained hypophosphorylated. When differentiation was induced by insulin + IBMX + Dex,

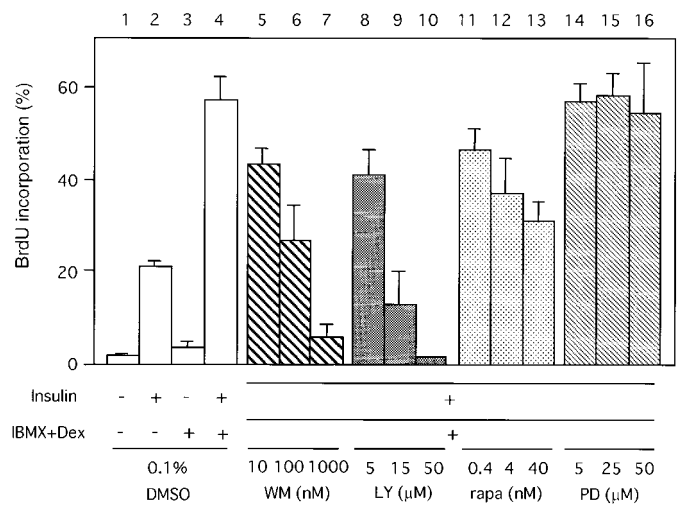


**FIG. 2.** DNA synthesis on day 1 is inhibited by the PI 3-kinase inhibitors and rapamycin, but not the MEK inhibitor. 3T3-L1 cells were stimulated with the indicated combination of insulin and IBMX + dexamethazone. When the cells were stimulated with insulin + IBMX + dexamethazone, the effects of pretreatment with wortmannin (lanes 5–7), LY294003 (lanes 8–10), rapamycin (lanes 11–13), or PD98059 (lanes 14–16) were examined. These chemical inhibitors were added to the cells 15 min before the stimulation with insulin + IBMX + dexamethazone. Twenty-four hours after the stimulation, DNA synthesis was assessed by BrdU incorporation into the cells as described under Materials and Methods. The results were shown as mean ± SE of three independent experiments.

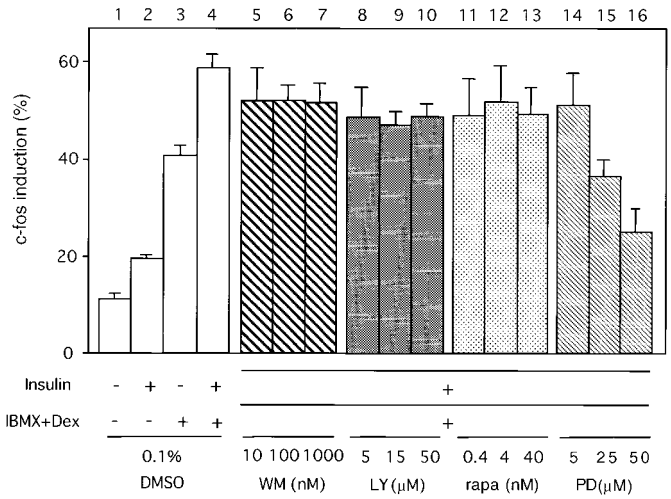


**FIG. 3.** c-fos induction is inhibited by the MEK inhibitor, but not the PI 3-kinase inhibitors or rapamycin. 3T3-L1 cells were stimulated with the indicated combination of insulin and IBMX + dexamethazone. When the cells were stimulated with insulin + IBMX + dexamethazone, they were pretreated with wortmannin (lanes 5–7), LY294003 (lanes 8–10), rapamycin (lanes 11–13), or PD98059 (lanes 14–16). The chemical inhibitors were added to the cells 15 min before the stimulation with insulin + IBMX + dexamethazone. Ninety minutes after the adipogenic stimulation, c-fos induction was evaluated by immunostaining as described under Materials and Methods. The results were shown as mean ± SE of three independent experiments.

pretreatment with wortmannin, LY294003 or rapamycin inhibited hyperphosphorylation of Rb. In contrast, the MEK inhibitor did not prevent appearance of hyperphosphorylated form of Rb.

## DISCUSSION

In the current study, we showed that adipocyte differentiation, clonal expansion and phosphorylation of Rb were stimulated only in the presence of insulin and were inhibited by PI 3-kinase inhibitors or rapamycin, i.e., they were all dependent on the same insulin signaling pathway. These findings suggest a novel mechanism of insulin-induced adipocyte differentiation that insulin stimulates Rb phosphorylation via the activation of PI 3-kinase and mTOR, resulting in the adipocyte differentiation through clonal expansion in 3T3-L1 cells.

Several recent studies have demonstrated that Rb is important for differentiation of certain cell types including adipocytes (3, 13, 17– 19). The results of Rb-knock-out mouse suggest that the existence of Rb is necessary for the cells to complete adipocyte differentiation. Although Rb is reported to stimulate adipocyte differentiation by binding directly to C/EBP$\beta$ and enhancing its transcriptional activity (13), little is known about the role of Rb during the differentiation process. The role of Rb as a tumor suppressor gene product is to suppress G1 progression of cell cycle. Phosphorylation of Rb inactivates its function and leads to progression of the cell cycle. Recently, it has been demonstrated that Rb is important for the clonal expansion observed in the process of adipocyte differentiation (13, 17–19). We observed that the time course of BrdU incorporation and Rb phosphorylation was coincident with each other (data not shown), and that they were dependent on the activation of the same insulin signals. These results also suggest that Rb phosphorylation plays an important role for the stimulation of clonal expansion. Furthermore, clonal expansion has been reported to be an essential process to complete adipocyte differentiation (4, 5, 10, 19, 20). In this study, full differentiation was observed only when insulin stimulated BrdU incorporation, and it was suppressed when PI 3-kinase inhibitors or rapamycin inhibited BrdU incorporation. Therefore, these results supports the importance of clonal expansion in adipocyte differentiation. Taken together, we may suggest it is one of the indispensable processes for the differentiation of 3T3-L1 cells that Rb exists and is phosphorylated by insulin resulting in the stimulation of clonal expansion.

Insulin is known to be one of the factors that stimulate differentiation into adipocytes (1, 2, 21). Studies

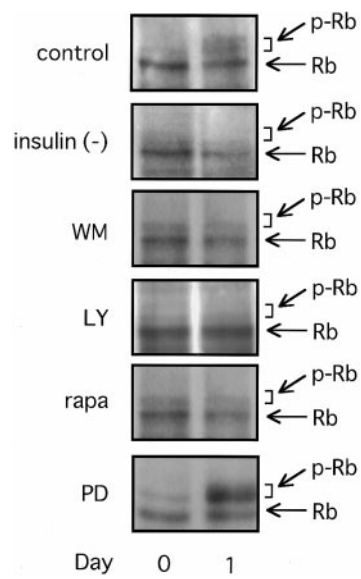


**FIG. 4.** Rb phosphorylation is inhibited by the PI 3-kinase inhibitors and rapamycin, but not the MEK inhibitor. Adipocyte differentiation was enhanced by IBMX + dexamethazone in the presence (control) or absence of insulin (insulin (−)). When differentiation was enhanced by insulin + IBMX + dexamethazone, the cells were pretreated with wortmannin (WM), LY294003 (LY), rapamycin (rapa) or PD98059 (PD). These chemical inhibitors were added to the cells 15 min before the initiation of differentiation. Twenty-four hours after the stimulation, samples were prepared and analyzed by Western blotting with anti-Rb antibody as described under Materials and Methods. Hyperphosphorylated and hypophosphorylated forms of Rb are shown as p-Rb and Rb, respectively. Representative films are shown.

have been conducted to clarify which insulin signals are necessary for adipocyte differentiation, and the importance of PI 3-kinase (6–9, 22, 23), Akt (8, 9), and mTOR (10), have been reported. One study demonstrated that PI 3-kinase activation was important for the translation of adipocyte-specific genes through which differentiation was promoted (7). Since the translation of adipocyte-specific genes does not occur immediately after the initiation of differentiation and insulin is not present in the media during the period when such genes are translated, these results indicate the importance of PI 3-kinase activation in the relatively later phase of differentiation which is independent of insulin stimulation. However, in our current study, adipocyte differentiation was induced only in the presence of insulin and it was inhibited by PI 3-kinase inhibitors or rapamycin when they were added for the initial 4 days of adipocyte differentiation. Their inhibitory effects on adipocyte differentiation were not observed when inhibitors were added after the day 4 (data not shown). Therefore, our current study demonstrates the importance of PI 3-kinase and mTOR which are activated just after the initiation of differentiation induced by insulin. One study demonstrated the role of PI 3-kinase which was activated immediately after the initiation of adipocyte differentiation induced by IGF-1 (23). They showed that the transcription of adipocyte-specific genes was stimulated in a PI 3-kinase-dependent manner in the primary cultures of brown adipocytes. However, in contrast to our results, they showed that PI 3-kinase inhibitors do not inhibit IGF-1-induced DNA synthesis or cell cycle progression. Therefore, the role of PI 3-kinase in adipocyte differentiation may be different depending on the cell types.

Rb is phosphorylated by an active complex of cyclin-dependent protein kinase (Cdk) such as Cdk4 and Cdk6 and G1 cyclins such as cyclin D1 and cyclin D2. Akt, a serine/threonine kinase regulated by PI 3-kinase was reported to enhance the translation of cyclin D mRNA (24). Akt also regulates mTOR (25, 26), which in turn regulates the release of eIF4E by phosphorylation of 4EBP-1, which is also essential for the translation of cyclin D mRNA (27, 28). Therefore, the inhibition of Rb phosphorylation by the PI 3-kinase inhibitors and rapamycin may be at least in part through inhibition of translation of cyclin D mRNA by the inactivation of Akt and/or mTOR. Rb phosphorylation may also be controlled by the degradation of a cyclin-dependent kinase inhibitor, as a recent report indicated that the degradation of p27 by calpain regulates Rb phosphorylation, clonal expansion and adipocyte differentiation (19). This finding supports the notion that Rb phosphorylation plays a key role in clonal expansion and adipocyte differentiation.

## ACKNOWLEDGMENT

This study was supported in part by a grant-in-aid for Scientific Research from the Ministry of Education, Science, Sports and Culture, Japan, to T.H.

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