

# AICAR, an activator of AMPK, inhibits adipogenesis via the WNT/ $\beta$ -catenin pathway in 3T3-L1 adipocytes

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**Abstract.** AMP-activated protein kinase (AMPK) is known to sense the cellular energy state and regulates various cellular energy metabolism pathways through its activation by AMP, an indicator of a low-energy state. 5-Aminoimidazole-4-carboxamide-1- $\beta$ -D-ribofuranoside (AICAR), an activator of AMPK, efficiently inhibited the adipogenesis of 3T3-L1 cells. To elucidate its possible mechanism of action, the expression levels of  $\beta$ -catenin and other members of the WNT/ $\beta$ -catenin pathway were analyzed during the adipogenesis of 3T3-L1 cells in the presence or absence of AICAR. It was found that AICAR significantly enhanced  $\beta$ -catenin expression and its nuclear accumulation. Transfection of  $\beta$ -catenin small interfering RNA (siRNA) significantly prevented the effects of AICAR on the expression of various genes. The expression of the major genes of adipogenesis including the peroxisome proliferator-activated receptor (PPAR) $\gamma$ , the CCAAT/enhancer binding protein (C/EBP) $\alpha$ , the fatty acid binding protein (FABP)4 and lipoprotein lipase (LPL), which were all reduced by AICAR treatment, were significantly recovered in  $\beta$ -catenin siRNA-transfected cells. Among the members of the WNT/ $\beta$ -catenin pathway, the expression of low density lipoprotein receptor-related protein (LRP)6, dishevelled (DVL)2 and DVL3 were significantly up-regulated by AICAR treatment, whereas the expression of AXIN was down-regulated. The present study provides compelling evidence that AICAR inhibits adipogenesis through the modulation of the WNT/ $\beta$ -catenin pathway.

## Introduction

Adipogenesis is a complex cellular process involving changes in gene expression, metabolism and cell morphology, resulting in intracellular fat accumulation. Adipogenesis is induced by

hormones such as insulin and dexamethasone, which rapidly induce various upstream regulators of adipogenesis, interactions of which in the early stage of adipogenesis lead to the induction of major transcription factors of adipogenesis, such as the peroxisome proliferator-activated receptor (PPAR) $\gamma$  and the CCAAT/enhancer binding protein (C/EBP) $\alpha$ . PPAR $\gamma$  and C/EBP $\alpha$  act synergistically by forming a positive feedback loop between them, resulting in the up-regulation of lipid-metabolizing enzymes including lipoprotein lipase (LPL), fatty acid binding protein (FABP)4 and fatty acid synthase (1,2).

Wingless-type MMTV integration site (WNT) is a family of secreted glycoproteins that induce intracellular signaling pathways to regulate various cellular processes including proliferation, differentiation and development (3-6).  $\beta$ -catenin plays a pivotal role as a transcriptional co-activator in the canonical WNT/ $\beta$ -catenin pathway. Binding of WNT to the frizzled (FZ) receptor and to the low density lipoprotein receptor-related protein (LRP) co-receptor activates dishevelled (DVL) to inhibit AXIN and glycogen synthase kinase (GSK)3, which leads to the stabilization and nuclear translocation of  $\beta$ -catenin (7). Recently, the WNT/ $\beta$ -catenin pathway was implicated in the inhibition of adipogenesis, and it was reported that  $\beta$ -catenin suppresses adipogenesis by inhibiting the expression and activity of PPAR $\gamma$  and C/EBP $\alpha$  (8,9).

AMP-activated protein kinase (AMPK) is a serine/threonine protein kinase that senses the cellular energy state and regulates various cellular energy metabolism pathways through its activation by AMP, an indicator of a low-energy state (10,11). If the ATP/ADP ratio is decreased by ATP consumption, ADP is converted to AMP by adenylate kinase, resulting in activation of AMPK. The activated AMPK not only down-regulates anabolic ATP-consuming processes such as lipogenesis and gluconeogenesis, but also up-regulates catabolic ATP-producing processes such as lipid  $\beta$ -oxidation (12). AMPK is a heterotrimeric protein composed of the catalytic subunit  $\alpha$  and two regulatory subunits,  $\beta$  and  $\gamma$  (13). AMPK kinase (AMPKK), an upstream kinase of AMPK, phosphorylates threonine 172 of the AMPK subunit  $\alpha$ , which is required for AMPK activation (10). Activated AMPK phosphorylates and inactivates acetyl-CoA carboxylase (ACC), thereby leading to an increase of lipid  $\beta$ -oxidation (10,14). The product of ACC, malonyl-CoA, is a potent inhibitor of  $\beta$ -oxidation (15). When AMPK phosphorylates and inactivates ACC, the cellular concentration of malonyl-CoA is decreased, resulting in decreased inhibition

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of  $\beta$ -oxidation (16). Activated AMPK also inhibits glucose-activated fat synthesis by suppressing the expression of lipogenic enzymes in liver cells (17,18). 5-Aminoimidazole-4-carboxamide-1- $\beta$ -D-ribofuranoside (AICAR), an activator of AMPK, enhances the phosphorylation and activation of AMPK both *in vivo* and *in vitro* (19,20). AICAR is metabolized in the cytoplasm to ZMP, which has a structure very similar to AMP. Binding of ZMP to the AMPK subunit  $\gamma$  induces an allosteric conformational change of AMPK heterotrimers, and subsequently enhances the phosphorylation of the AMPK subunit  $\alpha$  by AMPKK. Recently, it was reported that AICAR inhibits fat accumulation in 3T3-L1 adipocytes by suppressing the expression of PPAR $\gamma$  and C/EBP $\alpha$  (20,21). However, the molecular mechanism of this suppressive effect was not fully elucidated. The present study reports that AICAR suppresses the expression of PPAR $\gamma$  and C/EBP $\alpha$  through the modulation of the WNT/ $\beta$ -catenin pathway.

## Materials and methods

**Chemicals and reagents.** Cell culture reagents were obtained from Life Technologies (Grand Island, NY, USA). Anti-C/EBP $\alpha$ , anti-C/EBP $\beta$ , anti-DVL2 and anti-AXIN antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-PPAR $\gamma$ , anti-FABP4 and secondary antibodies were purchased from Cell Signaling (Beverly, MA, USA). Anti- $\beta$ -catenin antibody was purchased from BD Transduction Laboratories (Lexington, KY, USA). Control small interfering RNA (siRNA) and  $\beta$ -catenin siRNA were purchased from Santa Cruz Biotechnology. Lipofectamine RNAiMAX transfection reagent was purchased from Invitrogen (Carlsbad, CA, USA). AICAR and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

**Cell culture.** 3T3-L1 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Two days after reaching confluence (day 0), 3T3-L1 cells were cultured for 2 days in differentiation-induction medium consisting of Dulbecco's Modified Eagle's Medium (DMEM) containing 1  $\mu$ g/ml insulin, 0.25  $\mu$ M dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine and 10% fetal bovine serum. The cells were then maintained in differentiation medium, which consisted of DMEM containing 1  $\mu$ g/ml insulin and 10% fetal bovine serum (maintenance medium). This medium was changed every 2 days until the cells were harvested. To test the effects of AICAR on adipogenesis, AICAR was added to the differentiation induction and maintenance media until the cells were harvested. Differentiated cells in each well of 6-well plates were harvested in 500  $\mu$ l of phosphate-buffered saline (PBS), frozen and sonicated. The triglyceride contents of cell lysates were measured using the TG-S reaction kit (Asan Pharm., Seoul, Korea). Lipid droplets in the cells were stained with Oil Red O as previously described (22).

**Real-time reverse transcription polymerase chain reaction (RT-PCR).** Total RNA was extracted using the RNeasy kit (Qiagen, Hilden, Germany). PCR primer and probe sequences for C/EBP $\alpha$  were kindly provided (23). Assay-on-demand gene expression products (Applied Biosystems, Foster City, CA, USA) were used for the other genes: FABP4, Mm00445880\_

m1; LPL, Mm00434764\_m1; PPAR $\gamma$ , Mm00440945\_m1; WNT10B, Mm00442104\_m1; LRP6, Mm00999795\_m1; DVL1, Mm00438592\_m1; DVL2, Mm00432899\_m1; DVL3, Mm00432914\_m1;  $\beta$ -catenin, Mm00483039\_m1. The level of 18S rRNA was used as an endogenous control as previously described (23). Briefly, for each sample, the levels of each mRNA were normalized to the abundance of 18S rRNA, and ratios of normalized mRNA amounts of each samples to that of preadipocytes (day 0) were determined using the comparative Ct method (24).

**Preparation of total cell lysates and Western blotting.** Cultured and differentiated cells were lysed with ice-cold RIPA buffer containing 25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS) and a protease inhibitor cocktail (Sigma-Aldrich) to obtain total cell lysates. Each lysate was centrifuged at 14,000 rpm for 20 min at 4°C to remove insoluble materials, and the protein concentrations were determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA). Protein extracts (50  $\mu$ g) were resolved by 10% SDS-polyacrylamide gel electrophoresis and electrotransferred to nitrocellulose membranes at 150 mA for 1 h. The membranes were blocked for 2 h at room temperature with PBS containing 5% skim milk and 0.1% Tween-20, and then incubated with a 1:1,000 dilution of primary antibody overnight at 4°C. This was followed by incubation with a 1:1,000 dilution of a horseradish peroxidase-conjugated anti-rabbit secondary antibody for 1 h at room temperature. Peroxidase activity was visualized using an enhanced chemiluminescence kit (Pierce).

**Preparation of nuclear extracts.** 3T3-L1 cells were scraped in PBS and centrifuged for 10 min at 2,000  $\times$  g. The pelleted cells were suspended in buffer A containing 25 mM Tris-HCl (pH 7.5), 50 mM KCl, 2 mM MgCl<sub>2</sub>, 1 mM EDTA and 5 mM dithiothreitol, and homogenized in a tightly-closed homogenizer. Pellets containing nuclei were isolated by centrifugation at 14,000  $\times$  g for 5 min at 4°C. High salt extraction of nuclear proteins was performed by incubating the nuclei with buffer B containing 25 mM Tris-HCl (pH 7.5), 420 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 0.5 mM EDTA and 25% sucrose for 30 min on ice. After a 30 min centrifugation at 20,000  $\times$  g, the supernatants were used as nuclear extracts.

**Transfection of siRNA.** Two days after reaching confluence, 3T3-L1 cells were cultured in a serum-free medium for 1 h and transfected with 60 nM of the target gene or control siRNA using the Lipofectamine RNAiMAX transfection reagent. After 6 h, the transfected cells were differentiated according to the differentiation protocol. After 4 days, total RNA and protein extracts were prepared and analyzed by real-time RT-PCR and Western blot analysis, respectively.

**Statistical analysis.** All experimental data are expressed as means  $\pm$  standard error (SE) from at least three independent experiments. Statistical significances for differences between experimental groups were determined using the unpaired t-test. All analyses were performed using SPSS v. 17 (SPSS, Chicago, IL, USA).

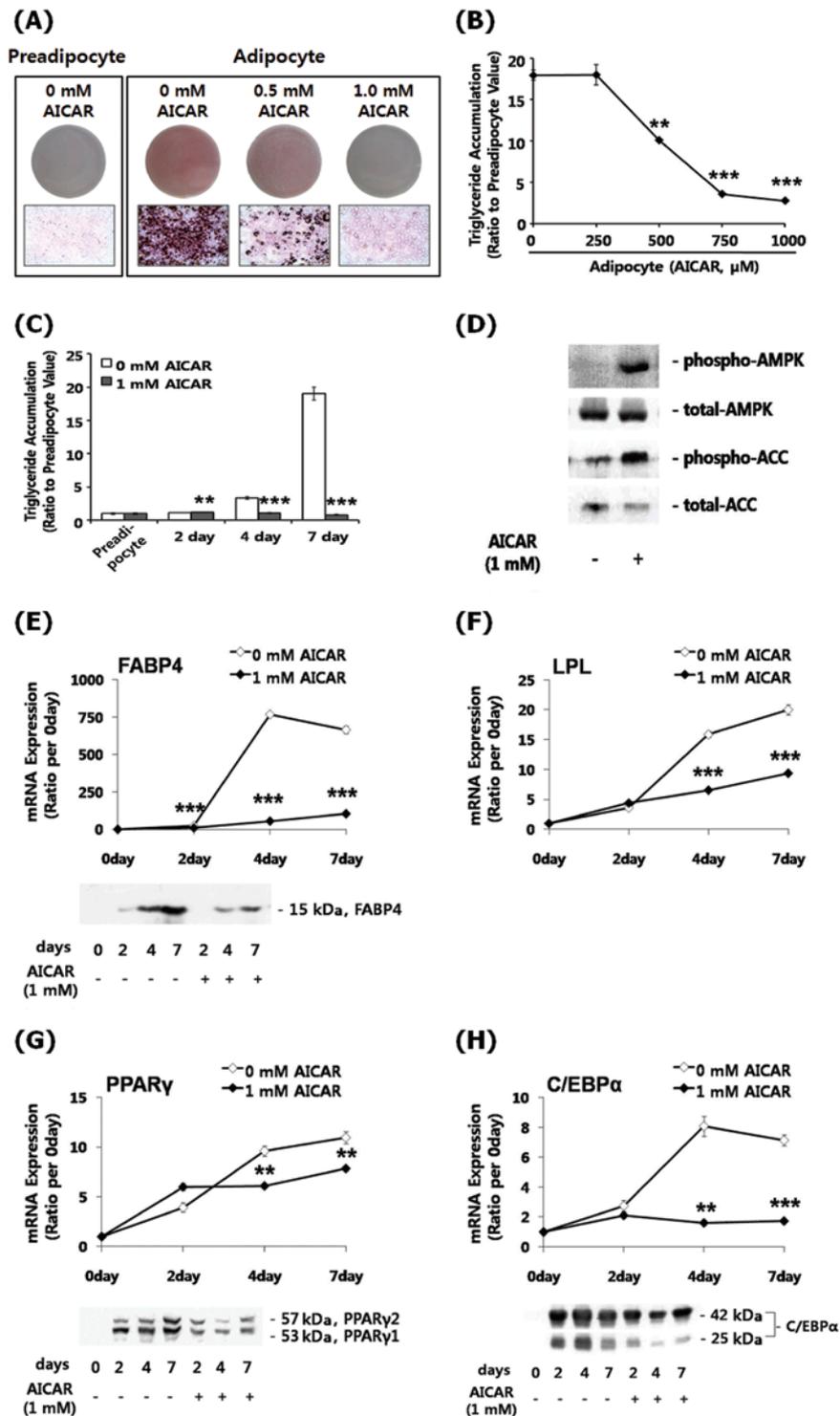


Figure 1. AICAR activates AMPK and inhibits adipogenesis in 3T3-L1 cells. (A) Inhibitory effects of AICAR on lipid droplet formation. Lipid droplets were stained with Oil Red O on day 7 of adipocyte differentiation and examined using light microscopy. (B) Inhibition of intracellular triglyceride accumulation by AICAR treatment. The effects of various concentrations of AICAR on triglyceride contents were measured on day 7 of differentiation. (C) Time course experiments for the inhibitory effects of 1 mM AICAR on intracellular triglyceride accumulation. The triglyceride contents of 3T3-L1 cells differentiated for 2, 4 and 7 days in the presence or absence of 1 mM AICAR are presented. (D) Increased phosphorylation of AMPK and its substrate, ACC, by treatment with 1 mM AICAR in 3T3-L1 cells on day 7 of differentiation. (E-H) Effects of 1 mM AICAR treatment on the expression of mRNA or protein levels of adipogenic genes. AICAR significantly decreased the expression of the lipid metabolizing enzymes, FABP4 (E) and LPL (F), as well as the major transcription factors, PPAR $\gamma$  (G) and C/EBP $\alpha$  (H). \*\* $p < 0.01$ , \*\*\* $p < 0.001$  compared with untreated adipocytes on the same differentiation day.

**Results**

*AICAR activates AMPK and inhibits adipogenesis in 3T3-L1 cells.* Adipogenesis of 3T3-L1 cells was significantly inhibited

by AICAR treatment. To visualize the anti-adipogenic effects of AICAR, lipid droplets in differentiated 3T3-L1 adipocytes were stained with Oil Red O dye. Formation of lipid droplets during adipocyte differentiation was dose-dependently inhibited

by AICAR treatment (Fig. 1A). AICAR treatment also significantly inhibited intracellular triglyceride accumulation in a dose-dependent manner (Fig. 1B). AICAR effectively inhibited fat accumulation at 0.5-1 mM concentrations, in agreement with previous reports (20,21). The inhibitory effect of AICAR on triglyceride accumulation was also evident in a time course experiment (Fig. 1C). In this experimental condition, AICAR increased the phosphorylation of both AMPK and its substrate, ACC, which providing clear evidence of AMPK activation by AICAR treatment (Fig. 1D). Next, the mRNA and protein expression levels were determined for lipid-metabolizing enzymes and major transcription factors of adipogenesis. Expressions of lipid-metabolizing enzymes, such as FABP4 and LPL, were markedly increased during adipogenesis, but their expression was significantly down-regulated by AICAR treatment (Fig. 1E and F). Also, the mRNA and protein levels of PPAR $\gamma$  and C/EBP $\alpha$ , which are transcription factors required for the expression of lipid metabolizing enzymes, were significantly down-regulated by AICAR treatment (Fig. 1G and H). These results provide clear evidence that the AMPK activator, AICAR, inhibits the entire adipogenic process including the expression of both lipid-metabolizing enzymes and the major transcription factors of adipogenesis.

*AICAR up-regulates the expression and nuclear accumulation of  $\beta$ -catenin.* The present interest in  $\beta$ -catenin was prompted by reports of its crucial role as a negative regulator of adipogenesis (8,9). Expression of  $\beta$ -catenin was significantly decreased during adipogenesis of 3T3-L1 cells, but AICAR significantly up-regulated  $\beta$ -catenin mRNA (Fig. 2A) and protein (Fig. 2B) expression. AICAR also increased the nuclear accumulation of  $\beta$ -catenin during adipogenesis (Fig. 2C). These results support the possibility that AICAR inhibits adipogenesis by up-regulating the expression and nuclear accumulation of  $\beta$ -catenin.

*$\beta$ -catenin siRNA transfection attenuates the anti-adipogenic effects of AICAR.* To confirm the role of  $\beta$ -catenin on the anti-adipogenic effects of AICAR, 3T3-L1 cells were transfected with  $\beta$ -catenin siRNA in the presence or absence of AICAR treatment.  $\beta$ -catenin levels, which were up-regulated by AICAR treatment, were significantly decreased in  $\beta$ -catenin siRNA-transfected cells compared to control siRNA-transfected cells (Fig. 3A). In this experimental condition, mRNA and protein expressions of PPAR $\gamma$  and C/EBP $\alpha$ , which were both down-regulated by AICAR treatment, were significantly increased by  $\beta$ -catenin siRNA transfection (Fig. 3B and C).  $\beta$ -catenin siRNA transfection, partially but significantly, increased the expression of lipid-metabolizing enzymes such as LPL and FABP4, which were down-regulated by AICAR treatment (Fig. 3D and E). These results support the notion that the anti-adipogenic effects of AICAR, including the suppression of major transcription factors and lipid-metabolizing enzymes, are at least partially mediated by  $\beta$ -catenin.

*AICAR induces the activation of the WNT/ $\beta$ -catenin pathway.* To elucidate the effects of AICAR on the WNT/ $\beta$ -catenin pathway, the expressions of the major genes involved in this pathway were analyzed during the adipogenesis of 3T3-L1

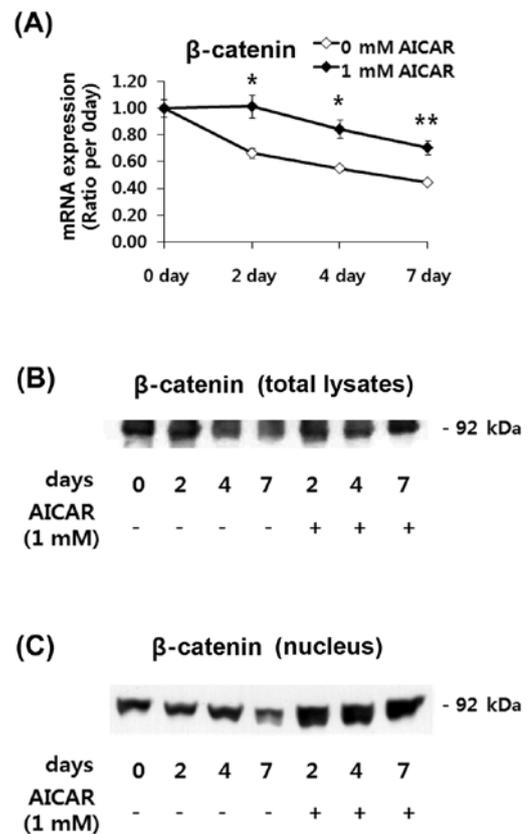


Figure 2. AICAR up-regulates the expression and nuclear accumulation of  $\beta$ -catenin. (A) Expression of  $\beta$ -catenin mRNA in differentiated 3T3-L1 cells for 0, 2, 4 and 7 days in the presence or absence of 1 mM AICAR. (B) Expression of  $\beta$ -catenin protein in whole cell lysates during adipogenesis for 0, 2, 4 and 7 days in the presence or absence of 1 mM AICAR. (C) Effects of AICAR on the levels of  $\beta$ -catenin protein in nuclear extracts of differentiated 3T3-L1 cells. \* $p < 0.05$ , \*\* $p < 0.01$  compared to untreated adipocytes on the same differentiation day.

cells in the presence or absence of AICAR. WNT10B, a potent inhibitor of adipogenesis (8), was markedly reduced during adipogenesis, and was not affected by AICAR treatment (Fig. 4A). On the contrary, the mRNA expression of LRP6 was significantly up-regulated by AICAR treatment on day 2 and on day 4 of adipogenesis (Fig. 4B). The DVL family of proteins, which suppresses the AXIN-APC-GSK3 complex in the cytoplasm, showed differential modulations among its members. The expression of DVL1 was not clearly modulated by AICAR treatment, whereas DVL2 and DVL3 were significantly up-regulated by AICAR treatment (Fig. 4C-F). The expression of AXIN, which induces  $\beta$ -catenin degradation, was down-regulated by AICAR treatment (Fig. 4G). The collective data demonstrate the up-regulation of LRP6 and DVL2/3, which are positive regulators of  $\beta$ -catenin, and the down-regulation of AXIN, a negative regulator of  $\beta$ -catenin, and also provide experimental support for the suggestion that AICAR modulates the WNT/ $\beta$ -catenin pathway to inhibit adipogenesis, as summarized in Fig. 5.

## Discussion

Two studies have reported that AICAR inhibits adipocyte differentiation of 3T3-L1 cells and restores metabolic alterations

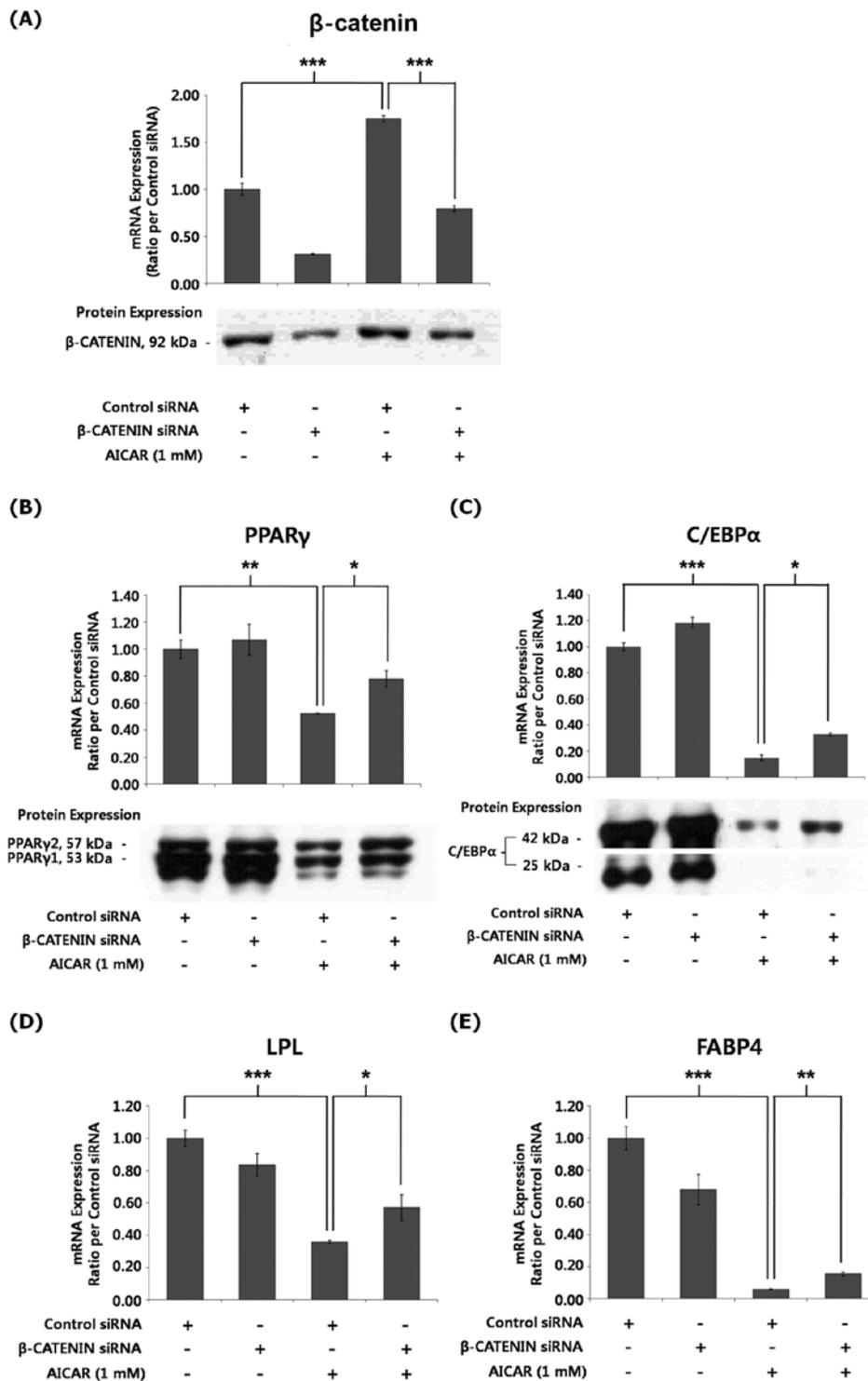


Figure 3.  $\beta$ -catenin siRNA transfection attenuates the anti-adipogenic effects of AICAR. (A) Effects of  $\beta$ -catenin siRNA on the levels of  $\beta$ -catenin mRNA and protein during adipogenesis of 3T3-L1 cells in the presence or absence of AICAR. (B and C) Effects of  $\beta$ -catenin siRNA on the mRNA and protein expression of the major transcription factors of adipogenesis, PPAR $\gamma$  (C) and C/EBP $\alpha$  (D) were determined in the presence or absence of AICAR. (D and E) Effects of  $\beta$ -catenin siRNA on the mRNA expression of the lipid-metabolizing enzymes, LPL (D) and FABP4 (E). The mRNA levels were expressed as the ratios per the level of control siRNA-treated cells without AICAR treatment. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

in diet-induced obese mice (20,21). However, until now, its mechanism of action have not been completely elucidated.

As shown in Fig. 1, AICAR not only induced the activation of AMPK, but also inhibited lipid metabolizing enzymes and major transcription factors of adipogenesis. The expression level of C/EBP $\beta$ , which is an upstream regulator of PPAR $\gamma$

and C/EBP $\alpha$ , was not altered by AICAR, suggesting that AICAR did not affect the upstream part of the adipogenesis pathway (data not shown). In the present study, to discover the repressor of PPAR $\gamma$  and C/EBP $\alpha$  induced by AICAR, the levels of  $\beta$ -catenin, a negative regulator of adipogenesis (23,25), were evaluated. AICAR increased the expression

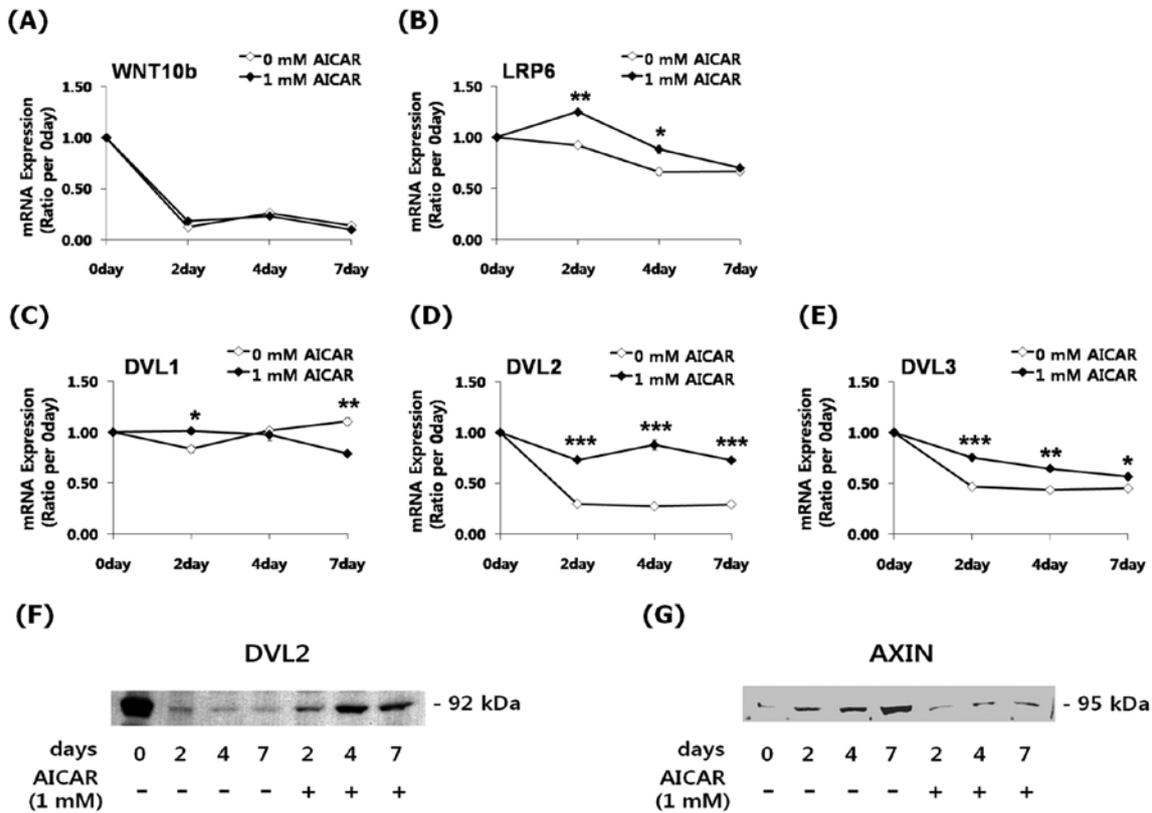


Figure 4. Modulation of the WNT/ $\beta$ -catenin pathway by AICAR during adipogenesis of 3T3-L1 cells. (A) Effects of AICAR on the mRNA expression of WNT10B. (B) Effects of AICAR on the mRNA expression of LRP6. (C-E) Effects of AICAR on the mRNA expression of the DVL family members, DVL1 (C), DVL2 (D) and DVL3 (E). (F) Effects of AICAR on the protein expression level of DVL2. (G) Effects of AICAR on the protein expression level of AXIN. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  compared with untreated adipocytes on the same differentiation day.

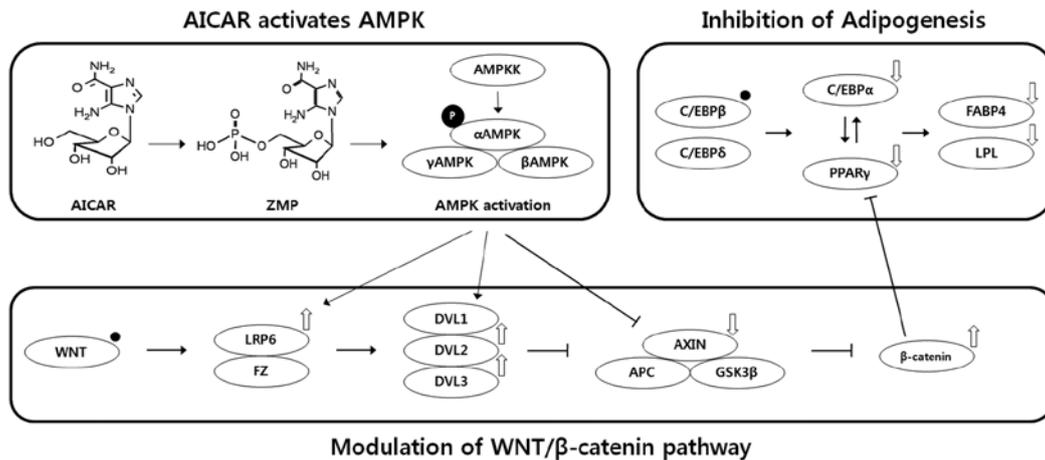


Figure 5. A possible mechanism for the anti-adipogenic effects of AICAR. AICAR increases nuclear  $\beta$ -catenin levels through the modulation of the WNT/ $\beta$ -catenin pathway and inhibits the expression of the various genes involved in adipogenesis.

and nuclear accumulation of  $\beta$ -catenin (Fig. 2). Reduction of  $\beta$ -catenin expression by its siRNA transfection coincided with a significantly increased expression of PPAR $\gamma$  and C/EBP $\alpha$  as well as with a partial but significant enhancement of LPL and FABP4 expression (Fig. 3). These results are consistent with the involvement of  $\beta$ -catenin in the anti-adipogenic effects of AICAR.

There are two reported mechanisms for the anti-adipogenic effects of  $\beta$ -catenin. Firstly,  $\beta$ -catenin directly suppresses the

activity of PPAR $\gamma$  through the binding interaction between the LEF binding domain of  $\beta$ -catenin and a catenin binding domain of PPAR $\gamma$  (26). Secondly,  $\beta$ -catenin indirectly inhibits PPAR $\gamma$  and C/EBP $\alpha$  by the induction of its target genes such as cyclin D1, Myc and PPAR $\delta$ , which inhibit the expression and activity of PPAR $\gamma$  (27-30).

To investigate the effects of AICAR on upstream genes of the WNT/ $\beta$ -catenin pathway, their expression levels were analyzed during adipogenesis in the presence or absence of

AICAR. WNT10B, which is located in the most upstream position of the WNT/ $\beta$ -catenin pathway, is a potent anti-adipogenic factor (25). In this study, however, no changes were observed in the expression of WNT10B by AICAR treatment (Fig. 4). On the contrary, upon treatment of AICAR, expression of LRP6, DVL2 and DVL3 were significantly up-regulated, while AXIN expression was down-regulated. Reportedly, cytokines such as interleukin (IL)-6 and tumor necrosis factor (TNF)- $\alpha$  impaired adipogenesis via the WNT/ $\beta$ -catenin pathway through differing mechanisms: TNF- $\alpha$  markedly enhanced WNT10B expression, whereas IL-6 had no effect on WNT10B expression. Both IL-6 and TNF- $\alpha$  commonly up-regulated LRP6, DVLs and  $\beta$ -catenin, and down-regulated AXIN (23). Showing a similar mechanism with IL-6, AICAR induced activation of the WNT/ $\beta$ -catenin pathway without altering WNT10B expression. It can be suggested that the anti-adipogenic effects of AICAR were mediated by the up-regulation of LRP6 and DVL2/3 and by down-regulation of AXIN (Figs. 4 and 5). There are two models for the signal transduction from the FZ-LRP receptor complex to  $\beta$ -catenin. In the first model, FZ-dependent activation of DVLs inhibits the AXIN-APC-GSK3 complex formation from stabilizing  $\beta$ -catenin. In the second model, LRP inactivates or degrades AXIN independently of DVL (7). The present data support both models of signal transduction even though further studies are warranted.

The present study provides compelling evidence that AICAR inhibits adipogenesis via the WNT/ $\beta$ -catenin pathway (Fig. 5). During the adipogenesis of 3T3-L1 cells, AICAR increased both the expression and nuclear accumulation of  $\beta$ -catenin, which is a potent inhibitor of adipogenesis. The best explanation for this is that AICAR activates signal transduction through the WNT/ $\beta$ -catenin pathway via LRP6, DVL2/3 and AXIN.

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