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AMP-activated protein kinase (AMPK) cross-talks with canonical Wnt signaling via phosphorylation of β-catenin at Ser 552

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ABSTRACT

AMP-activated protein kinase (AMPK) is a key regulator of energy metabolism; its activity is regulated by a plethora of physiological conditions, exercises and many anti-diabetic drugs. Recent studies show that AMPK involves in cell differentiation but the underlying mechanism remains undefined. Wingless Int-1 (Wnt)/β-catenin signaling pathway regulates the differentiation of mesenchymal stem cells through enhancing β-catenin/T-cell transcription factor 1 (TCF) mediated transcription. The objective of this study was to determine whether AMPK cross-talks with Wnt/β-catenin signaling through phosphorylation of β-catenin. C3H10T1/2 mesenchymal cells were used. Chemical inhibition of AMPK and the expression of a dominant negative AMPK decreased phosphorylation of β-catenin at Ser 552. The β-catenin/TCF mediated transcription was correlated with AMPK activity. *In vitro*, pure AMPK phosphorylated β-catenin at Ser 552 and the mutation of Ser 552 to Ala prevented such phosphorylation, which was further confirmed using [γ-^{32}P]ATP autoradiography. In conclusion, AMPK phosphorylates β-catenin at Ser 552, which stabilizes β-catenin, enhances β-catenin/TCF mediated transcription, expanding AMPK from regulation of energy metabolism to cell differentiation and development via cross-talking with the Wnt/β-catenin signaling pathway.

1. Introduction

AMP-activated protein kinase (AMPK) is a key player in the development of obesity and type 2 diabetes (T2D) [1,2]. It is a serine-threonine kinase consisting of a catalytic subunit (α) and two regulatory subunits (β and γ). AMPK regulates energy metabolism in cells. Its activation is associated with phosphorylation of AMPKα subunit at Thr 172. Protein phosphatase 2C (PP2C) dephosphorylates the Thr 172 phosphorylation of AMPKα subunit, inactivating AMPK [3]. An accumulating body of evidences suggests the involvement of AMPK in mesenchymal stem cell (MSC) differentiation [4–6]. In 3T3-L1 cells, activation of AMPK inhibits adipogenesis [6,7]. Activation of AMPK promotes myogenesis [8]. Our previous studies found that enhanced adipogenesis from MSC was associated with down-regulation of AMPK activity [9–11]. However, mechanisms linking AMPK to MSC remain undefined.

Canonical Wnt/β-catenin signaling pathway is required for early embryonic myogenesis [12]. Activation of the Wnt signaling pathway enhances myogenesis and inhibits adipogenesis in cultured MSCs [13] while down-regulation promoting adipogenesis [14–16]. Wnt/β-catenin signaling suppresses MSC commitment to the adipogenic lineage and terminal differentiation by blocking induction of peroxisome proliferator-activated receptor (PPAR) γ, an effect mediated by β-catenin [17]. Ser 552 of β-catenin is previously identified as a phosphorylation site by protein kinase A (PKA) [18,19] and protein kinase B (Akt) [20]. We used GPS 2.0 phosphorylation prediction system [21] and this site was also predicted to be a site for AMPK phosphorylation. We hypothesized that AMPK cross-talks with canonical Wnt signaling through phosphorylation of β-catenin to regulate adipogenesis and MSC differentiation. Our results show that AMPK phosphorylates β-catenin at Ser 552, which enhances β-catenin/TCF mediated transcription, linking AMPK to Wnt/β-catenin signaling pathway to regulate adipogenesis.

2. Materials and methods

2.1. Cell culture

Mouse C3H10T1/2 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% antibiotics at 37 °C. Cell differentiation was induced as described previously [22–24]. Briefly, C3H10T1/2 cells were treated with 10 μM 5′-azacytidine for 3 days, and seeded at 30% confluence in

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24-well plates. Cells were grown in adipogenic medium (1 μg/ml insulin, 0.1 μg/ml dexamethazone, 27.8 μg/ml isobutylmethylaxanthine and 10 μM troglitazone) for 3 days, repeated once. Then, medium was changed to DMEM medium supplemented with insulin (1 μg/ml) only for 2 days, and repeated once.

2.2. Oil-Red O staining

C3H10T1/2 cells and muscle cryosections were fixed in 4% paraformaldehyde for 10 min at room temperature, rinsed three times with PBS and stained with 0.2% (w/v) Oil-Red O (Sigma Chemical Co., Saint Louis, MO) for 10 min as described previously [25], and then destained with 60% ethanol.

2.3. Transfection

Transfection was performed using Lipofectamine (Invitrogen, Carlsbad, CA) according to the instruction from the manufacture. Briefly, C3H10T1/2 cells were seeded on special plates to reach 90% confluence; 1 day before transfection, cells were changed to no antibiotic medium, and the DNA:Lipofectamine ratio at 1:3 was used. Transfection efficiency was monitored by the co-transfection of a GFP plasmid. Cells were incubated at 37 °C in 5% CO_2.

2.4. DNA mutagenesis

Wild-type (WT) β-catenin was obtained from Addgene (plasmid 16518) [26]. The Ser 552 was mutated to Ala (S552A) using QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA).

2.5. In vitro kinase assays

The kinase reaction were done by mixing purified β-catenin with or without AMPK in AMPK assay buffer (pH 7.0) containing 2 μCi of [γ-^{32}P]ATP, 40 mM HEPES, 0.2 mM AMP, 80 mM NaCl, 0.8 mM EDTA, 0.8 mM DTT, 5 mM $MgCl_2$ and 0.2 mM ATP, for 25 min at room temperature [27]. Reaction was stopped by adding an equal volume of 2× SDS–PAGE loading buffer and boiling for 5 min. Then, samples were separated with 10% SDS–PAGE and transferred to nitrocellulose membranes for autoradiography.

2.6. β-Catenin RNA interference

RNA interference was conducted according to a kit from Santa Cruz Immunoprecipitation was conducted as previously described.

2.7. Immunoprecipitation

Immunoprecipitation was conducted as previously described [28]. Immunoprecipitated samples were analyzed by SDS–PAGE and immunoblotting using an antibody against phospho-β-catenin at Ser 552.

2.8. Luciferase reporter activity assay

To measure the transcriptional activity of T-cell factor (TCF)/lymphoid-enhancer binding factor (LEF), C3H10T1/2 cells seeded in 24-well plates were transfected in triplicates with a TCF/LEF reporter (TOP-Flash, Addgene plasmid 12456) or a control vector (FOP-Flash, Addgene plasmid 12457) [29] along with WT (Addgene plasmid 16518) [26] or mutant S552R β-catenin, and AMPKα2 WT or AMPKα2 K45R vectors (Addgene plasmids 15991 and 15992) [30]. β-Galactosidase vector (Promega, Madison, WI) was transfected as an internal control. Twenty-four hours following transfection, cells were lysed and the luciferase activity was measured with Bright-Glo Luciferase Assay System (Promega, Madison, WI) normalized to β-galactosidase activity.

2.9. Antibodies and chemicals

Antibodies against β-catenin, phospho-β-catenin at Ser 552, β-tubulin, PPARγ and histone H3 were purchased from Cell Signaling (Danvers, MA). IRDye 800CW goat anti-rabbit secondary antibody and IRDye 680 goat anti-mouse secondary antibody were purchased from Li-COR Biosciences (Lincoln, NE). AICAR (5-amino-imidazole-carboxamide ribonucleoside), Compound C (6-[4-(2-piperidin-1-ylethoxy)phenyl)]-3-pyridin-4-ylpyrazolo-[1,5-*a*]-pyrimidine, Akt inhibitor IV and PKA inhibitor 14–22 amide were purchased from Calbiochem, Inc. (San Diego, CA). Cycloheximide was purchased from Sigma (Saint Louis, MO).

2.10. Immunoblotting analyses

Cells were homogenized and subjected to SDS–PAGE and immunoblotting analyses as previously described using an Odyssey Infrared Imaging System (Li-COR Biosciences, Lincoln, NE). Band density was normalized according to the β-tubulin content [11,31].

2.11. Quantitative real-time PCR

Total RNA was extracted using Trizo (Sigma, Saint Louis, MO) and cDNAs were synthesized by reverse transcription kit (Bio-Rad, Hercules, CA). Real-time PCR (RT-PCR) was carried out using CFX RT-PCR detection system (Bio-Rad, Hercules, CA) with SYBR Green RT-PCR kit from Bio-Rad (Hercules, CA) and the following cycle parameters: 20 s at 95 °C, 20 s at 55 °C, and 20 s at 72 °C for 36 cycles. Primer sequences and their respective PCR fragment length were as follows: cyclin D1 (111 bp): 5′-GCGTACCCTGACACC AATCT-3′, and reverse, 5′-ATCTCCTTCTGCACGCACTT-3′; PPARγ (140 bp) 5′-GCCTGCGGAAGCCCTTTGGT-3′, and reverse, 5′-CAGCAA GCCTGGGCGGTCTC-3′; β-actin (180 bp) forward, 5′-TGCTGTCCCTG TATGCCTCT-3′, and reverse, 5′-TGTAGCCACGCTCGGTCA-3′. After amplification, a melting curve (0.01 C/s) was used to confirm product purity and agarose gel electrophoresis were performed to confirm that only a single product of right size was amplified.

2.12. Statistics

For cell culture studies, at least three independent experiments were conducted. All data were expressed as means ± standard errors (SE). Data from each time point were analyzed independently using the GLM procedure of SAS (SAS Inst., Inc., Cary, NC) and Tukey's Studentized Range test was used for multi-comparison to determine significant difference among means ($P < 0.05$).

3. Results

3.1. AMPK phosphorylates β-catenin at Ser 552

To determine whether the Ser 552 of β-catenin could serve as a substrate for AMPK, we mutated Ser 552 to Ala and performed *in vitro* kinase assays using WT β-catenin or β-catenin S552A. β-Catenin was purified by immunoprecipitation, and then incubated with or without pure AMPK. AMPK catalyzed the phosphorylation of WT β-catenin, and the mutation at Ser 552 to Ala abolished this effect (Fig. 1A). The weak band of phosphorylation detected in the S552R β-catenin sample should be due to nonspecific phosphorylation of β-catenin by AMPK. To further determine whether this phosphorylation was at Ser 552, immunoblotting

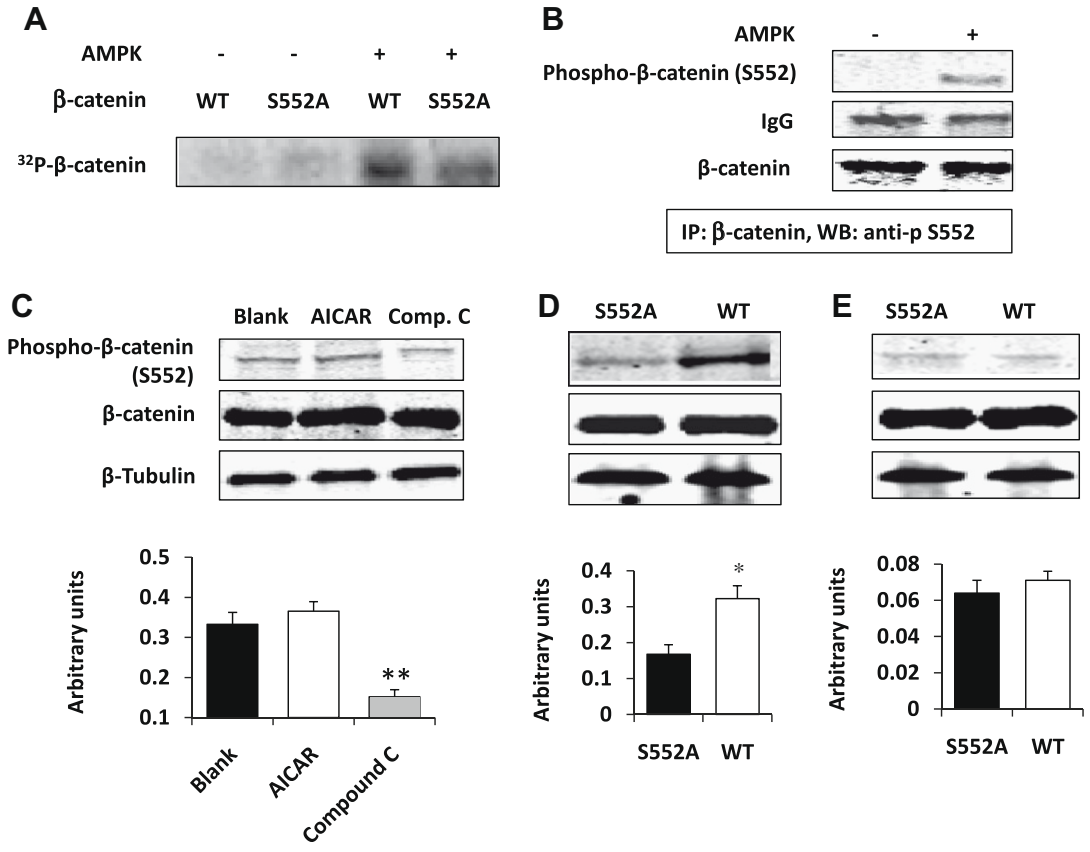

Fig. 1. AMP-activated protein kinase phosphorylates β-catenin at Ser 552 *in vitro* and in C3H10T1/2 cells. Wild-type (WT) and S552A mutated β-catenin were over-expressed in C3H10T1/2 cells and immunoprecipitated, which were then incubated with pure AMPK in the presence of [γ-^{32}P]ATP for 30 min; the representative image of autoradiography shows AMPK phosphorylated β-catenin *in vitro* (A). WT and K45R mutated AMPK were transfected in C3H10T1/2 cells for 6 h, and then, β-catenin was immunoprecipitated and the presence of phosphorylated β-catenin at Ser 552 was determined; the western blotting image shows AMPK phosphorylated β-catenin at Ser 552 *in vivo* (B). C3H10T1/2 cells were treated with vehicle only, AICAR and Compound C for 6 h and the presence of phosphorylated β-catenin at Ser 552 was detected by immunoblotting; the image shows that Compound C reduced β-catenin phosphorylation (C). C3H10T1/2 cells were over-expressing either S552A or WT β-catenin. Simultaneously, cells were over-expressing WT AMPK or K45R mutated AMPK. Images show that the mutation of Ser 552 of β-catenin to Ala prevented phosphorylation by over-expression of WT AMPK (D), and the expression of AMPK K45R, which is constantly inactive, prevented β-catenin Ser 552 phosphorylation (E).

was carried with an antibody specific to phosphorylated β-catenin at Ser 552, and S552 phosphorylation was detected (Fig. 1B).

If AMPK phosphorylates β-catenin, alteration of AMPK activity would change the phosphorylated β-catenin content. To test this notion, AICAR (0.25 mM) and Compound C (2 μM) were used to treat cells in order to alter AMPK activity as described previously [32,33]. AMPK inhibition by Compound C reduced the phosphorylation of β-catenin content (Fig. 1C). However, the effect of AICAR on β-catenin phosphorylation was not significant, which could be due to the low dose of AICAR used in this study. We used a low dose (0.25 mM) here instead of 1.0 mM commonly used in other studies in order to prevent possible side effects [28]. Another possibility is that normal cellular AMPK activity is sufficient to induce β-catenin phosphorylation and thus enhanced AMPK activation does not have additional effect.

Previous studies show that both Akt and PKA also phosphorylate β-catenin at Ser 552 [19,20]. To determine whether the observed changes in β-catenin phosphorylation could be caused by Akt or PKA, cells were treated with Akt inhibitor IV (10 μM), PKA inhibitor 14–22 amide (10 μM) or both as describe previously [34,35]. After inhibition of Akt, PKA, or both, β-catenin remained phosphorylated and such phosphorylation was associated with AMPK activity (data not shown), showing that AMPK induced β-catenin phosphorylation.

We further transfected cells with AMPKα2 WT and its dominant negative form-AMPKα2 (K45R) together with β-catenin (WT) or its mutant (S552A), to see whether ectopic expression of AMPK have the same effect on β-catenin. When cells were transfected with AMPKα2 WT, it phosphorylated β-catenin (WT) at Ser 552; the S552A mutation abolished such phosphorylation (Fig. 1D). As expected, when cells were transfected with dominant negative form of AMPKα2, the phosphorylated β-catenin was almost undetectable on both WT and S552A β-catenin (Fig. 1E), clearly showing that this phosphorylation was mediated by AMPK, not other kinases. In addition, mock transfection with empty vector alone did not affect β-catenin phosphorylation. Taken together, these data showed that β-catenin is phosphorylated by AMPK at Ser 552.

3.2. Phosphorylation of β-catenin increased transcriptional activity

Nuclear localized β-catenin interacts with transcriptional factors of the TCF/LEF-1 family to regulate downstream target gene expression. To test the effect of β-catenin phosphorylation by AMPK on TCF/LEF-1 transcriptional activity, the TCF/LEF-1 luciferase reporter TOP-Flash or control vector FOP-Flash were co-transfected with WT or S552A β-catenin, and together with AMPKα2 WT or K45R. As shown in Fig. 2, when cells were transfected with FOP-Flash, there was no change in the transcriptional activity of both AMPKα2 WT and K45R. In TOP-Flash group, AMPKα2 WT increased transcriptional activity of WT β-catenin compared with S552A β-catenin, showing the phosphorylation of β-catenin at Ser 552 is necessary for the enhancement of TCF/LEF-1 transcriptional

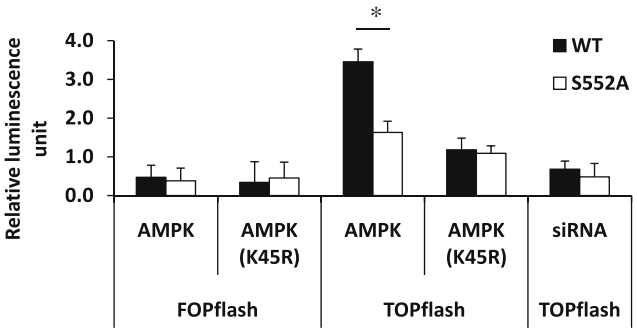

Fig. 2. Phosphorylation of β-catenin at Ser 552 increased TCF/LEF-1 transcriptional activity. C3H10T1/2 cells were transfected with wild-type (WT) or K45R AMPK, and WT or S552R mutated β-catenin for 24 h. The TCF/LEF-1 transcription factors mediates β-catenin transcriptional activity, which was analyzed by the generation of luminescence. Data show that WT AMPK activated TCF/LEF-1 transcriptional activity (TOP-Flash) while K45R AMPK did not (A). No activation of transcriptional activity was observed in the negative control (FOP-Flash). Knockdown β-catenin by RNA interference prevented activation, showing activation of TCF/LEF-1 transcription was mediated by β-catenin (A). (*$P < 0.05$; mean ± SE; $n = 3$).

activity (Fig. 2). In addition, AMPKα2 K45R abolished this effect, showing AMPK mediates the enhancement of transcriptional activity (Fig. 2). We also used siRNA to knockout β-catenin which abolished TCF/LEF-1 transcriptional activity (Fig. 2), showing the detected transcriptional activity was β-catenin specific.

To see whether the increased transcriptional activity results in the up-regulation of the expression of its downstream target gene, cyclin D1 mRNA level was analyzed. Cyclin D1 is a well-known target gene of Wnt/β-catenin signaling pathway [36]. Cells transfected with AMPKα2 WT increased cyclin D1 mRNA expression compared to K45R transfection, consistent with the enhancement of TCF/LEF-1 transcriptional activity (Fig. 3A). Mutation of the phosphorylation site on β-catenin to Ala abolished this effect (Fig. 3A), again showing that the phosphorylation at Ser 552 of β-catenin by AMPK is necessary for the enhancement of TCF/LEF-1 transcriptional activity.

PPARγ is also regulated by Wnt/β-catenin signaling [37]. Transfection of cells with WT β-catenin, AMPK inhibited PPARγ expression, which was not observed in cells transfected with S552A mutated β-catenin, elucidating that AMPK mediates PPARγ expression through phosphorylation of β-catenin at Ser 552 (Fig. 3B). Because PPARγ is a critical regulator and marker of adipogenesis, the protein level of PPARγ was further analyzed. Inhibition of AMPK by transfecting AMPK K45R increased PPARγ protein content (Fig. 3C). Consistent with this, the adipogenesis was much higher in cells transfected with constant negative AMPK compared to the wild-type AMPK (Fig. 3D).

4. Discussion

Obesity and T2D are closely linked metabolic complications, both of which are increasing at alarming rates [38]. AMPK has a key role in the development of obesity and T2D [39]. Activation of AMPK inhibits adipogenesis [4,5]. Activation of AMPK promotes myogenesis [8]. However, the mechanisms linking AMPK to MSC differentiation remains unclear. Wnt/β-catenin signaling pathway regulates morphogenesis during early developmental stages. Activation of Wnt signaling stabilizes β-catenin, which enters nuclei

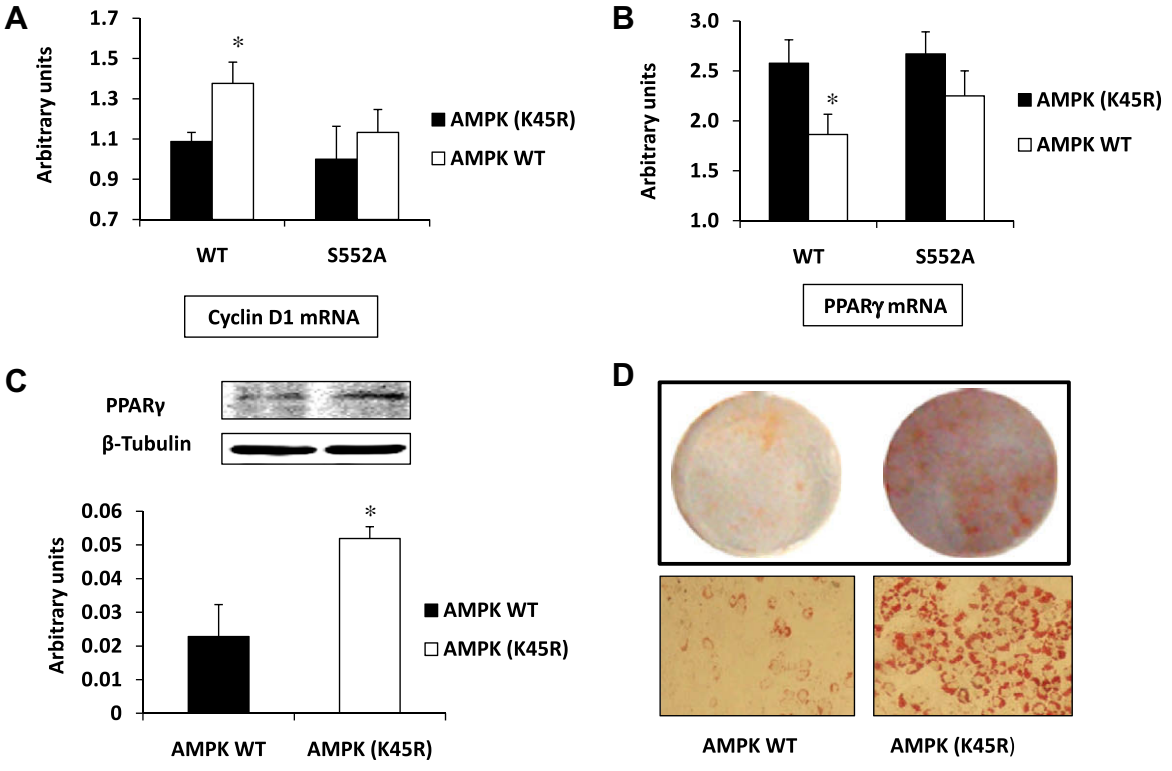

Fig. 3. Phosphorylation of β-catenin at Ser 552 mediated downstream mRNA expression and adipogenesis. C3H10T1/2 cells were transfected with wild-type (WT) or K45R AMPK, and WT or S552R mutated β-catenin for 24 h and total mRNA was extracted. The cyclin D1 and peroxisome proliferator-activated receptor (PPAR) γ mRNA expressions were measured by RT-PCR. Data show that cyclin D1 mRNA expression was enhanced by AMPK WT compared to K45R mutant, but the mutation of S552R of β-catenin prevented enhancement of cyclin D1 expression by AMPK (A). Similarly, PPARγ mRNA expression as inhibited by ectopic expression of WT AMPK, but the mutation of S552R of β-catenin prevented it (B). Ectopic AMPK K45R expression enhanced PPARγ protein concentration in above treated cells compared to WT AMPK (C). C3H10T1/2 cells were incubated in adipogenic medium for 14 days, and the ectopic AMPK K45R expression enhanced adipogenesis compared to WT AMPK (D). (*$P < 0.05$; mean ± SE; $n = 3$).

and binds to TCF/LEF transcription factors to induce Wnt target genes [40]. β-Catenin is a primary mediator of the canonical Wnt/β-catenin signaling pathway [41,42]. Down-regulation of Wnt/β-catenin signaling was linked to the promotion of adipogenesis [14,15].

We hypothesized that AMPK inhibits adipogenesis through Wnt/β-catenin signaling pathway via phosphorylation of β-catenin. We treated C3H101/2 cells with chemicals to either activate or inhibit AMPK, as well as ectopic expression of wild-type and mutated AMPKα subunit, and AMPK inhibition reduces Ser 552 phosphorylation in β-catenin. The phosphorylation of β-catenin by AMPK was further confirmed by *in vitro* kinase assays. Ser 552 is a site previously identified as a phosphorylation site by protein kinase A (PKA) [18,19] and Akt [20]. We used a specific inhibitor of PKA as well as Akt to exclude the possibility that such phosphorylation was mediated by these two kinases.

To analyze the biological functions of this phosphorylation, we analyzed the effect of this phosphorylation on the activity of β-catenin/TCF mediated transcription using a TCF/LEF reporter construct. The β-catenin/TCF mediated transcription was dramatically enhanced due to β-catenin Ser 552 phosphorylation. Therefore, AMPK induces β-catenin phosphorylation at Ser 552, which stabilizes β-catenin and enhances β-catenin/TCF mediated transcription, inhibiting adipogenesis.

The next question is whether β-catenin phosphorylation is one of the main mechanisms for AMPK to inhibit adipogenesis. Using Ser 552 mutated β-catenin (S552A), AMPK was not effective to regulate β-catenin/TCF mediated PPARγ transcription and adipogenesis, strongly suggesting that Ser 552 is a major mechanism for AMPK to regulate adipogenesis from MSC.

Our observation that AMPK mediates adipogenesis via phosphorylation of β-catenin has important physiological implications. AMPK has a central role in the regulation of energy metabolism and is regulated by numerous factors [43]. First, obesity epidemic is becoming increasingly serious and it is known that obesity inhibits AMPK [44,11]. In addition, AMPK is modulated by adipokines such as leptin, adiponectin and resistin, and cytokine, such as interleukin-6 [45]. Furthermore, AMPK is activated and regulates energy metabolism during exercise [46,47]. Therefore, obesity, inflammation and exercise likely affect adipogenesis through the cross-talking of AMPK with Wnt/β-catenin signaling, exerting long-term effects on tissue development and physiological functions. Because both AMPK and Wnt/β-catenin signaling are widely expressed in various tissues and conserved in animal kingdom, such cross-talking likely has broad biological effects waiting to be discovered.

Acknowledgments

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