RACK1 is required for adipogenesis

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The running title: RACK1 is required for adipogenesis

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Abstract

Adipose tissue plays a critical role in metabolic diseases and the maintenance of energy homeostasis. RACK1 has been identified as an adaptor protein involved in multiple intracellular signal transduction pathways and diseases. However, whether it regulates adipogenesis remains unknown. Here, we reported that RACK1 is expressed in 3T3-L1 cells and murine white adipose tissue, and RACK1 knockdown by shRNA profoundly suppressed adipogenesis by reducing the expression of PPARγ and C/EBPβ. 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 demonstrated that RACK1 is a novel factor required for adipocyte differentiation by emerging Wnt/β-Catenin signaling and PI3K-Akt-mTOR-S6K signaling pathway(s).

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

RACK1: Receptor of activated protein kinase C

WAT: white adipose tissue

BAT: brown adipose tissue

C/EBP: CCAAT/enhancer-binding protein

PPARγ: peroxisome proliferator-activated receptor γ

SCD1: stearoyl-CoA desaturase-1

Glut4: glucose transporter 4

GSK-3β: glycogen synthase kinase-3β

LRP: low density lipoprotein receptor-related protein

Tcf/Lef: T-cell factor/lymphoid enhancer factor

DAPI: 4,6-diamidino-2-phenylindole

KLF: Krüppel-like factor

GR: glucocorticoid receptor

STAT: signal transducer and activator of transcription

RXR: retinoid X receptor

JAK: Janus kinase

SREBP: sterol response element-binding protein
51 PI3K: phosphatidylinositol-3 kinase
52 Akt: protein kinase B, also known as PKB
53 S6K: ribosomal S6-kinase
54 mTOR: mammalian target of rapamycin
55 IGF-1R: insulin growth factor-1 receptor
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 (28). White adipose tissue (WAT), the predominant type of fat in adult humans, primarily serves to store excess energy in the form of lipids, whereas brown adipose tissue (BAT) functions mainly to dissipate energy in the form of heat (7). Excessive WAT accumulation leads to obesity, which increases the risks of diabetes, cardiovascular diseases and some cancers (19). Conversely, lipoatrophy, a paucity of adipose tissue, is also associated with insulin-resistant diabetes and several other disorders (24). Therefore, understanding the signaling pathways regulating adipogenesis is crucial for developing rational therapies to prevent and treat these disorders.

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, CCAAT/enhancer-binding protein β (C/EBPβ) and C/EBPδ are rapidly and transiently induced. These proteins then stimulate the expression of the two key adipogenic transcription factors, C/EBPα and peroxisome proliferator-activated receptor γ (PPARγ), through Krüppel-like factor 5 (KLF5) and the recruitment of a transcriptional activation complex including the transcription factors glucocorticoid receptor (GR), signal transducer and activator of transcription 5A (STAT5A) and retinoid X receptor (RXR) and a co-activator complex (32). C/EBPα and PPARγ act synergistically to induce expression of various
adipocyte-specific genes, including 422/aP2, SCD1, and Glut4 (29). Besides this primary drivers of gene induction during adipogenesis, there are other transcription factors include Janus kinase 2 (JAK2), signal transducer and activator of transcription 3 (STAT3), early growth response protein-2 (KROX20), KLF4 and sterol response element-binding protein-1c (SREBP1c) that directly induce C/EBPβ or PPARγ expression and promote adipogenesis (3, 5, 16, 36).

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, glycogen synthase kinase-3β (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 low density lipoprotein receptor-related protein (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 T-cell factor/lymphoid enhancer factor (Tcf/Lef) transcription factors, leading to activation of target genes (13). Wnt signaling maintains preadipocytes in an undifferentiated state through inhibition of C/EBPα and PPARγ (30). Overexpression of Wnt10b in 3T3-L1 preadipocytes stabilizes β-catenin and blocks adipogenesis. Transgenic mice overexpressing Wnt-10b 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).
Receptor of activated protein kinase C (RACK1, GNB2L1) is a 36-kilodalton cytosolic protein with a propeller-like structure of 7 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 deficit (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-1R, 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 (14, 15). 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 white adipose tissue. 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(s). Our findings highlight RACK1 as a new mediator of obesity and obesity-related metabolic disorders.
Materials and Methods

Cell culture and adipocyte differentiation.

3T3-L1 preadipocytes (American Type Culture Collection, Manassas, VA) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% calf serum. Cells were induced to differentiate into mature adipocytes as described previously (30). Briefly, 2 days after reaching confluence (day 0), cells were induced to differentiate into adipocytes with DMEM containing 10% fetal bovine serum (FBS), 0.5 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, and 1 μg/ml insulin (DMI) until day 3. Then the cells were 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 pSiren-RetroQ vector (Clontech, Mountain View, CA). The retroviral packaging cell line ECO was transfected with retroviral vectors using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). The cell lines constructed by two RNAi sequences targeting RACK1 are 5’-GCAAGATCATTGTAGATGA-3’ (shRACK1-1#) and 5’-GCTAAAGACCAACCACATT-3’ (shRACK1-2#) respectively. A non-target sequence 5’-GCGAAAGATGATAAGCTAA-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 (Invivogen, San Diego, CA).

**Oil Red-O staining.**

Lipid accumulation was visualized by staining with Oil Red-O (30). Cells were washed three times with phosphate-buffered saline (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/EBPβ (no. sc-56637), cyclin D1 (no. sc-20044), PI3K p85α (no. sc-1637) and β-actin (no. sc-81178) were obtained from Santa Cruz (Dallas, TX). Antibodies against Akt (no. 9272), pAkt T473 (no. 9271s), and S6K p70 (no. 2708), PPARγ (no. 2435) and FABP4 (no. 2120) were obtained from Cell Signaling Technology (Danvers, MA). Antibodies against RACK1 (no. 610678) and β-catenin (no. 610154) were obtained from BD (Franklin Lakes, NJ). Antibody against active β-catenin was obtained from Millipore (no. 05-665; Temecula, CA). Cells were lysed in lysis buffer (50 mM Tris-HCl pH7.4, 150 mM NaCl, 1 mM EDTA, 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 was performed as described previously (4). 3T3-L1 adipocytes were fixed with 4% paraformaldehyde and then permeabilized with 0.2% Triton-X100. After blocking, cells were incubated with anti-β-Catenin antibodies. Cy3 labeled secondary antibodies were used to detect the primary antibody. Nuclei were stained with 4, 6-diamidino-2-phenylindole (DAPI). Micrographs were captured using a fluorescence microscope (Nikon Ti-E, 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 below 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. In order 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. 1B 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 PPARγ, 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 knockdown 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 preadipocytes 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 (MDI). Lipid accumulation was visualized by staining with Oil Red-O. The control 3T3-L1 cells could efficiently differentiate into mature 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/EBPβ, PPARγ 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/EBPα and PPARγ 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. 3A 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. 3C and D). Consistently, active β-Catenin level was also dramatically elevated in shRACK1 cells, indicating that depletion of RACK1 activates Wnt signaling (Fig. 3C and D). Similar results were obtained in immunofluorescent staining of β-Catenin (Fig. 3E). Depletion of RACK1 also increased the 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 (Fig. 2C, Fig. 3C-E). Overall, these results supports that RACK1 regulates adipocyte differentiation through β-Catenin signaling.

**RACK1 Knockdown suppressed PI3K-Akt- mTOR-S6K signaling**

IGF-1R is a predominant receptor of insulin in the early stage of adipocytes differentiation (33). The downstream factors of the IGF1 signaling pathway, including PI3K, Akt and mTOR, are known to induce PPARγ 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.

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/EBPβ and PPARγ, 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-1R 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 represses 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 pathway(s).

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 knock-out 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 great interest for supporting this hypothesis.
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Author Contributions

Wenyong Xiong and Yan Li designed the research, Qinghua Kong, Lan Gao, Yuhui Xu and Pianchou Gongpan performed the experiments, Qinghua Kong, Lan Gao and Wenyong Xiong wrote the paper.

Conflict of interest

The authors declare no conflict of interest.

References


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Figure Legends

Fig. 1. Expressions of RACK1 in differentiating 3T3-L1 cells and murine adipose tissue. (A) RACK1 is expressed in WAT of mice and 3T3-L1 preadipocyte cells. Epidydimal WAT were prepared from 2-month-old C57BL/6J mice. The position of molecular weight marker (kDa) is as 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 the means SD values of at least three independent experiments.

Fig. 2. RACK1 is required for adipocyte differentiation of 3T3-L1 preadipocytes. 3T3-L1 preadipocytes were infected with a retrovirus carrying control shRNA (con)
or a retrovirus carrying RACK1 shRNA (1# or 2#), selected with puromycin, and
differentiated with MDI. (A) The RACK1 protein levels were significantly reduced by
RNAi. Two RNAi sequences that target RACK1 were used. (B) Quantification of
RACK1 protein levels in (A). (C) 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/EBPβ,
PPARγ and FABP4.

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). (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 4,6-diamidino-2-phenylindole (DAPI). Bar: 100 μm. (F)
Western blot analysis of Cylin D1 from indicated cells at day 6. *P < 0.05, **P <
0.01.

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.
A

WAT 3T3-L1 kDa

RACK1

β-Actin

B

Days of differentiation

-2 0 3 5 7 kDa

RACK1

FABP4

β-Actin

C

RACK1 Protein Levels

D-2 D0 D3 D5 D7

0 1 2 3 4

bar graph indicating protein levels across days.