RACK1 is required for adipogenesis

Qinghua Kong,1* Lan Gao,1,2* Yanfen Niu,1,3 Pianchou Gongpan,1,2 Yuhui Xu,1,2 Yan Li,1,4* and Wenyong Xiong1,4*

1State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, China; 2Graduate University of the Chinese Academy of Sciences, Beijing, China; 3Biomedical Engineering Research Center, Kunming Medical University, Kunming, China; and 4Yunnan Key Laboratory of Natural Medicinal Chemistry, Kunming, China

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RACK1 is an interacting protein of IGF-IR, which is a pre- dominant receptor of insulin in early stages of adipogenesis. Therefore, RACK1 depletion in mice causes lethality at gastrulation, and RACK1 knockdown profoundly suppresses adipogenesis by reducing the expression of PPAR-γ and C/EBP-β. Consequently, stabilized β-catenin and targets this protein for ubiquitin-mediated degradation by the proteasome. In the absence of Wnt, RACK1 functions as an insulin-like mediator of adipocyte differentiation by Wnt/β-catenin signaling and PI3K-Akt-mTOR-S6K signaling pathways.

RACK1; 3T3-L1; adipogenesis; Wnt; PI3K-Akt-mTOR-S6K

Adipose tissue is an important metabolic organ that plays crucial roles in maintaining energy homeostasis as well as in obesity, whole body insulin sensitivity, and other metabolic diseases. Adipose tissue and that RACK1 knockdown by shRNA profoundly suppresses adipogenesis by reducing the expression of PPAR-γ and C/EBP-β. Depletion of RACK1 increased β-catenin protein levels and activated Wnt signaling. Furthermore, RACK1 knockdown also suppressed the PI3K-Akt-mTOR-S6K signaling pathway by reducing the PI3K p85α, pAkt T473, and S6K p70. Taken together, these results demonstrate that RACK1 is a novel factor required for adipocyte differentiation by emerging Wnt/β-catenin signaling and PI3K-Akt-mTOR-S6K signaling pathways.

RACK1; 3T3-L1; adipogenesis; Wnt; PI3K-Akt-mTOR-S6K

Adipogenesis involves the sequential activation of a cascade of transcription factors that coordinate the expression of genes responsible for the adipogenic phenotype (8, 27). In response to adipogenic stimuli, C/EBP-β and C/EBP-α are rapidly and transiently induced. These proteins then stimulate the expression of the two key adipogenic transcription factors, C/EBP-α and PPAR-γ, through KLF5 and the recruitment of a transcriptional activation complex including the transcription factors GR, STAT5A, and RXR and a coactivator complex (32).

Wnts are a family of secreted proteins that act through paracrine and autocrine mechanisms to regulate many aspects of cell fate and development (13, 21). β-Catenin plays a pivotal role as a transcriptional coactivator in the canonical Wnt pathway. In the absence of Wnt, GSK-3β phosphorylates β-catenin and targets this protein for ubiquitin-mediated degradation by the proteasome. In the presence of Wnt, activation of Frizzled receptors and LRP coreceptors leads to inhibition of GSK-3β activity, resulting in stabilization of cytoplasmic β-catenin. Subsequently, stabilized β-catenin translocates into the nucleus and binds to the Tcf/Lef tranflection factors, leading to activation of target genes (13). Wnt signaling maintains adipocytes in an undifferentiated state through inhibition of C/EBP-α and PPAR-γ (30). Overexpression of Wnt10b in 3T3-L1 preadipocytes stabilizes β-catenin and blocks adipogenesis. Transgenic mice overexpressing Wnt10b in adipose tissues show a 50% decline in total body fat and resist high-fat diet-induced WAT accumulation (22). In contrast, disruption of Wnt/β-catenin signaling leads to spontaneous adipocyte differentiation (2, 30).

RACK1 (GNB2L1) is a 36-kDa cytosolic protein with a propeller-like structure of seven WD40 repeats (1, 26). Through association with a large number of kinases and receptors (11, 23), it serves as a scaffold protein and plays pivotal roles in a variety of cellular processes including regulation of protein translation, cellular stress, tissue development, mammalian circadian clock, and cancer progression (23, 25, 31). RACK1 depletion in mice causes lethality at gastrulation, and heterozygous young females have a transient growth defect (35). In gastric cancer cells, RACK1 is found to regulate Wnt signaling pathway by stabilizing the β-catenin destruction complex (4). RACK1 is ubiquitously expressed and is highly conserved among all eukaryotic species (23). Recently, RACK1 has been identified as a glycogen particle-associated protein in 3T3-L1 adipocytes, which prompt that RACK1 may play a role in triacylglycerol synthesis regulation (34). Besides, RACK1 is an interacting protein of IGF-IR, which is a predominant receptor of insulin in early stages of adipogenesis and can modulate this receptor signaling pathway, including downstream factors PI3K p85α and phosphorylation of Akt.
However, the function of RACK1 in adipogenesis has not been explored to date.

We present here the first report that RACK1 is expressed in 3T3-L1 cells and murine WAT. RACK1 protein levels increase during differentiation of 3T3-L1 preadipocyte cells. Knockdown of RACK1 by RNAi profoundly suppresses adipogenesis of 3T3-L1 cells. Depletion of RACK1 increases β-catenin protein levels and activates Wnt signaling. Furthermore, RACK1 knockdown also reduces PI3K p85α, pAkt T473, and S6K p70. Our results suggest that RACK1 is a novel factor required for adipocyte differentiation and that it exerts its functions by regulating PI3K-Akt-mTOR-S6K and Wnt/β-catenin signaling pathway(s). Our findings highlight RACK1 as a new mediator of obesity and obesity-related metabolic disorders.

Glossary

<table>
<thead>
<tr>
<th>Term</th>
<th>Description</th>
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<tbody>
<tr>
<td>Akt</td>
<td>Protein kinase B, also known as PKB</td>
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<tr>
<td>BAT</td>
<td>Brown adipose tissue</td>
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<tr>
<td>C/EBP</td>
<td>CCAAT/enhancer-binding protein</td>
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<td>DAPI</td>
<td>4,6-Diamidino-2-phenylindole</td>
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<tr>
<td>FABP4</td>
<td>Fatty acid-binding protein 4</td>
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<td>Glut4</td>
<td>Glucose transporter 4</td>
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<td>GR</td>
<td>Glucocorticoid receptor</td>
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<tr>
<td>GSK-3β</td>
<td>Glycogen synthase kinase 3β</td>
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<tr>
<td>IGF-IR</td>
<td>Insulin-like growth factor I receptor</td>
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<td>JAK</td>
<td>Janus kinase</td>
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<tr>
<td>KLF</td>
<td>Krüppel-like factor</td>
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<tr>
<td>LRP</td>
<td>Low-density lipoprotein receptor-related protein</td>
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<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
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<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase 3-kinase</td>
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<tr>
<td>PPAR-γ</td>
<td>Peroxisome proliferator-activated receptor γ</td>
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<td>RACK1</td>
<td>Receptor of activated protein kinase C</td>
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<tr>
<td>RXR</td>
<td>Retinoid X receptor</td>
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<tr>
<td>S6K</td>
<td>Ribosomal S6-kinase</td>
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<tr>
<td>SCD1</td>
<td>Stearoyl-CoA desaturase-1</td>
</tr>
<tr>
<td>SREBP</td>
<td>Sterol response element-binding protein</td>
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<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
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<tr>
<td>Tcf/Lef</td>
<td>T-cell factor/lymphoid enhancer factor</td>
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<td>WAT</td>
<td>White adipose tissue</td>
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MATERIALS AND METHODS

Cell culture and adipocyte differentiation. 3T3-L1 preadipocytes (American Type Culture Collection, Manassas, VA) were cultured in DMEM with 10% calf serum. Cells were induced to differentiate into adipocytes with DMEM containing 10% FBS, 0.5 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, and 1 μg/ml insulin until day 3. The cells were then cultured with DMEM containing 10% FBS and 1 μg/ml insulin for 2 days. After that, the medium was replaced with DMEM containing 10% FBS every other day. Acquisition of the adipocyte phenotype began on day 3 and was maximal by day 6.

Retroviral constructs and infection. Stable knockdown of RACK1 was achieved by retroviral expression of shRNA from the pSirenaRetroQ vector (Clontech, Mountain View, CA). The retroviral packaging cell line ECO was transduced with retroviral vectors using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). The cell lines constructed by two RNAi sequences targeting RACK1 are: 5'-GCAGATCATATTGAGATGA-3' (shRACK1-1) and 5'-GCTAAAGACCAACCACATT-3' (shRACK1-2), respectively. A nontarget sequence 5'-GGCAAGATGATAAGCTAA-3' was used as a scramble control (shCon). After 48 h of transfection, the medium containing retroviruses was collected and used to infect 3T3-L1 preadipocytes. The infected cells were selected with 7.5 μg/ml puromycin (Invitrogen, San Diego, CA).

Oil Red O staining. Lipid accumulation was visualized by staining with Oil Red O (30). Cells were washed three times with PBS and then fixed for 30 min with 3.7% formaldehyde. Oil Red O (0.35% in isopropanol) was diluted with water (3:2) and incubated with the fixed cells for 30 min at room temperature. Cells were washed with water, and the stained fat droplets in the cells were visualized with an inverted microscope and photographed.

Antibodies and Western blotting. Antibodies against C/EBP-β (no. sc-56637), cyclin D1 (no. sc-20044), PI3K p85α (no. sc-1637), and β-actin (no. sc-81178) were obtained from Santa Cruz Biotechnology (Dallas, TX). Antibodies against Akt (no. 9272), pAkt T473 (no. 9271s), S6K p70 (no. 2708), PPAR-γ (no. 2435), and FABP4 (no. 2120) were obtained from Cell Signaling Technology (Danvers, MA). Antibodies against RACK1 (no. 610678) and β-catenin (no. 610154)

![Fig. 1. Expression of RACK1 in differentiating 3T3-L1 cells and murine adipose tissue. A: RACK1 is expressed in WAT of mice and 3T3-L1 preadipocyte cells. Epididymal WAT was prepared from 2-mo-old C57BL/6J mice. The position of molecular weight marker (kDa) as is indicated. B: RACK1 protein levels increase during differentiation of 3T3-L1 preadipocyte cells. Whole cell protein lysates were extracted at the indicated days of differentiation from 3T3-L1 and subjected to immunoblot analyses with antibodies against RACK1 and the adipocyte differentiation marker FABP4. C: quantification of RACK1 protein levels in B. The data are presented as means ± SD values of at least 3 independent experiments.](http://ajpcell.physiology.org/)
were obtained from BD Bioscience (Franklin Lakes, NJ). Antibody against active β-catenin was obtained from Millipore (no. 05–665; Billerica, MA). Cells were lysed in lysis buffer (50 mM Tris·HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100) containing 1 mM NaF, 1 mM Na3VO4, and protease inhibitor cocktail. Lysates were subjected to SDS-PAGE and Western blotting with indicated antibodies.

Immunofluorescent staining. Immunofluorescent staining was performed as described previously (4). 3T3-L1 adipocytes were fixed with 4% paraformaldehyde and then permeabilized with 0.2% Triton X-100. After being blocked, cells were incubated with anti-β-catenin antibodies. Cy3-labeled secondary antibodies were used to detect the primary antibody. Nuclei were stained with DAPI. Micrographs were captured using a fluorescence microscope (Nikon Ti-E, Tokyo, Japan).

Data analysis and statistics. Protein bands of Western blot were quantified using Metamorph software (Molecular Devices, Sunnyvale, CA). Student’s t-test was used to compare the differences between two groups. A P value <0.05 was considered to show statistically significant difference (or statistical difference).

RESULTS

Expression of RACK1 in differentiating 3T3-L1 cells and murine adipose tissue. Previous studies have shown that RACK1 is ubiquitously expressed and plays roles in a wide range of biological processes (23). However, its function in adipogenesis remains unknown. 3T3-L1 preadipocytes are well characterized in vitro models of adipocyte differentiation and have been extensively used to study the mechanisms of adipogenesis (12). Upon exposure to hormonal stimuli, 3T3-L1 preadipocytes can reliably differentiate into mature fat cells. To investigate the potential role of RACK1 in adipogenesis, we first analyzed the expression of RACK1 in 3T3-L1 cells. Western blot analysis demonstrated that RACK1 is obviously expressed in 3T3-L1 preadipocytes (Fig. 1A), and its protein levels dramatically increase during differentiation of 3T3-L1 preadipocytes into mature adipocytes (Fig. 1, B and C). RACK1 is highly induced after day 0 and keeps high protein levels throughout the induction process. We also examined the expression of RACK1 in mouse adipose tissue. As shown in Fig. 1A, RACK1 is also obviously detected in WAT of adult mice. Together, these data suggest that RACK1 is expressed in adipose tissue and 3T3-L1 adipocytes and might be involved in the adipocyte differentiation.

RACK1 is required for adipocyte differentiation. The above results demonstrate that RACK1 is strongly induced during differentiation of 3T3-L1. To our knowledge, many essential factors governing adipogenesis are induced during adipocyte differentiation, such as PPAR-γ, C/EBPs, and KLF4 (20). Thus we hypothesize that RACK1 may also function as a novel factor required for adipocyte differentiation. To test this hypothesis, we performed loss-of-function experiments by knocking down of endogenous RACK1. 3T3-L1 preadipocytes were transfected with retroviruses expressing RACK1 shRNA (shRACK1) or control shRNA (shCon) and then selected with puromycin. Finally, 3T3-L1 preadipocyte cell lines with constitutive knockdown of RACK1 were established. As shown in Fig. 2A, endogenous RACK1 protein was substantially decreased by RNAi. Both of the two shRACK1 targets can reduce the expression of RACK1 by ~70% (Fig. 2B). No major differences were observed in morphology or viability between shRACK1 and shCon 3T3-L1 cells.

To evaluate the impact of reduced RACK1 levels on adipogenesis, these stable cell lines were induced to differentiate into mature adipocytes in the presence of full-induction cocktail (methylxanthine, dexamethasone, and insulin). Lipid accumulation was visualized by staining with Oil Red O. The control 3T3-L1 cells could efficiently differentiate into mature adipocytes at day 6 (Fig. 2C). Knockdown of RACK1 leads to reduced lipid accumulation. The cells seeded in 6-cm dishes were stained with Oil Red O to visualize the degree of lipid accumulation at day 6 of differentiation. Bar, 100 μm. D: knockdown of RACK1 reduces the expression of the adipogenic markers C/EBP-β, PPAR-γ, and FABP4.
adipocytes at day 6 after induction (Fig. 2C). In contrast, knockdown of RACK1 almost completely blocked lipid accumulation, as assessed by Oil Red O staining in Fig. 3C. To further investigate the molecular mechanisms underlying this effect, we analyzed the expression of adipogenic markers by Western blot. As expected, depletion of RACK1 reduced expression of C/EBP-β, PPAR-γ, and FABP4 in these cells (Fig. 2D). Taken together, these data demonstrate that RACK1 is required for adipogenesis.

RACK1 knockdown increases β-catenin levels and activates Wnt signaling in 3T3-L1. Wnts are a family of secreted proteins that act through paracrine and autocrine mechanisms to regulate many aspects of cell fate and development (22). Wnt signaling maintains preadipocytes in an undifferentiated state through inhibition of C/EBP-α and PPAR-γ and, thereby, adipogenesis (6, 30). RACK1 was reported to regulate the Wnt signaling pathway in gastric cancer cells (4). Therefore, we hypothesized that RACK1 may also influence Wnt signaling in 3T3-L1 cells. We first examined the expression of β-catenin and RACK1 during adipocyte differentiation. Interestingly, Western blot results demonstrate that the protein levels of β-catenin and RACK1 were inversely correlated during adipogenesis (Fig. 3, A and B). RACK1 expression was induced after day 0 and its levels increased with the progression of differentiation, whereas the levels of both total and active β-catenin decreased as differentiation progressed. This is in accordance with the previous finding that Wnt signaling needs to be suppressed for adipogenesis to occur (2). Furthermore, it was found that knockdown of RACK1 increased β-catenin expression (Fig. 3, C and D). Consistently, active β-catenin level was also dramatically elevated in shRACK1 cells, indicating that depletion of RACK1 activates Wnt signaling (Fig. 3, C and D). Similar results were obtained in immunofluorescent staining of β-catenin (Fig. 3E). Depletion of RACK1 also increased the protein levels of cyclin D1 in differentiated cells (Fig. 3F). This suggests that RACK1 may play a critical role in regulating Wnt signaling during adipogenesis.

Fig. 3. Knockdown of RACK1 increases β-catenin protein levels and activates Wnt signaling in 3T3-L1. A: expression of RACK1 and β-catenin during adipogenesis. B: quantification of indicated protein levels in A. KD, knockdown. C: knockdown of RACK1 elevates the total and active β-catenin. Samples from cells expressing control shRNA or RACK1 shRNA were extracted at the indicated days of differentiation and subjected to immunoblot analyses. LiCl was added into the control group at day 0. D: quantification of indicated protein levels in C. E: immunostaining of β-catenin at day 6. Cy3-labeled secondary antibodies were used to detect the primary antibody. Nuclei were stained with DAPI. Bar, 100 μm. F: Western blot analysis of cyclin D1 from indicated cells at day 6. *P < 0.05, **P < 0.01.
expression of cyclin D1, a downstream target of Wnt signaling (Fig. 3F). Lithium chloride (LiCl) is known to activate canonical Wnt signaling by inhibiting GSK-3β and consequently stabilizing cytosolic β-catenin (18). In our experiments, knockdown of RACK1 yielded similar results to those obtained with LiCl treatment; Wnt signaling was activated through upregulation of β-catenin expression, thereby inhibiting the differentiation of 3T3-L1 preadipocytes (Figs. 2C and 3, C–E). Overall, all these results support that RACK1 regulates adipocyte differentiation through β-catenin signaling.

**RACK1 knockdown suppressed PI3K-Akt-mTOR-S6K signaling.** IGF-IR is a predominant receptor of insulin in the early stage of adipocyte differentiation (33). The downstream factors of the IGF-I signaling pathway, including PI3K, Akt, and mTOR, are known to induce PPAR-γ and essential for adipogenesis (9, 10, 17). The previous literatures showed that RACK1 is an IGF-1R-interacting protein that can modulate this receptor signaling pathway including the expression of PI3K p85α and phosphorylation of Akt (14, 15). Thus we speculated that RACK1 may also regulate adipogenesis through PI3K-Akt-mTOR-S6K signaling pathway.

To test this hypothesis, we analyzed the protein levels of PI3K p85α, pAkt T473, and S6K p70 during adipogenesis. As shown in Fig. 4, the two bands between 70 and 100 kDa showed similar trends in reduction upon knockdown of RACK1. As expected, RACK1 knockdown reduced the levels of PI3K p85α, pAkt T473, and S6K p70. LiCl treatment had similar results as those obtained by knockdown of RACK1. Therefore, besides the activation of Wnt signaling, knockdown of RACK1 also suppresses the PI3K-Akt-mTOR-S6K signaling.

![Fig. 4. Knockdown of RACK1 affects the protein levels of PI3K p85α, pAkt T473, and S6K p70. Samples from cells expressing control shRNA or RACK1-shRNA (2#) and cells treated with LiCl were extracted at the indicated days of differentiation and subjected to immunoblot analyses. LiCl was added into the control group at day 0.](http://ajpcell.physiology.org/)

**DISCUSSION**

RACK1 is ubiquitously expressed and is highly conserved among all eukaryotic species (23). It serves as a scaffold protein and functions in a variety of cellular processes. In this study, we have revealed a novel role of RACK1 in adipocyte differentiation. RACK1 protein levels increase during differentiation of 3T3-L1 preadipocytes. RACK1 knockdown profoundly suppressed adipocyte differentiation of 3T3-L1 cells and inhibited the induction of C/EBP-β and PPAR-γ, suggesting that RACK1 functions upstream of these two crucial adipogenic transcription factors. We also found that RACK1 knockdown led to elevation of β-catenin activity, as well as the suppression of PI3K-Akt-mTOR-S6K signaling. Wnt signaling maintains preadipocytes in an undifferentiated state, and differentiation of preadipocytes into adipocytes requires suppression of the Wnt/β-catenin pathway (6). Overexpression of Wnt10b in 3T3-L1 preadipocytes stabilizes β-catenin and blocks adipogenesis, while disruption of Wnt/β-catenin signaling leads to spontaneous adipocyte differentiation (2, 22). Additionally, IGF-IR functions as an insulin receptor in the early adipogenesis, and the downstream factors of PI3K-Akt-mTOR-S6K signaling pathway are required for adipogenesis (33). Inhibition of PI3K and loss of Akt1 or Akt2 repress adipocyte differentiation (9, 10). Altogether, it is plausible that RACK1 controls adipogenesis in 3T3-L1 cells by regulating PI3K-Akt-mTOR-S6K and Wnt/β-catenin signaling pathways.

RACK1 is also expressed in the WAT of mice (Fig. 1A), indicating that RACK1 might also function in the development of adipose tissue in vivo. Genetically modified mice are useful models for studying the function of genes in vivo. Recently, it has been reported that RACK1 depletion in mice causes lethality at gastrulation and a transient growth deficit in heterozygous young females (35). As RACK1 plays essential roles in a number of cellular processes, it is reasonable that loss of RACK1 in mice leads to embryonic death. Thus the role of RACK1 in adipocyte development should be investigated using conditional RACK1 knockout mice.

In summary, our study demonstrates that RACK1 is a novel factor required for adipocyte differentiation. The identification of factors regulating adipogenesis has important implications for obesity research, and the present results suggest a novel approach for studying obesity and its associated metabolic disorders, through modulation of RACK1 expression. Further in vivo study would be of great interest for supporting this hypothesis.

**GRANTS**

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**

REFERENCES