

RPTP μ tyrosine phosphatase promotes adipogenic differentiation via modulation of p120 catenin phosphorylation

Won Kon Kim^a, Hyeyun Jung^a, Eun Young Kim^a, Do Hyung Kim^a, Yee Sook Cho^b,
Byoung Chul Park^a, Sung Goo Park^a, Yong Ko^c, Kwang-Hee Bae^a, and Sang Chul Lee^a

^aMedical Proteomics Research Center and ^bDevelopment and Differentiation Research Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-806, Republic of Korea; ^cDivision of Life Science and Genetic Engineering, College of Life and Environmental Sciences, Korea University, Seoul 136-701, Republic of Korea

ABSTRACT Adipocyte differentiation can be regulated by the combined activity of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). In particular, PTPs act as key regulators in differentiation-associated signaling pathways. We recently found that receptor-type PTP μ (RPTP μ) expression is markedly increased during the adipogenic differentiation of 3T3-L1 preadipocytes and mesenchymal stem cells. Here, we investigate the functional roles of RPTP μ and the mechanism of its involvement in the regulation of signal transduction during adipogenesis of 3T3-L1 cells. Depletion of endogenous RPTP μ by RNA interference significantly inhibited adipogenic differentiation, whereas RPTP μ overexpression led to an increase in adipogenic differentiation. Ectopic expression of p120 catenin suppressed adipocyte differentiation, and the decrease in adipogenesis by p120 catenin was recovered by introducing RPTP μ . Moreover, RPTP μ induced a decrease in the cytoplasmic p120 catenin expression by reducing its tyrosine phosphorylation level, consequently leading to enhanced translocation of Glut-4 to the plasma membrane. On the basis of these results, we propose that RPTP μ acts as a positive regulator of adipogenesis by modulating the cytoplasmic p120 catenin level. Our data conclusively demonstrate that differentiation into adipocytes is controlled by RPTP μ , supporting the utility of RPTP μ and p120 catenin as novel target proteins for the treatment of obesity.

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INTRODUCTION

Protein phosphorylation plays major roles in a wide range of cellular processes. It occurs at serine, threonine, and tyrosine residues. In particular, tyrosine phosphorylation is an important regulatory mechanism in eukaryote physiology and human disease (Hunter,

1987; Mustelin *et al.*, 2002a, 2002b). Reversible tyrosine phosphorylation, which is governed by the balanced activity between protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs), regulates important signaling pathways involved in the control of proliferation, adhesion, and differentiation. Although equal and balanced activities of PTKs and PTPs have been reported in many physiological processes, the findings of several studies contribute to the emerging idea that PTPs have specific, active, and even dominant roles in tyrosine phosphorylation (Mustelin *et al.*, 2002a, 2002b; Mustelin and Tasken, 2003). Moreover, several PTPs can act as biochemical “on” or “off” switches and as key regulators in many signaling pathways (Hunter, 2000).

Adipogenesis involves the formation of adipocytes from mesenchymal progenitor cells, and it is controlled by the sequential activation of transcription factors (Gregoire *et al.*, 1998; Rosen and Spiegelman, 2000; Rosen *et al.*, 2000; Rosen and MacDougald, 2006). Subsequently, these transcription factors are regulated via

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Address correspondence to: Kwang-Hee Bae (khbae@kribb.re.kr) or Sang Chul Lee (lesach@kribb.re.kr).

Abbreviations used: aP2, fatty acid-binding protein 4; FBS, fetal bovine serum; LAR, leukocyte common antigen-related; MSC, mesenchymal stem cell; PBS, phosphate-buffered saline; PTKs, protein tyrosine kinases; PTPs, protein tyrosine phosphatases; RPTP μ , receptor-type PTP μ ; shRNA, short hairpin RNA.

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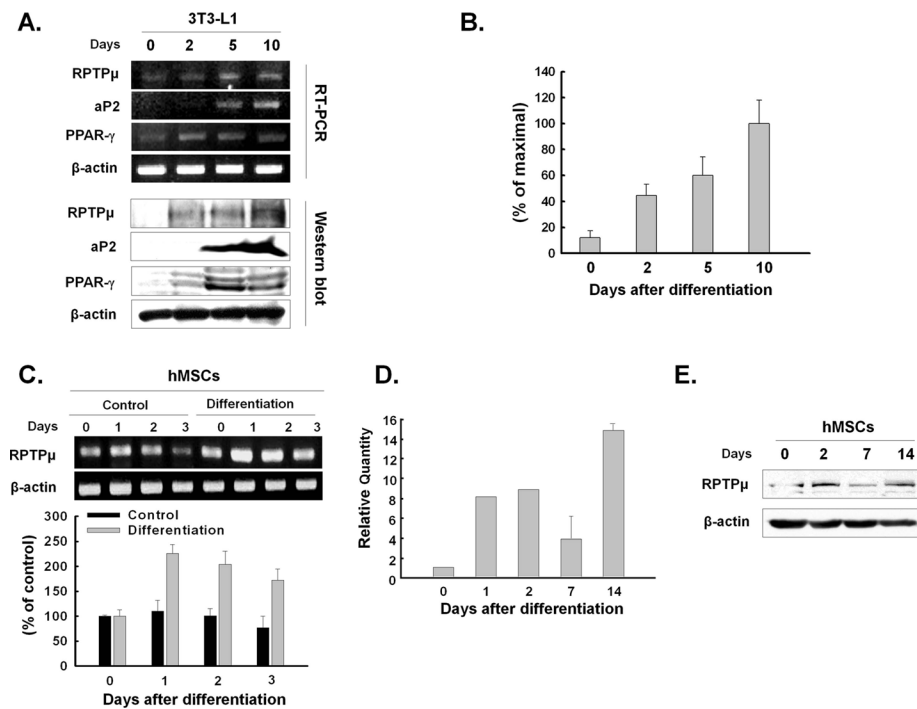


FIGURE 1: RPTP μ expression is dramatically increased in both 3T3-L1 preadipocytes and human MSCs during adipogenic differentiation. (A) Analysis of RPTP μ mRNA (top) and protein (bottom) expression during the adipocyte differentiation of 3T3-L1 preadipocytes. (B) Densitometric analysis of RPTP μ protein expression during adipocyte differentiation of 3T3-L1 cells. (C) Analysis of RPTP μ mRNA expression during early stages of adipogenesis in human MSCs (hMSCs) with RT-PCR (top) and densitometry (bottom). (D) Analysis of RPTP μ mRNA expression during adipocyte differentiation of hMSCs with real-time PCR. (E) Analysis of RPTP μ protein expression during adipocyte differentiation of hMSCs. For the RT-PCR analysis, total RNA was extracted on the indicated days of differentiation and was used to synthesize cDNA for RT-PCR. aP2 and PPAR- γ were used as positive controls for adipocyte differentiation, and β -actin was used as a loading control. For Western blot analysis, whole-cell lysates were extracted on the indicated days of differentiation and were subjected to analysis using antibodies against RPTP μ (23820, Abcam) and adipocyte differentiation markers, such as aP2 and PPAR- γ . β -Actin was used as the loading control.

the activation of various adipogenic signaling pathways. Recently several reports have suggested that adhesion-mediated signaling also participates in adipogenic differentiation (Li and Xie, 2007; Luo *et al.*, 2008; Park *et al.*, 2009). For instance, Li and Xie (2007) showed that focal adhesion kinase is an essential regulator in the adipogenic differentiation of 3T3-L1 cells. Similarly, Luo *et al.* (2008) reported that disruption of the focal adhesion complex enhances adipocyte differentiation in mesenchymal progenitor cells, suggesting that adipocyte differentiation is induced by the disassembly of cell-matrix proteins. In addition, cytoskeletal disassembly promotes adipocyte differentiation of mouse embryonic stem cells (Feng *et al.*, 2010). β -Catenin, which is essential for cell adhesion and migration, is well known as an antiadipogenic factor, and its expression is down-regulated during adipogenesis (Kennell and MacDougald, 2005; Hou *et al.*, 2006; Kawai *et al.*, 2007; Prestwich and Macdougald, 2007). The expression of another adhesion-related protein, p120 catenin, is also decreased during adipogenic differentiation in 3T3-L1 preadipocytes (Hou *et al.*, 2006), indicating that p120 catenin may inhibit adipogenic differentiation, similar to the action of β -catenin. Furthermore, the mechanism of action of other p120 catenin-like adipogenic signaling molecules, such as insulin, insulin-like growth factor-I, and β -catenin (White and Kahn, 1994; White and Yenush, 1998; Piedra *et al.*, 2001), is mediated by phosphorylation and dephosphorylation at tyrosine residues (Keilhack *et al.*,

2000; Owens *et al.*, 2000; Zondag *et al.*, 2000). These findings collectively suggest that PTPs may have a pivotal role in adipogenesis. However, the functional role of PTPs in adipogenic differentiation is still poorly understood.

PTPs are a group of enzymes that remove phosphate groups from phosphorylated tyrosine residues on proteins. On the basis of their primary structure and catalytic domains, PTPs are divided into four distinct classes that encompass all 107 members of this group of enzymes (Alonso *et al.*, 2004). Individual PTPs may be expressed by all cell types, or their expression may be strictly specific to certain tissues, including insulin-sensitive tissues (Drake and Posner, 1998; Goldstein *et al.*, 1998). Several PTPs, such as PTP-BL, PTP-RQ and leukocyte common antigen-related (LAR), have important roles in adipogenesis (Glondou-Lassis *et al.*, 2009; Jung *et al.*, 2009; Kim *et al.*, 2009). Previously, we demonstrated that LAR and PTP-RQ tyrosine phosphatases participate in adipogenic differentiation by modulating insulin receptor phosphorylation and the intracellular level of phosphatidylinositol phosphates, respectively (Jung *et al.*, 2009; Kim *et al.*, 2009). This strongly implies the intimate involvement of PTPs in adipogenesis.

Recently we also found that the expression of receptor-type PTP μ (RPTP μ) is increased during adipogenesis. RPTP μ is a key regulator in tyrosine phosphorylation of the cadherin-catenin complex (Lilien and Balsamo, 2005; Ostman *et al.*, 2006), and this phosphatase binds to and directly dephosphorylates p120 catenin (Zondag *et al.*, 2000), indicating the possibility that RPTP μ affects adipogenic differentiation through the regulation of p120 catenin phosphorylation. However, the effect of PTP μ on adipogenesis has not yet been reported. Moreover, the relationship between p120 catenin and adipogenic differentiation had not been extensively examined until now. Therefore, the present study was undertaken to investigate the mechanism of RPTP μ in adipogenic differentiation in the 3T3-L1 preadipocyte cell line.

RESULTS

Expression of RPTP μ during adipogenic differentiation of 3T3-L1 preadipocytes and human mesenchymal stem cells

To establish whether RPTP μ is involved in regulating adipogenic differentiation, we examined the change in the expression level of RPTP μ during the adipogenesis of 3T3-L1 cells. The RPTP μ mRNA and protein levels were significantly increased after differentiation of 3T3-L1 cells into adipocytes (Figure 1, A and B). Expression of the adipogenic markers fatty acid-binding protein 4 (aP2) and PPAR- γ was increased during adipogenesis of 3T3-L1 preadipocytes (Figure 1A). In addition, RPTP μ mRNA and protein levels were markedly increased from the early stages of adipogenesis in human mesenchymal stem cells (MSCs) (Figure 1, C–E), although expression analysis showed an oscillation pattern. These results clearly show that RPTP μ expression increases during adipogenic

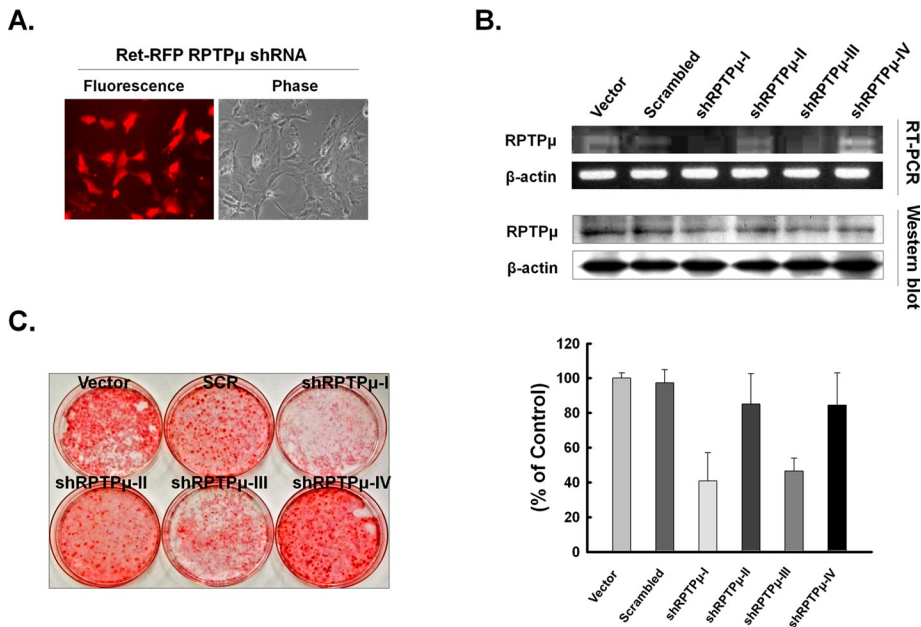


FIGURE 2: RPTP μ shRNA suppresses adipogenic differentiation. (A) Direct monitoring of RFP expression under a fluorescence microscope after enrichment using a FACS sorter (BD Biosciences FACSARIA). 3T3-L1 cells were infected with a retrovirus containing vector alone (pSIREN-RetroQ-DsRed), scrambled insert, or four different RPTP μ shRNA constructs. Infected cells were then selected with a FACS sorter. (B) Knockdown of RPTP μ shRNA was confirmed by RT-PCR (top) and Western blot (middle) analysis (using antibodies against RPTP μ ; 23820, Abcam). Quantification of RPTP μ protein expression was performed using densitometric analysis (bottom). (C) RPTP μ -depleted cells were induced to differentiate into mature adipocytes for 10 d by MDI medium. Ten days later, cells were stained with Oil-Red-O to visualize the degree of lipid accumulation.

differentiation in both human MSCs and the 3T3-L1 preadipocyte cell line.

Effect of RPTP μ knockdown on adipogenic differentiation of 3T3-L1 preadipocytes

To determine whether endogenous RPTP μ influences differentiation into adipocytes, four different short hairpin RNA (shRNA) constructs (RPTP μ shRNA I–IV) were produced and tested for their effectiveness in suppressing RPTP μ expression in 3T3-L1 preadipocytes using a retrovirus expression system (Clontech pSIREN-RetroQ-DsRed). Most of the infected cells were found to be red fluorescent protein (RFP) positive by fluorescence microscopy after fluorescence-activated cell sorting (FACS) sorting (Figure 2A). The knockdown of endogenous RPTP μ expression was additionally confirmed by RT-PCR and Western blot, which showed that the shRNA RPTP μ -I and III constructs efficiently decreased the endogenous RPTP μ expression (Figure 2B). On the other hand, RPTP μ expression was not significantly changed by the shRNA RPTP μ -II and IV constructs. Retrovirally transduced 3T3-L1 preadipocytes were induced to differentiate into adipocytes in growth medium containing MDI (see *Materials and Methods*) for 2 d, followed by further differentiation and maturation in growth medium supplemented with insulin only for 10 d. As shown in Figure 2C, fat accumulation was visualized by staining the lipid droplets with Oil-Red-O. The depletion of RPTP μ (using the shRNA RPTP μ -I and III constructs) dramatically inhibited the differentiation of these cells into mature adipocytes as compared with the differentiation of the control and scrambled retrovirus-infected cells (Figure 2C). Generally, the degree of adipogenic differentiation correlated with the expression level of RPTP μ (compare Figure 2, B and C), confirming

that RPTP μ expression is certainly involved in adipogenesis.

Effect of ectopic expression of RPTP μ on adipogenic differentiation

To further clarify the role of RPTP μ in adipogenesis, 3T3-L1 cells were infected with the FLAG-tagged RPTP μ using a retrovirus system (RPTP μ IRES-GFP). The RPTP μ -CS (catalytic inactive mutant; catalytic Cys1129 was replaced with Ser) and a control vector were used as negative controls. Infected cells were isolated using a FACS sorter (BD Biosciences FACSARIA) and were further grown. Most of the enriched cells were green fluorescent protein (GFP) positive under a fluorescence microscope (Figure 3A). Overexpression of wild-type and mutant RPTP μ proteins was verified by Western blot analysis using anti-RPTP μ antibody (Figure 3B). Expression of the RPTP μ protein was continuously detected in mature adipocytes (Figure 3E). The enriched cells were induced to differentiate into mature adipocytes, and lipid accumulation was analyzed by Oil-Red-O staining 10 d after culturing with the differentiation medium (Figure 3C). Overexpression of wild-type RPTP μ resulted in an enhanced lipid accumulation as compared with that of control cells and those expressing the RPTP μ mutant

protein (Figure 3, C and D), suggesting that the phosphatase activity is critical in up-regulating the adipogenic differentiation of 3T3-L1 cells. Consistent with these results, the expression levels of aP2 increased in mature adipocytes upon ectopic RPTP μ expression (Figure 3E).

Effect of p120 catenin on adipogenic differentiation

In view of the finding that RPTP μ binds and directly dephosphorylates p120 catenin (Zondag *et al.*, 2000), we initially investigated whether p120 catenin regulates the adipogenic differentiation of 3T3-L1 cells. Consistent with a previous report (Hou *et al.*, 2006), p120 catenin and β -catenin expression markedly decreased during adipogenesis (Figure 4A). In general, p120 catenin exists in equilibrium between two states, either bound to the membrane-proximal region of cadherin or free in the cytoplasm. Accordingly, we examined the subcellular localization of p120 catenin during adipogenesis. As shown in Figure 4B, the p120 catenin level was dramatically reduced in the membrane fraction, whereas the cytoplasmic pool of p120 was relatively retained at the early adipogenesis phase. Next we stably expressed the full-length p120 catenin by using a retrovirus system (pLZRS-MS-IRES-GFP) in 3T3-L1 cells. The infection of cells was confirmed by fluorescence microscopy (Figure 4C) and Western blotting (Figure 4D). Cells overexpressing p120 catenin were induced into mature adipocytes for 10 d, and the cells were stained with Oil-Red-O to visualize the degree of lipid accumulation. Overexpression of p120 catenin resulted in a lower degree of lipid accumulation as compared with the control vector in 3T3-L1 preadipocytes (Figure 4, E and F). These results suggest that p120 catenin may be involved in adipocyte differentiation as a negative regulator.

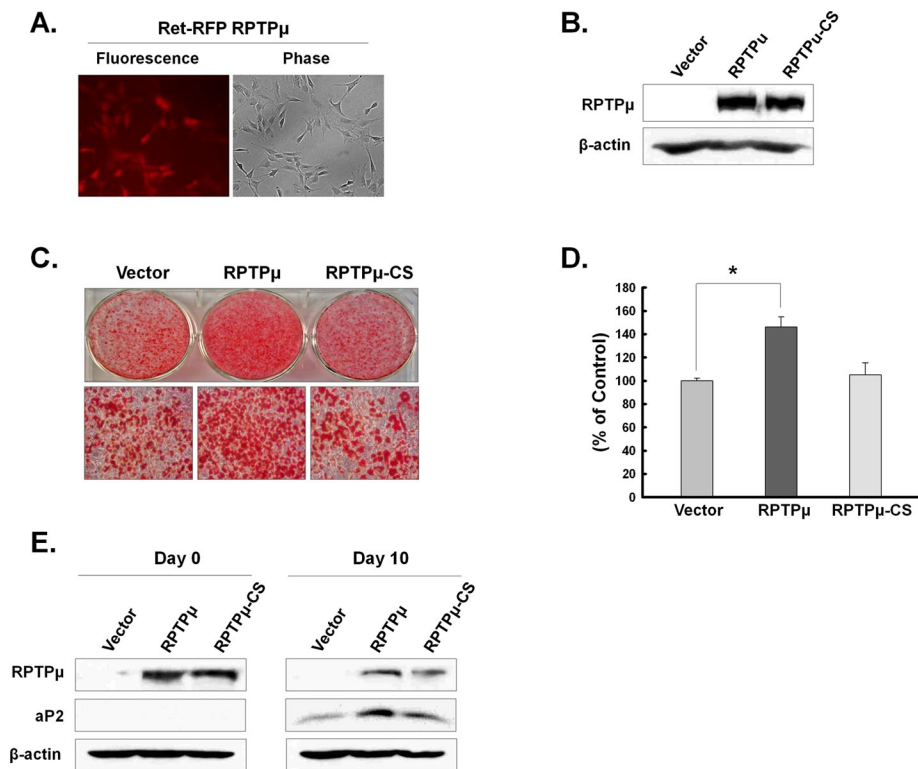


FIGURE 3: Overexpression of RPTP μ promotes adipogenic differentiation of 3T3-L1 cells. Cells were infected with retroviruses expressing FLAG-tagged RPTP μ or the RPTP μ -CS mutant (catalytically inactive; catalytic D1-Cys1129 was replaced with Ser). Infected cells were selected via FACS sorting. (A) GFP expression was monitored directly using fluorescence microscopy. (B) Expression of RPTP μ was confirmed by Western blot analysis using anti-RPTP μ antibody (MAB3740, Millipore). (C) Two days postconfluence (day 0), cells overexpressing wild-type or mutant RPTP μ were induced to differentiate into mature adipocytes with MDI for 10 d. Cells were stained with Oil-Red-O to visualize lipid droplets at 10 d postinduction. (D) Quantification of stained cells was performed using dye extraction buffer. The data represent the mean percentage levels \pm SD as compared with the control vector ($n = 3$; * $p < 0.05$). (E) Whole-cell lysates were prepared on day 0 or 10 for Western blot analysis of RPTP μ , aP2, and β -actin. β -Actin was used as an internal control.

Effects of RPTP μ on adipogenic differentiation in cells overexpressing p120 catenin

To clarify the functional role of RPTP μ in adipogenesis, we reinfected the p120 catenin-expressing 3T3-L1 cells with RPTP μ or mutant RPTP μ (by using pRetro-IRES-RFP). Most of the reinfected cells were detected as both GFP and RFP positive by fluorescence microscopy (Figure 5A). Overexpression of p120 catenin and RPTP μ expression were confirmed by Western blot analysis (Figure 5B). Of interest, the reintroduction of wild-type RPTP μ in the p120 catenin-overexpressing cells induced the partial recovery of adipogenic differentiation. By contrast, the reintroduction of RPTP μ -CS caused no significant change in the differentiation rate of the cells, indicating the crucial involvement of RPTP μ phosphatase activity in adipogenesis (Figure 5, C and D).

RPTP μ regulates adipogenic differentiation via p120 catenin dephosphorylation

On the basis of a report that RPTP μ is a key mediator of p120 catenin dephosphorylation (Zondag *et al.*, 2000), it is proposed that RPTP μ regulates adipogenesis by controlling p120 catenin phosphorylation. Under our experimental conditions, phosphorylation of p120 catenin was dramatically decreased during adipogenesis in RPTP μ -expressing 3T3-L1 cells as compared with the control cells

(Figure 6A). However, the p120 catenin phosphorylation level was not changed by introduction of the RPTP μ -CS mutant. These data strongly indicate that RPTP μ phosphatase activity is associated with the dephosphorylation of p120 catenin. Next we examined the effect of RPTP μ on endogenous p120 catenin distribution. Our results clearly supported a crucial role of RPTP μ in phosphorylation-dependent distribution of p120 catenin (Figure 6B). Specifically, RPTP μ dephosphorylated p120 catenin, subsequently leading to a lower level of cytoplasmic protein compared with that observed with the vector control and RPTP μ -CS.

Because cytoplasmic p120 catenin is related to Glut-4 trafficking (Hou *et al.*, 2006), we next examined Glut-4 membrane trafficking in p120 catenin- and RPTP μ -coexpressing 3T3-L1 cells. Intracellular Glut-4 vesicles are translocated to the plasma membrane when stimulated by insulin. Consistent with a previous report (Hou *et al.*, 2006), Glut-4 membrane trafficking was significantly inhibited by p120 catenin overexpression. Of note, the reintroduction of wild-type RPTP μ in p120 catenin-overexpressing cells induced a recovery of Glut-4 membrane translocation in differentiated 3T3-L1 adipocytes (Figure 6C, top). Analogous to the result obtained with immunofluorescence staining, flow cytometry analysis also confirmed that overexpression of p120 catenin inhibited Glut-4 membrane trafficking, and coexpression of p120 catenin and RPTP μ resulted in an increase in Glut-4 membrane trafficking as compared with p120 catenin-expressing cells in differentiated 3T3-L1 cells (Figure 6C, bottom). Consistently, this effect was diminished upon introduction of mutant RPTP μ -CS in lieu of wild-type RPTP μ .

DISCUSSION

Our understanding of the molecular regulation of adipogenic differentiation has been remarkably advanced by the recent finding that several PTPs are critically involved in adipogenesis (Glondou-Lassis *et al.*, 2009; Jung *et al.*, 2009; Kim *et al.*, 2009). We recently found that the expression level of RPTP μ phosphatase is significantly up-regulated during the differentiation of 3T3-L1 preadipocytes. We previously demonstrated that LAR and PTP-RQ tyrosine phosphatases function as negative regulators during adipocyte differentiation (Jung *et al.*, 2009; Kim *et al.*, 2009). In the present study, we investigated the functional roles of the tyrosine phosphatase RPTP μ in the adipogenic differentiation of 3T3-L1 preadipocytes.

Enforced expression of wild-type RPTP μ in 3T3-L1 preadipocytes significantly promoted adipogenesis (Figure 3, C and D), clearly implying that RPTP μ is crucial for adipocyte differentiation. In addition, RPTP μ overexpression, but not introduction of the RPTP μ -CS mutant, induced increased aP2 expression, indicating that RPTP μ functions by up-regulating this key adipogenic factor. Moreover, silencing of RPTP μ with shRNA led to dramatic inhibition of adipogenic

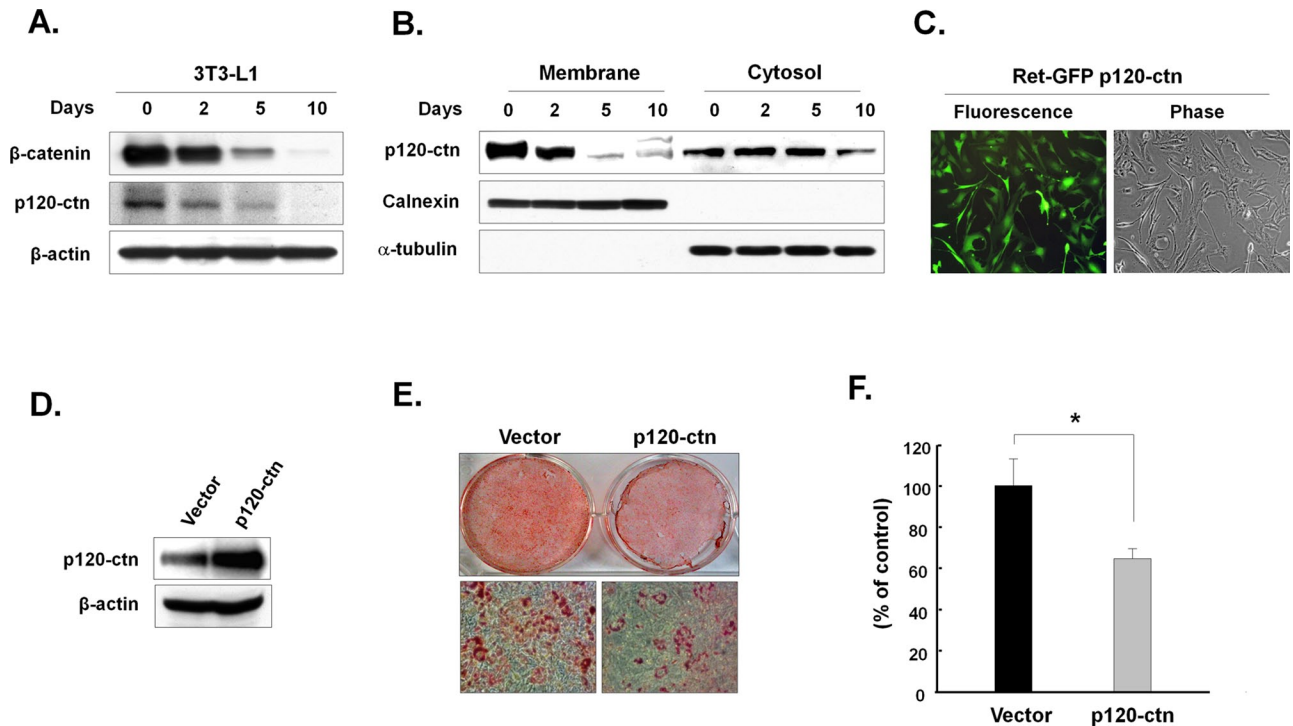


FIGURE 4: p120 catenin inhibits adipogenic differentiation of 3T3-L1 cells. (A) Analysis of β -catenin and p120 catenin protein levels during adipogenic differentiation of 3T3-L1 cells. (B) Subcellular localization of p120 catenin during adipogenesis of 3T3-L1 cells. Cells were induced to differentiate into mature adipocytes using a standard procedure. For fractionation analysis, whole-cell lysates were extracted on the indicated days of differentiation and fractionated, as described in *Materials and Methods*. Equal amounts of membrane and cytoplasmic proteins were analyzed by Western blot, using the anti-p120 catenin antibody. Loading control and fraction purity were confirmed after reprobing using antibodies against the membrane marker protein calnexin and cytoplasmic marker protein α -tubulin. (C) Full-length p120 catenin was inserted into the MCS of the pLZRS-MS-IRES-GFP vector, and infected cells were selected using a FACS sorter. Enriched cells (GFP positive) were confirmed using fluorescence microscopy. (D) Western blot analyses were performed to confirm overexpression of p120 catenin. (E) 3T3-L1 preadipocytes expressing p120 catenin were induced to differentiate into adipocytes via the adipogenic program for 10 d. Cells were stained with Oil-Red-O to visualize lipid droplets at 10 d postinduction. (F) Quantification of stained cells was performed using dye extraction buffer. The data represent mean percentage levels \pm SD, compared with the control vector ($n = 3$; * $p < 0.05$).

differentiation, suggesting that RPTP μ is a potent proadipogenic regulator (Figure 2C).

The cadherin-associated molecule β -catenin is a well-known antiadipogenic factor, and its expression is down-regulated during adipogenesis (Kennell and MacDougald, 2005; Hou *et al.*, 2006; Kawai *et al.*, 2007; Prestwich and Macdougald, 2007). The expression of another cadherin-associated molecule, p120 catenin, is also decreased during adipogenic differentiation in 3T3-L1 preadipocytes (Hou *et al.*, 2006), suggesting a negative role for this protein in adipogenesis. The present study clearly shows reduced p120 catenin expression during adipogenic differentiation. In addition, overexpression of p120 catenin resulted in low lipid accumulation, indicating that p120 catenin has a role similar to that of β -catenin with respect to its antiadipogenic properties. Of note, the introduction of RPTP μ in p120 catenin-expressing cells resulted in a significant change in the differentiation rate of the cells (Figure 5, C and D), indicating that RPTP μ enhances adipogenic differentiation by regulation of p120 catenin-dependent inhibition of adipogenesis.

RPTP μ functions as a cell-cell adhesion receptor (Brady-Kalnay *et al.*, 1993; Gebbink *et al.*, 1993). On the basis of a report that RPTP μ directly interacts with p120 catenin and controls the tyrosine phosphorylation state of p120 catenin (Zondag *et al.*, 2000), we speculated that RPTP μ promotes adipogenesis by modulating p120

catenin signaling through its tyrosine phosphatase activity. Consistent with a previous observation (Zondag *et al.*, 2000), RPTP μ markedly reduced p120 catenin tyrosine phosphorylation following MDI treatment during adipocyte differentiation. This result indicates that p120 catenin tyrosine phosphorylation has the capacity to influence the antiadipogenic effect of p120 catenin. The p120 catenin protein exists in equilibrium between two states—either bound to the membrane-proximal region of cadherin or free in the cytoplasm (Papkoff, 1997; Ozawa and Kemler, 1998; Noren *et al.*, 2000; Thoreson *et al.*, 2000), although p120 catenin is also found at low levels in the nucleus. Our results indicate that p120 catenin distribution depends on its tyrosine phosphorylation state, which is regulated by phosphatase activity of RPTP μ . In adipocytes, insulin increases glucose transport by stimulating the translocation of Glut-4 from intracellular sites to the plasma membrane (Saltiel and Kahn, 2001). It has been reported that cytoplasmic p120 catenin inhibits Glut-4 membrane trafficking by the regulation of Rho and Rac (Hou *et al.*, 2006). Our results also clearly show that cytoplasmic p120 catenin suppresses Glut-4 membrane translocation, and reduced Glut-4 membrane trafficking by p120 catenin is recovered by RPTP μ overexpression, suggesting that RPTP μ -mediated regulation of adipogenesis functions by increasing the membrane trafficking activity. Furthermore, the marginal effect of the inactive RPTP μ mutant strongly supports

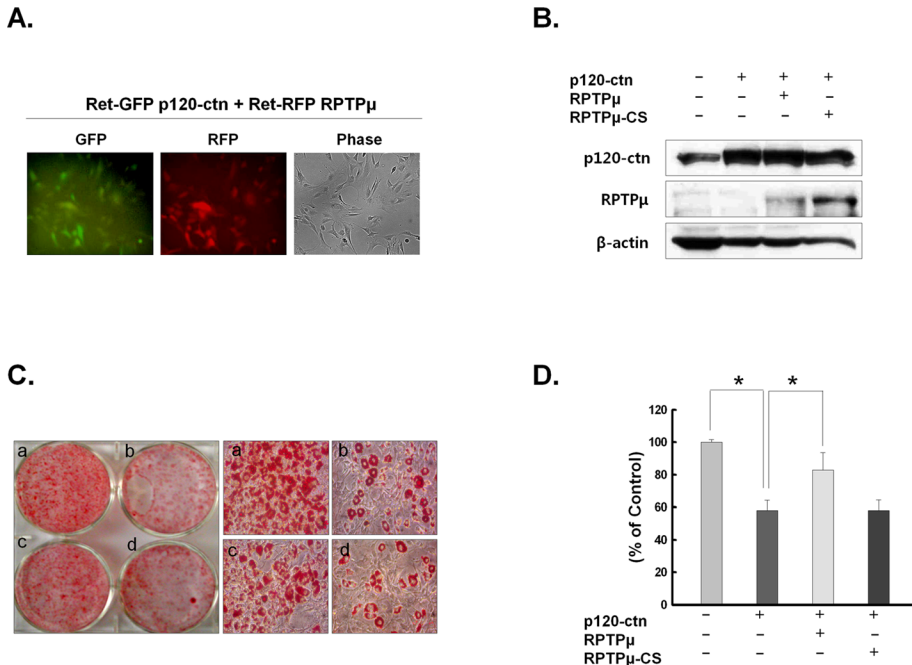


FIGURE 5: Effects of p120 catenin and RPTP μ on adipogenic differentiation of 3T3-L1 cells. Cells overexpressing p120 catenin were reinfected with wild-type RPTP μ or mutant RPTP μ vector (pRetro-IRES-RFP). Reinfected cells were selected with a FACS sorter. (A) Expression of GFP and RFP was monitored using fluorescence microscopy. (B) Expression of p120 catenin and RPTP μ (MAB3740, Millipore) was confirmed by Western blot analysis. (C) p120 catenin- and RPTP μ -coexpressing 3T3-L1 cells were induced to undergo adipogenic differentiation for 10 d according to standard procedures. Cells were stained with Oil-Red-O to visualize lipid accumulation (a, control vector; b, p120 catenin; c, p120 catenin plus RPTP μ ; d, p120 catenin plus RPTP μ -CS). (D) Quantification of stained cells was performed. The data represent mean percentage levels \pm SD, compared with the control vector ($n = 3$; * $p < 0.05$, ** $p < 0.01$).

the importance of tyrosine phosphorylation(s) in the control of the p120 catenin function in membrane trafficking. In view of the partial restoration of adipogenesis upon the introduction of RPTP μ , we cannot exclude the possibility of involvement of other p120 catenin-based mechanisms in the antiadipogenic effects. However, data from the present study clearly suggest that RPTP μ functions as a positive regulator of adipogenesis through modulating the cytoplasmic p120 catenin level via its phosphatase activity

Adipocyte and osteoblast differentiation are controlled in a reciprocal pattern. Our previous study showed that LAR tyrosine phosphatase inhibits adipogenesis but induces osteoblastogenesis (Kim *et al.*, 2009). Accordingly, we speculated that the enhancement of adipogenic differentiation by RPTP μ may occur via the inhibition of osteogenic differentiation, similar to the action of LAR phosphatase. However, we did not detect any change in the osteogenic differentiation of MC3T3-E1 preosteoblast cells following RPTP μ overexpression (Supplemental Figure S1). Thus it is unlikely that RPTP μ is involved in the regulation of osteoblast differentiation.

In summary, the present study demonstrates that RPTP μ levels are increased during adipogenesis, and increased RPTP μ expression promotes adipogenesis. RPTP μ directly suppresses p120 catenin phosphorylation, which leads to decreased p120 catenin localization in the cytoplasm, which, in turn, increases Glut-4 plasma membrane translocation and enhances adipogenesis (Figure 7). Our results collectively indicate that adipogenic differentiation is controlled by RPTP μ and p120 catenin, supporting the utility of these proteins as therapeutic targets for the treatment of obesity. Until now, this has not been extensively investigated with regard to the expression analysis of RPTP μ in adipose tissue (Koop *et al.*, 2003). In addition, there

is no report about adipose tissue defects in RPTP μ -knockout (KO) mice (Koop *et al.*, 2005). Therefore, to explore the detailed functions of RPTP μ in adipogenesis at an organism level, it is necessary to perform more extensive investigation of RPTP μ KO mice.

MATERIALS AND METHODS

Cell culture and adipogenic differentiation

The preadipocyte cell line 3T3-L1, derived from mouse embryonic fibroblasts, was purchased from the American Type Culture Collection (Manassas, VA). The cells were cultured in growth medium (high-glucose DMEM containing a 1% antibiotic-antimycotic solution and 10% bovine calf serum [Life Technologies-Invitrogen, Carlsbad, CA]) at 37°C in a humidified atmosphere with 5% CO₂. Human MSCs were purchased from Cambrex Bio Science (Walkersville, MD) and were maintained in MSC growth medium (MSCGM; Cambrex Bio Science) at 37°C in a humidified atmosphere with 5% CO₂. GP2-293 packaging cells were grown in DMEM containing a 1% antibiotic-antimycotic solution and 10% fetal bovine serum (FBS). 3T3-L1 cells were induced to differentiate into mature adipocytes as described previously (Kim *et al.*, 2008, 2009; Jung *et al.*, 2009). Confluent 3T3-L1 cells were incubated in differentiation medium composed of DMEM, 10% FBS, and MDI (a differentiation cocktail of 0.5 mM 3-isobutyl-1-methylxanthine, 1 μ M dexamethasone, and 10 μ g/ml insulin [Sigma-Aldrich, St. Louis, MO]). After 48 h, the medium was switched to a maintenance medium composed of DMEM, 10% FBS, and 10 μ g/ml insulin. The medium was replenished every other day. Adipogenic differentiation of MSCs was induced as recommended by the manufacturer (Cambrex Bio Sciences).

Oil-Red-O staining

Cultured cells were washed twice with phosphate-buffered saline (PBS) and fixed with 10% formalin for 30 min at room temperature. Then, the cells were washed with distilled water and stained for 30 min at room temperature with 0.3% filtered Oil-Red-O solution in 60% isopropanol (Sigma-Aldrich). The stained cells were washed with distilled water, and micrographs were obtained. To extract the incorporated Oil-Red-O dye, absolute isopropanol was added to the stained cell culture dish, and the dish was shaken at room temperature for 30 min. Triplicate samples were read at 510 nm using a GeneQuant 1300 spectrophotometer (GE Healthcare, Piscataway, NJ; Kim *et al.*, 2009).

Construction of retroviral vectors and transduction

To construct 3T3-L1 cells that stably express a FLAG-tagged mouse RPTP μ (clone BC_079621, acquired from ImaGene, Berlin, Germany), a retrovirus-mediated infection system was used. For expression of RPTP μ , DNA encoding the FLAG-tagged RPTP μ was inserted into the MCS of the pRetroX-IRES-ZsGreen1 or pRetroX-IRES-DsRed vector (Clontech, Mountain View, CA). Mouse p120 catenin cDNA in the pLZRS-MS-IRES-GFP retroviral vector was kindly provided by A.

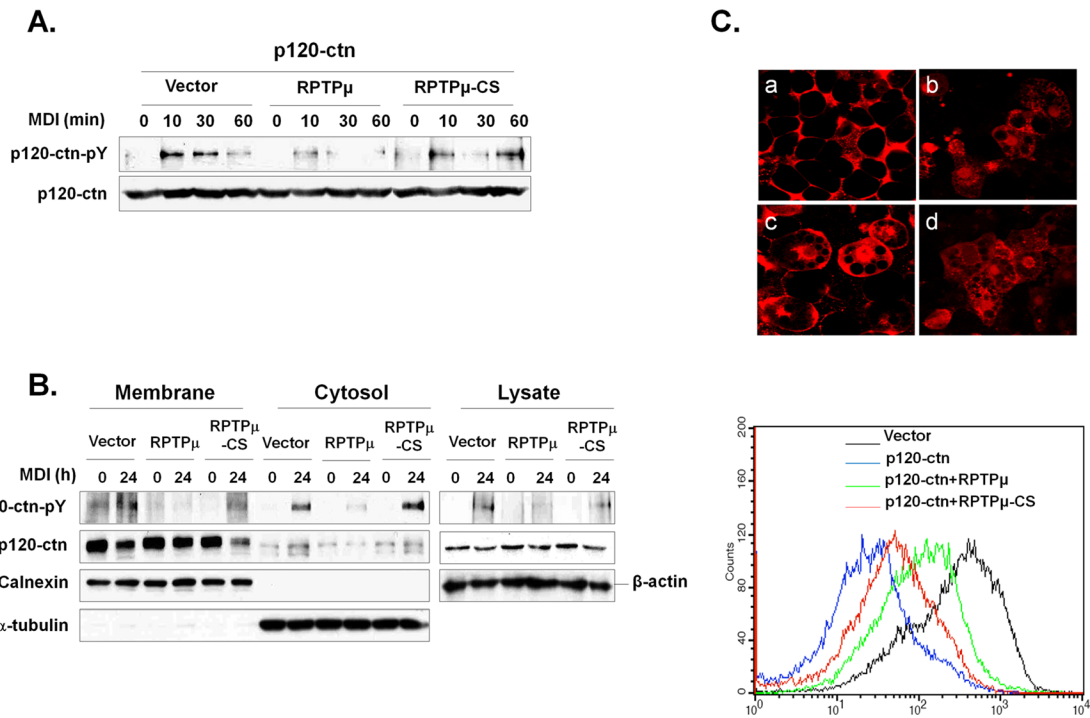


FIGURE 6: RPTP μ inhibits the cytoplasmic p120 catenin level via regulation of p120 catenin phosphorylation. (A) After obtaining a confluent culture of 3T3-L1 cells overexpressing p120 catenin plus RPTP μ or mutant RPTP μ -CS, cells were serum starved for 12 h and treated with MDI for the indicated times. Total cell lysates containing equal amounts of protein were immunoprecipitated using agarose conjugated with anti-phosphotyrosine antibodies (4G10 and PY20), and Western blot analysis was performed with anti-p120 antibody. The same membrane was reprobbed and immunoblotted with the anti-p120 catenin antibody. (B) Analysis of endogenous p120 catenin distribution by RPTP μ . Confluent 3T3-L1 cells overexpressing wild-type RPTP μ or mutant RPTP μ -CS were treated with MDI for 24 h and fractionated (see *Materials and Methods*). Equal amounts of fractionated proteins were immunoprecipitated using agarose conjugated with anti-phosphotyrosine (4G10 and PY20) antibodies, and Western blot analysis was performed with anti-p120 catenin antibody. Loading control and fraction purity were measured with anti-calnexin and anti- α -tubulin antibodies. (C) Analysis of Glut-4 membrane trafficking in differentiated adipocytes. p120 catenin- and RPTP μ -coexpressing 3T3-L1 cells were induced to differentiate into mature adipocytes for 10 d. Cells were serum starved for 3 h and treated with 100 nM insulin for 30 min. Cells were stained with the anti-Glut-4 antibody, and Glut-4 staining was confirmed with confocal microscopy (a, control vector; b, p120 catenin; c, p120 catenin plus RPTP μ ; d, p120 catenin plus RPTP μ -CS) (top) and FACS analysis (bottom).

Reynolds (Vanderbilt University, Nashville, TN). For viral production, GP2-293 cell lines were transfected using Lipofectamine 2000 (Life Technologies-Invitrogen). The details of the transfection and transduction methods were described previously (Jung *et al.*, 2009; Kim *et al.*, 2009). Infected cells were selected using a FACSria cell sorter (BD Biosciences, San Diego, CA) and further maintained in growth medium. The ectopic expression of FLAG-tagged RPTP μ and p120 catenin was confirmed by Western blot analysis. As a negative control, an RPTP μ mutant (RPTP μ -CS; inactive mutant; catalytic Cys replaced with Ser) was constructed.

RNA interference

For the knockdown of RPTP μ in 3T3-L1 cells, the pSIREN-RetroQ-DsRed Express retroviral vector (Clontech) was used. The shRNA was designed by selecting a target sequence specific for the mouse sequence by using the mission shRNA system of Sigma-Aldrich. The following gene-specific sequences were used: shRPTP μ -I: top, 5'-GATCCGCCACTGCACATCCGTAGTTATTTCAAGAGAATAACTACGGATGTGCAGTGGTTTTTTG-3' and bottom, 5'-AATTCAAAAAACCCTGCACATCCGTAGTTAT-TCTCTTGAAATAACTACGGATGTGCAGTGGCG-3'; shRPTP μ -II: top, 5'-GATCCGGCTGCCTCTTT GATGAACCATTTC AAGA-GAATGGTTCATCAAAGAGGCAGCTTTTTTTG-3' and bottom,

5'-AATTCAAAAAAGCTGCCTCTTTGATGAACCATTCTCTT-GAAATGGTTCATCAAAGAGGCAGCCG-3'; shRPTP μ -III: top, 5'-GATC CGCCTATGAATTTGAGACACCTTTTCAAGAGAAAGG TGTCTCAAATTCATAGGTTTTTTG-3' and bottom, 5'-AATTC AAAAACTATGAATTTGAGACACCTTTCTCTTGAAAAGGTGTCT-CAAATTCATAGGCG-3'; and shRPTP μ -IV: top, 5'-GATCCGC-CCGAAATAGATTTATCCAATTCAAGAGATTGGATAAATCT-ATTTCCGGTTTTTTG-3' and bottom, 5'-AATTCAAAAAACC CGGAAATAGATTTATCCAATCTCTTGAATTGGATAAATCTATTT-CCGGCG-3'. Each shRNA sequence was annealed and subcloned according to the manufacturer's guidelines, and the nontargeting control shRNA (scrambled; SCR) was obtained from Clontech.

Cell fractionation

Cells were fractionated into membrane and cytoplasmic fractions using the Qproteome Cell Compartment kit (Qiagen, Valencia, CA). The purity of subcellular fractions was confirmed using antibodies against calnexin (Santa Cruz Biotechnology, Santa Cruz, CA; membrane marker) and α -tubulin (Sigma-Aldrich; cytoplasmic marker).

Immunoblotting and immunoprecipitation

The cells were washed three times with ice-cold PBS containing 1 mM sodium orthovanadate and were harvested in ice-cold RIPA or

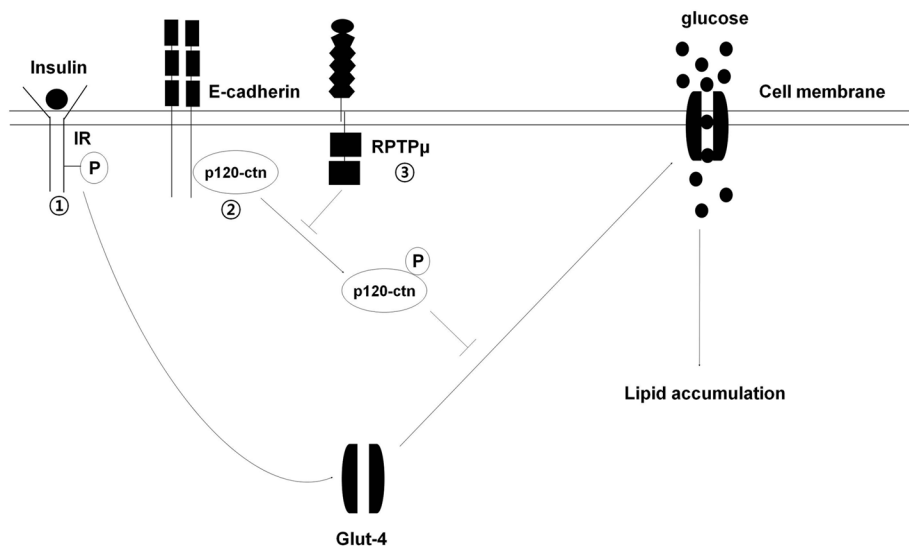


FIGURE 7: Proposed mechanism of RPTP μ -induced adipogenic differentiation. p120 catenin coimmunoprecipitates with cadherins. A key function of p120 catenin is to promote adhesion by binding and stabilizing cadherin at the cell surface (Peifer and Yap, 2003; Kowalczyk and Reynolds, 2004; Ishiyama *et al.*, 2010). Regulatory signals (probably mediated by receptor tyrosine kinases and PTPs) control the affinity of p120 catenin for cadherin, thereby regulating the internalization and/or recycling of cadherin complexes (Reynolds, 2010). 1) During adipogenesis, insulin is a positive regulator and induces Glut-4 membrane translocation, resulting in stimulation of glucose uptake into the cytoplasm. Subsequently, increased glucose flux may induce lipid accumulation during adipogenic differentiation. 2) p120 catenin exists in equilibrium between its membrane and cytoplasmic forms, and 3) this equilibrium may be regulated by p120 catenin phosphorylation. It has been known that cytoplasmic p120 catenin strongly inhibits Glut-4 membrane trafficking, thereby inhibiting adipogenesis. Our data clearly suggest that RPTP μ directly inhibits p120 catenin phosphorylation, inducing a decrease in cytoplasmic p120 catenin, which, in turn, leads to increased Glut-4 plasma membrane translocation and enhanced adipogenesis.

NP-40 lysis buffer containing a protease inhibitor and a phosphatase inhibitor cocktail (Roche, Basel, Switzerland). The protein concentrations were measured with a BCA Protein Assay Kit (Pierce, Rockford, IL). For analysis of endogenous p120 catenin phosphorylation, fractionated proteins were immunoprecipitated using agarose conjugated with anti-phosphotyrosine (4G10; Millipore, Billerica, MA) and platinum anti-phosphotyrosine (4G10 plus PY20; Millipore) antibodies. Antibodies (4G10 [20 μ l] and 4G10 plus PY20 [5–10 μ l]) were added to fractionated proteins (200–400 μ g). Mixtures were incubated overnight at 4°C. Immunoblotting and immunoprecipitation were performed as described previously (Kim *et al.*, 2009). Anti-RPTP μ was obtained from Abcam (Cambridge, MA) and Millipore, and the p120 catenin antibody was purchased from Cell Signaling Technology (Beverly, MA). The anti-FLAG and β -actin antibodies were purchased from Sigma-Aldrich. Secondary antibodies were purchased from Abcam, and the membranes visualized using the SuperSignal West Pico Chemiluminescent Substrate Kit (Pierce).

Immunofluorescence

Cells were induced to differentiate into mature adipocytes in six-well dishes containing glass coverslips. The cells were serum starved for 3 h and were treated with insulin for 30 min. Cells were fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences) for 30 min and then washed four times with PBS. Next the cells were blocked with Image-iT FX signal enhancer (Invitrogen) for 30 min and washed four times with PBS. The antibodies were diluted in 1% BSA-PBS. The cells were incubated with anti-Glut-4 (1:100, Abcam) for 1 h at room temperature and washed five times with PBS. The signal was

detected with a Cy5-conjugated secondary antibody (1:100; Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 h in a dark place. Finally, the cells were washed five times with PBS, mounted onto glass slides with Vectashield (Vector Laboratories, Burlingame, CA), and imaged using a Zeiss Axiovert fluorescence microscope (Carl Zeiss, Jena, Germany).

RNA extraction and real-time PCR

Total RNA was extracted from cultured cells using RNeasy minicolumns (Qiagen), and first-strand cDNA (cDNA) was synthesized using 1 μ g of total RNA as a template and AccuPower Cycle Script RT Premix (dT)20 (Bioneer, Daejeon, Korea) in a total volume of 20 μ l, according to the manufacturer's instructions. The primer sequences were as follows: mouse RPTP μ (forward, 5'-AGG AAA ATG ACA CCC ACT GC-3', and reverse, 5'-GTC AAG GTG CCC AAT CTT GT-3'); mouse PPAR- γ (forward, 5'-GAG CAC TTC ACA AGA AAT TAC C-3', and reverse, 5'-GAA CTC CAT AGT GGA AGC CT-3'); mouse aP2 (forward, 5'-GTG GGA ACC TGG AAG CTT GTC-3', and reverse, 5'-CTT CAC CTT CCT GTC GTC TGC-3'); and mouse RPTP μ (forward, 5'-CAT CCG TAA AGA CCT CTA TGC CAA-3', and reverse, 5'-ATG GAG CCA CCG ATC CAC A-3'). The PCR was performed with the Maxime PCR Premix Kit (Intron, Seoul, Korea).

For real-time PCR, the SYBR Premix Ex Tag (TaKaRa, Otsu, Japan) system was applied to detect the RPTP μ expression level using a Dice TP 800 Thermal Cycler (TaKaRa). Primers were used for human RPTP μ (forward, 5'-TTTGCTACCTTCCA-GTGCAG-3', reverse, 5'-CATCAATGCCCTGTAACC-AG-3') and β -actin (forward, 5'-AGGCCAGAGCAAGAGAGG-3', reverse, 5'-TACATGGCTGGGGTGTGAA-3'). Densitometric analysis of RPTP μ expression was performed using the ImageJ (National Institutes of Health, Bethesda, MD) program.

FACS analysis

Cells were cultured on six-well plates and differentiated into adipocytes. The cells were serum starved for 3 h and treated with 100 nM insulin. The cells were harvested with trypsin and then washed two times with FACS buffer (200 \times g for 5 min at 4°C). Next the cells were incubated with anti-Glut-4 antibody (1:50; diluted in 1% BSA-PBS; Abcam) for 30 min at 4°C and washed three times with FACS buffer. The signal was detected with a Cy5-conjugated secondary antibody (1:100; diluted in 1% BSA-PBS; Jackson ImmunoResearch Laboratories) for 30 min at 4°C. Finally, the cells were washed three times with FACS buffer, and the level of Glut-4 was determined by flow cytometry (FACSCalibur, BD Biosciences).

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