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ORIGINAL ARTICLE

PKA/AMPK signaling in relation to adiponectin's antiproliferative effect on multiple myeloma cells

EA Medina[1], K Oberheu[1], SR Polusani[1], V Ortega[1], GVN Velagaleti[1] and BO Oyajobi[2]

Obesity increases the risk of developing multiple myeloma (MM). Adiponectin is a cytokine produced by adipocytes, but paradoxically decreased in obesity, that has been implicated in MM progression. Herein, we evaluated how prolonged exposure to adiponectin affected the survival of MM cells as well as putative signaling mechanisms. Adiponectin activates protein kinase A (PKA), which leads to decreased AKT activity and increased AMP-activated protein kinase (AMPK) activation. AMPK, in turn, induces cell cycle arrest and apoptosis. Adiponectin-induced apoptosis may be mediated, at least in part, by the PKA/AMPK-dependent decline in the expression of the enzyme acetyl-CoA-carboxylase (ACC), which is essential to lipogenesis. Supplementation with palmitic acid, the preliminary end product of fatty acid synthesis, rescues MM cells from adiponectin-induced apoptosis. Furthermore, 5-(tetradecyloxy)-2-furancarboxylic acid (TOFA), an ACC inhibitor, exhibited potent antiproliferative effects on MM cells that could also be inhibited by fatty acid supplementation. Thus, adiponectin's ability to reduce survival of MM cells appears to be mediated through its ability to suppress lipogenesis. Our findings suggest that PKA/AMPK pathway activators, or inhibitors of ACC, may be useful adjuvants to treat MM. Moreover, the antimyeloma effect of adiponectin supports the concept that hypoadiponectinemia, as occurs in obesity, promotes MM tumor progression.

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INTRODUCTION

Multiple myeloma (MM) is an incurable malignancy of plasma cells that accumulate in the bone marrow. Obesity is associated with a 1.5- to 2-fold elevated risk of developing MM, suggesting that it has a role in myelomagenesis.[1–3] Several cytokines and growth factors that promote MM growth, such as tumor necrosis factor α, interleukin 6, MCP-1 and insulin,[4] are elevated in obesity[5] and may enhance tumor growth and progression. Adiponectin is an adipocyte-secreted cytokine, or adipokine, that circulates at very high concentrations (3–30 μg/ml).[6] Unlike other adipokines, such as leptin, circulating adiponectin levels are inversely proportional to an individual's fat mass;[7] low adiponectin concentrations have a role in obesity-associated type 2 diabetes and cardiovascular disease.[8] Adiponectin inhibits the proliferation of various cancer cell types[9] and suppresses tumor-associated angiogenesis.[10] Thus, the adipokine may function as a brake that restrains the progression and growth of tumors; hypoadiponectinemia may mechanistically link obesity and MM. Supporting a possible role for adiponectin deficiency in promoting myelomagenesis is the finding that circulating levels of the adipokine are decreased in patients with MM.[11] Moreover, tumor burden increased in immune-deficient and adiponectin-deficient (RAG2$^{-/-}$:Adipo$^{-/-}$) mice compared with immune-deficient (RAG2$^{-/-}$:Adipo$^{+/+}$) mice after injection of allogeneic 5TGM1 mouse MM cells.[12]

Adiponectin circulates in plasma as a low-molecular-weight trimer, a middle-molecular-weight hexamer and a high-molecular-weight 12- to 18-mer.[8] Adiponectin signals through AdipoR1 and AdipoR2, which interact with APPL1 (adapter protein-containing pleckstrin homology domain, phosphotyrosine binding domain, and leucine zipper motif), a critical adapter protein that links the receptors to various downstream mediators.[13] The cellular energy sensor AMP-activated protein kinase (AMPK) mediates many of adiponectin's effects on insulin-sensitive tissues. AMPK is activated when the intracellular AMP:ATP ratio rises in response to cellular stress or pharmacologic inducers; AMPK promotes catabolic pathways to generate more ATP and inhibits anabolic pathways.[14] However, the signaling mechanisms that adiponectin uses to mediate its various effects, including killing cancer cells, are diverse as well as cell- and tissue-dependent. Although AMPK appears to mediate adiponectin-induced inhibition of cancer cell growth for several epithelial cancers, such as breast, colon, liver and prostate, several other pathways are also involved in its antiproliferative effects. These pathways include cyclic AMP-dependent protein kinase (protein kinase A; PKA), signal transducer and activator of transcription 3 (STAT3), mitogen-activated protein kinases, β-catenin and phosphatidylinositol 3-kinase (PI3K)/AKT.[9] Understanding adiponectin's antiproliferative signaling mechanisms in human MM cells may elucidate novel therapeutic targets.

Plasma adiponectin levels exhibit only minor daily fluctuations.[6] Thus, MM cells are most likely exposed chronically to the adipokine *in vivo*. Therefore, we investigated signaling pathways that may mediate the effects of prolonged exposure to adiponectin on MM cell proliferation and survival. Herein, we show that adiponectin signals through the PKA/AMPK pathway to inhibit cell proliferation by inducing cell cycle arrest and apoptosis. These antiproliferative effects appear to be mediated, at least in part, by the PKA-dependent inhibition of AKT activity and the PKA/AMPK-dependent decline in the expression of the critical lipogenic enzyme acetyl-coenzyme A carboxylase (ACC). Our study further clarifies the regulation of MM cell growth and survival by adiponectin and offers a rationale for investigating the

[1]Division of Hematopathology, Department of Pathology, School of Medicine, University of Texas Health Science Center at San Antonio, San Antonio, TX, USA and [2]Department of Cellular and Structural Biology, University of Texas Health Science Center at San Antonio, San Antonio, TX, USA. Correspondence: Dr EA Medina, Division of Hematopathology, Department of Pathology, School of Medicine, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, TX 78229-3900, USA.
E-mail: medinae2@uthscsa.edu
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potential antitumor efficacy of adiponectin mimics, activators of PKA/AMPK, or antilipogenics in relevant animal models of MM.

MATERIALS AND METHODS

MM cells

Human MM cell lines (RPMI-8226, U266, MM1S and MM1R), the murine MM cell line 5TGM1 and primary patient MM cells were cultured in RPMI 1640 medium with L-glutamine (Corning, Tewksbury, MA, USA) supplemented with 10% fetal bovine serum (Sigma-Aldrich, St Louis, MO, USA) and 1% penicillin–streptomycin solution (Corning). We maintained cells at 37 °C in a humidified atmosphere of 5% CO_2. Primary patient MM cells were isolated from the bone marrow aspirates of patients using the EasySep Human CD138 Positive Selection kit according to the manufacturer's instructions (Stem Cell Technologies, Vancouver, BC, Canada). The institutional review board of the University of Texas Health Science Center at San Antonio (protocol #HSC20140072N), in accordance with the Declaration of Helsinki, approved use of the bone marrow samples.

Reagents

AICA-Riboside (AICAR), 1,1-dimethylbiguanide, hydrochloride (metformin) and compound C were from EMD Millipore (Billerica, MA, USA); Forskolin and H89 were from Cayman Chemical (Ann Arbor, MI, USA); palmitic acid, dimethyl sulfoxide and bovine serum albumin were from Sigma-Aldrich; and 5-(tetradecyloxy)-2-furancarboxylic acid (TOFA) was from Enzo Life Sciences (Farmingdale, NY, USA). Recombinant human full-length adiponectin was from Peprotech (Rocky Hill, NJ, USA) and Enzo Life Sciences. Antibodies were from Cell Signaling Technology (Danvers, MA, USA) unless otherwise stated: phospho-AMPKα (Thr172)(40H9); AMPKα; phospho-AKT (Ser473)(D9E) XP; phospho-AKT (Thr308); AKT (pan)(C67E7); phospho-Creb (Ser133); CREB (48H2); phospho-ACC (Ser79); ACC; cleaved caspase-3 (Asp175); and poly(ADP-ribose) polymerase. Anti-β-tubulin I was from Sigma-Aldrich. AdipoR1 and AdipoR2 antibodies were kind gifts from Dr Lily Dong (University of Texas Health Science Center at San Antonio, San Antonio, TX, USA). Donkey anti-rabbit horseradish peroxidase (HRP) and donkey anti-mouse HRP were from Jackson Immunoresearch (West Grove, PA, USA). Anti-actin (C-2) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Total live cell counts and cell proliferation assay

Total live cell counts were assessed using the Trypan blue exclusion test; unstained viable cells were counted with a hemocytometer. Cell proliferation was assessed (3×10^4 cells into 96-well microtiter plates) using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2 H-tetrazolium, inner salt (MTS) solution assay CellTiter 96 Aqueous One Solution Cell Proliferation Assay according to the manufacturer's instructions (Promega, Madison, WI, USA). Absorbance was measured at 490 nm on a microplate reader (Molecular Devices, Sunnyvale, CA, USA). Proliferation was expressed as the percentage of control calculated from the following equation: proliferation (% of control) = 100 × [OD (treatment) − OD (vehicle)]/OD (vehicle).

Analysis of cell cycle and apoptosis

Cell cycle analysis, which shows cells at different stages of the cell cycle on the basis of the amount of DNA present, was performed using DNA-binding dye propidium iodide and flow cytometry. Propidium iodide–Triton X-100 staining solution was prepared by adding 2 mg of DNase-free RNase A (Gene Link, Hawthorne, NY, USA) and 200 μl of 1 mg/ml of propidium iodide (Life Technologies, Grand Island, NY, USA) to 10 ml of 0.1% Triton X-100 (Sigma-Aldrich) in phosphate-buffered saline. For synchronization, cells were starved for 24 h in RPMI 1640 medium with 2% fetal bovine serum and then seeded at a density of 10^6 cells/ml into six-well plates. Cells were treated in triplicate as indicated in media containing 10% fetal bovine serum. At the end of the treatment period, we transferred 10^6 cells to a 5-ml round-bottom tube and then fixed the cells by adding 1 ml of ice-cold 70% ethanol. The tube was then centrifuged, cells were washed twice with phosphate-buffered saline, and they were stained with 1 ml of propidium iodide–Triton X-100 staining solution. Samples were incubated for 30 min and protected from light at room temperature, and the cell cycle profile was generated using an LSRII flow cytometer (BD Biosciences, San Jose, CA, USA) and FlowJo Software (Tree Star, Ashland, OR, USA) for analysis.

Apoptosis of cells was assessed using the Annexin V:PE Apoptosis Detection kit I (BD Biosciences) according to the manufacturer's instructions. In brief, we seeded 2×10^6 cells/well into six-well plates and then treated them as indicated. At the end of the treatment period, 10^6 cells were resuspended in binding buffer. A volume of 100 μl of the cell suspension was then incubated with 5 μl of annexin V:PE and 5 μl of 7-amino-actinomycin D at room temperature and protected from light for 15 min, after which binding buffer was added and the samples were analyzed using LSRII flow cytometry and FACSDiva software (BD Biosciences).

Western blot analysis

A total of 2×10^6 cells/well were seeded into six-well plates and then treated as indicated. At the end of the treatment period, cells were harvested and then washed with phosphate-buffered saline and lysates were generated using RIPA buffer (Sigma-Aldrich) and 1 × protease–phosphatase inhibitor cocktail (Cell Signaling Technology). Proteins (50 μg/lane) were separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline solution containing 0.1% Tween 20 (TBS-T) for 1 h at room temperature and then probed with the appropriate primary antibody overnight at 4 °C; primary antibodies were diluted in 5% bovine serum albumin (Cell Signaling) with TBS-T. Membranes were washed with TBS-T and then probed with species-specific secondary antibodies coupled to HRP diluted in bovine serum albumin with TBS-T. Protein bands were detected using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA).

PKA kinase activity assay

A total of 2×10^6 RPMI-8226 cells/well were seeded into six-well plates and then treated in triplicate as indicated. At the end of the treatment period, cells were harvested and then washed with phosphate-buffered saline, and lysates were generated using RIPA buffer containing protease and phosphatase inhibitors. Samples (30 μl) were added to a 96-well PKA substrate microtiter plate for assessment of PKA activity using the PKA Kinase Activity kit (Enzo Life Sciences) according to the manufacturer's instructions. Color was developed with tetramethylbenzidine substrate, which develops in proportion to PKA activity. Absorbance was measured at 450 nm on a microplate reader (Molecular Devices). PKA activity is expressed as the percentage of control calculated from the following equation: PKA activity (% of control) = 100 × [OD (treatment) − OD (vehicle)]/OD (vehicle).

Fatty acid preparation

Palmitic acid–bovine serum albumin complex was prepared as previously described.[15] This complex was added either alone or with treatments as indicated such that its final concentration was 100 μM.

Statistical analysis

Data are expressed as mean ± s.d. Microsoft Excel software was used to evaluate statistical significance with Student's *t*-test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Although adiponectin inhibits the proliferation of and/or kills a variety of solid cancer cell types, such as colon, prostate and breast,[9] its effects on hematopoietic cell types are less clear. Therefore, we initially sought to evaluate whether the adipokine could inhibit MM cell proliferation. Adiponectin mediates its effects through engaging its receptors AdipoR1 and/or AdipoR2.[9] All MM cell lines that were evaluated expressed both AdipoR1 and AdipoR2 (Figure 1a), and exposure of each cell line to adiponectin decreased proliferation (Figure 1b), as assessed using the MTS assay, which correlated closely with cell number counts determined with the Trypan Blue dye exclusion assay (Supplementary Figure S1). Adiponectin also inhibited the proliferation of MM cells in a concentration- and time-dependent manner (Figures 1c and d, respectively); the adipokine also inhibited the viability of primary $CD138^+$ MM cells isolated from patients' bone marrow sample (Figure 1e).

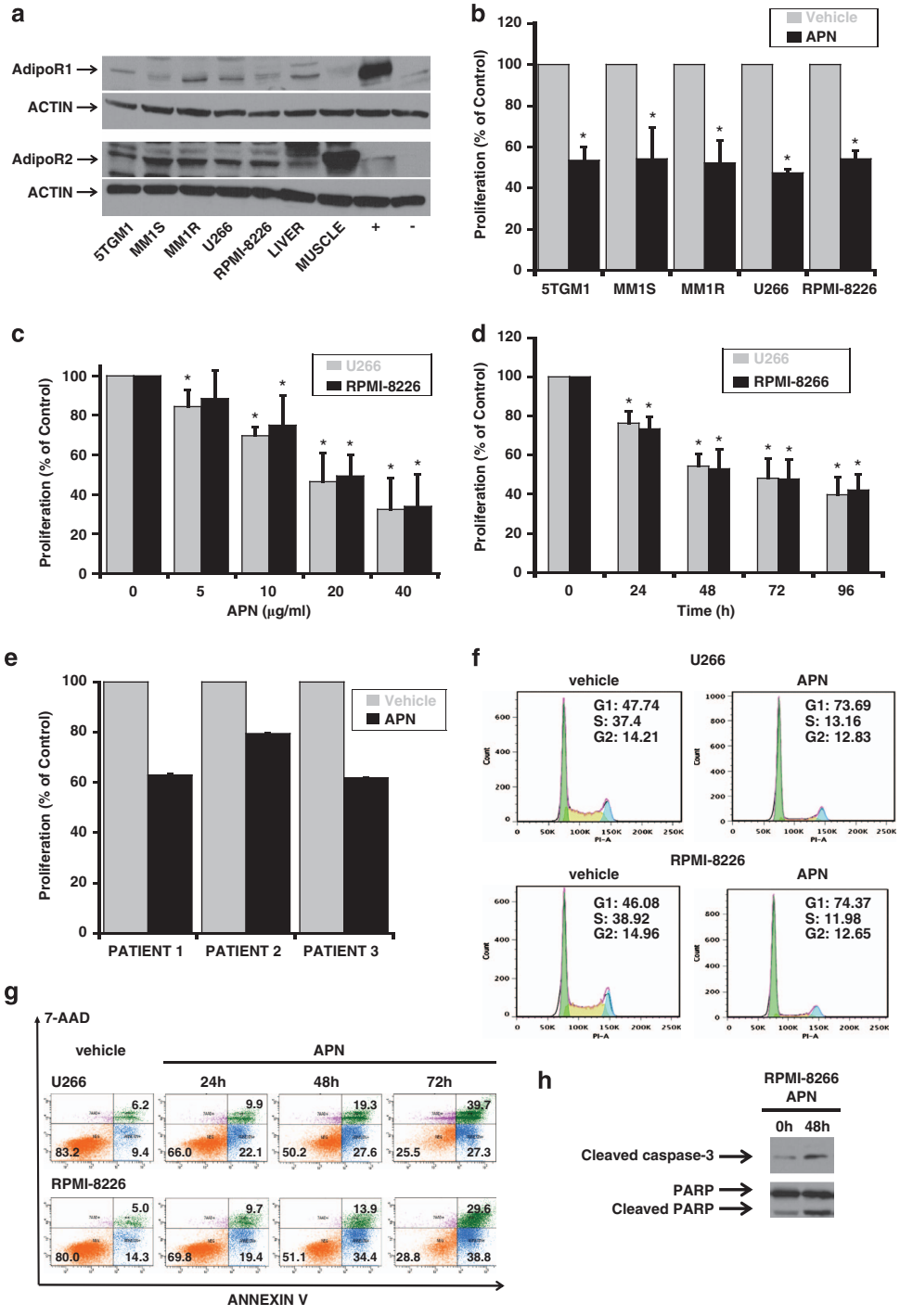

Figure 1. Adiponectin inhibits proliferation of MM cells. (**a**) Adiponectin receptor (AdipoR1 and AdipoR2) expression in mouse (5TGM1) and human (MM1S, MM1R, U266 and RPMI-8226) MM cell lines was evaluated by western blot. The plus sign represents a lane loaded with lysate from transfected C2C12 muscle cells overexpressing the corresponding receptor protein; the minus sign represents negative control C2C12 muscle cells. (**b**) Proliferation of 5TGM1, MM1S, MM1R, U266 and RPMI-8226 cells treated with 20 μg/ml of recombinant full-length adiponectin (APN) for 96 h. (**c**) Proliferation of U266 and RPMI-8226 cells exposed to increasing concentrations of APN (0–40 μg/ml) for 96 h. (**d**) Proliferation of U266 and RPMI-8226 cells exposed to 20 μg/ml of APN for 0–96 h. (**e**) Proliferation of primary patient MM cells exposed to 20 μg/ml of APN for 96 h. Proliferation in panels **b**–**e** was assessed by MTS assay. Data are expressed as mean ± s.d. of the percentage of control from at least two independent experiments. *$P < 0.05$ compared with untreated cells. (**f**) Cell cycle analysis by flow cytometric analysis of DNA content of U266 and RPMI-8226 cells exposed to 20 μg/ml of APN for 48 h (representative example of at least two experiments). (**g**) Flow cytometric analysis of the percentage of apoptotic cells by annexin V and 7-AAD staining after exposure of U266 and RPMI-8226 cells to 20 μg/ml of APN for 24–72 h (representative example of at least two independent experiments). (**h**) Western blot of cleaved caspase-3, PARP and cleaved PARP in RPMI-8266 cells exposed to 20 μg/ml of APN for 48 h.

To elucidate how adiponectin mediates its antiproliferative effects on MM cells, we used flow cytometry to examine the effect of adiponectin on cell cycle progression and apoptosis. Representative flow histograms depicting cell cycle distribution show that, compared with untreated U266 and RPMI-8226 cells, cells exposed to adiponectin accumulate in G_1/G_0 phase, with fewer cells present in S and G_2/M phases (Figure 1f). We then assessed apoptosis in RPMI-8226 and U266 cells exposed to adiponectin by flow cytometry after annexin V and 7-amino-actinomycin D staining. Exposure to adiponectin induced apoptosis (Figure 1g); adiponectin also increased the levels of the apoptotic markers cleaved caspase-3 and cleaved poly(ADP-ribose) polymerase in RPMI-8226 cells (Figure 1h). These results indicate that adiponectin exerts a direct antiproliferative effect on MM cells that is mediated, at least in part, through cell cycle arrest in G_1/G_0 phase and subsequent induction of apoptosis.

Adiponectin inhibits basal AKT activation and activates AMPK to induce cell cycle arrest and apoptosis of MM cells

AMPK mediates adiponectin-induced inhibition of growth of several epithelial cancers such as breast, colon, liver and prostate.[9] Adiponectin can in other cell types either activate or inhibit PI3K/AKT signaling, which has a central role in the survival of MM cells.[16] Therefore, we sought to evaluate whether adiponectin regulates AMPK and AKT activity in MM cells, as well as whether activators of AMPK could mimic adiponectin's effect to decrease MM cell survival and induce cell cycle arrest and apoptosis. We exposed RPMI-8226 and U266 cells to adiponectin and assessed levels of phosphorylation in the activation loop (Thr172) of AMPK and AKT phosphorylation (Ser473 and Thr308) by using immunoblotting as a surrogate measure of their activity. Adiponectin induced phosphorylation of AMPK, which started to increase by 6 h and peaked by 24 h. AKT Ser473 and Thr308 phosphorylation began to decline for both by 12 h and appeared to be maximally decreased by 48 h (Figure 2a). To activate AMPK, we treated RPMI-8226 and U266 cells with AICAR, a cell-permeable adenosine analog phosphorylated to the AMP analog ZMP[17] or metformin, which decreases ATP levels by inhibiting mitochondrial respiratory chain complex 1.[18] AICAR or metformin, as expected, activated AMPK (Supplementary Figure S2A) in MM cells, and exposure to these agents decreased proliferation in RPMI-8226 and U266 cells (Figure 2b). Similar to adiponectin, treatment with AICAR or metformin caused MM cells to accumulate in G_1/G_0 phase and cell numbers to decrease in S and G_2/M phases (Supplementary Figure S2B); exposure to these agents induced apoptosis (Supplementary Figure S2C). To examine whether AMPK activation has a role in downregulating AKT, we treated RPMI-8226 and U266 cells with AICAR and assessed levels of AKT phosphorylation (Ser473 and Thr308). In contrast to adiponectin, AICAR stimulated AKT phosphorylation (Supplementary Figure S2D). Thus, AMPK probably does not mediate the adiponectin-induced decline in AKT phosphorylation.

To more definitively establish AMPK's role in mediating adiponectin's antiproliferative effect, we next used a well-established pharmacological inhibitor of AMPK activity, compound C. Pretreating RPMI-8226 and U266 cells with compound C[19] and then exposing the cells to adiponectin suppressed the adipokine's ability to induce cell cycle arrest (Figures 2c and d) and apoptosis (Figures 2e and f). Thus, activating AMPK by using pharmacologic activators of the kinase (AICAR and metformin) mimicked adiponectin's ability to inhibit MM cell proliferation and induce cell cycle arrest and apoptosis, and pharmacologic AMPK inhibition suppressed the adipokine's ability to induce cell cycle arrest and apoptosis. Taken together, these data suggest that AMPK has a role in mediating adiponectin's antiproliferative effects on MM cells.

PKA mediates adiponectin-induced AKT inhibition, AMPK activation, cell cycle arrest, and apoptosis in MM cells

PKA is a tetrameric holoenzyme consisting of two catalytic and two regulatory subunits that dissociate when cyclic AMP binds the latter; the released catalytic subunits then phosphorylate cytosolic and nuclear substrate proteins. PKA mediates apoptosis in some hematologic malignancies,[20] and adiponectin activates PKA in various cell types, such as endothelial cells,[21] macrophages[22] and the human breast cancer cell line MCF-7.[23] Adiponectin can either activate or inhibit PI3K/AKT signaling,[24,25] which appears to have a central role in the survival of MM cells. PKA can also modulate AMPK[23,26] and AKT[27] activity. Therefore, we sought to evaluate whether adiponectin regulates PKA activity in MM cells and whether PKA is involved in mediating adiponectin-induced killing of MM cells. Immunoblotting for phosphorylated CREB (Ser133) as a measure of PKA activity in RPMI-8226 and U266 cells after exposure to adiponectin showed that phosphorylation began to increase in relation to control starting at 6 h and reached a maximum at 48 h (Figure 3a). The increase in CREB phosphorylation paralleled the increase in PKA activity in RPMI-8226 cells exposed to adiponectin; PKA activity began to increase at 30 min and continued to increase steadily thereafter (Supplementary Figure S3A). Next we sought to evaluate whether activating the PKA signaling pathway by using forskolin, which activates the kinase by increasing intracellular cyclic AMP, could mimic adiponectin's antiproliferative effects. As expected, treating RPMI-8226 and U266 cells with forskolin decreased proliferation (Supplementary Figure S3B), whereas exposure to the agent caused accumulation of cells in G_1/G_0 phase and cell numbers to decrease in S and G_2/M phases (Supplementary Figure S3C), and exposure to forskolin induced apoptosis (Supplementary Figure S3D); pretreating MM cells with the widely used pharmacological inhibitor H89, which competes with ATP for binding to the catalytic subunits,[28] inhibited these effects (Supplementary Figures S3B–D, respectively).

To establish PKA's role in signaling adiponectin's antiproliferative effect, we pretreated RPMI-8226 and U266 cells with H89 and then treated with adiponectin; doing so suppressed the adipokine's ability to induce cell cycle arrest (Figures 3b and c), whereas apoptosis induced by exposure to adiponectin was also dampened (Figures 3d and e). The extent of suppression of adiponectin's effect on cell cycle progression and apoptosis by H89 was comparable to its ability to suppress these same effects in response to forskolin (Figures 3c and d).

Although PKA mediates apoptosis for several cancer cell types, the precise downstream targets of PKA that lead to cell death remain unclear. Given AMPK activation's role in mediating adiponectin's antiproliferative effects and the adipokine's downregulation of AKT activity, which is essential to MM survival, we next asked whether PKA was involved in mediating adiponectin-induced AMPK activation and inhibition of AKT activity. To test this, we again used H89 to inhibit PKA activity and assess whether doing so would suppress adiponectin's ability to induce AMPK activation and decrease AKT activity. Figure 3f shows that pretreating RPMI-8226 cells with H89 before exposing them to adiponectin completely inhibited AMPK phosphorylation (Thr172) and suppressed its ability to decrease AKT phosphorylation (Ser473). Forskolin mimicked the effects of adiponectin on AMPK and AKT activation, and pretreatment of MM cells with H89 before exposure to forskolin suppressed its effects (Supplementary Figure S3E). Moreover, inhibiting AMPK with compound C before exposing MM cells to forskolin suppressed the ability of the PKA activator to induce cell cycle arrest and apoptosis (Supplementary Figures S3C and D, respectively). This finding suggests that AMPK acts downstream of PKA to mediate cell cycle arrest and apoptosis. Taken together, these data indicate that PKA activation at least partly mediates the antiproliferative effect of adiponectin on human MM cells, which ultimately inhibits basal AKT activity and AMPK activation.

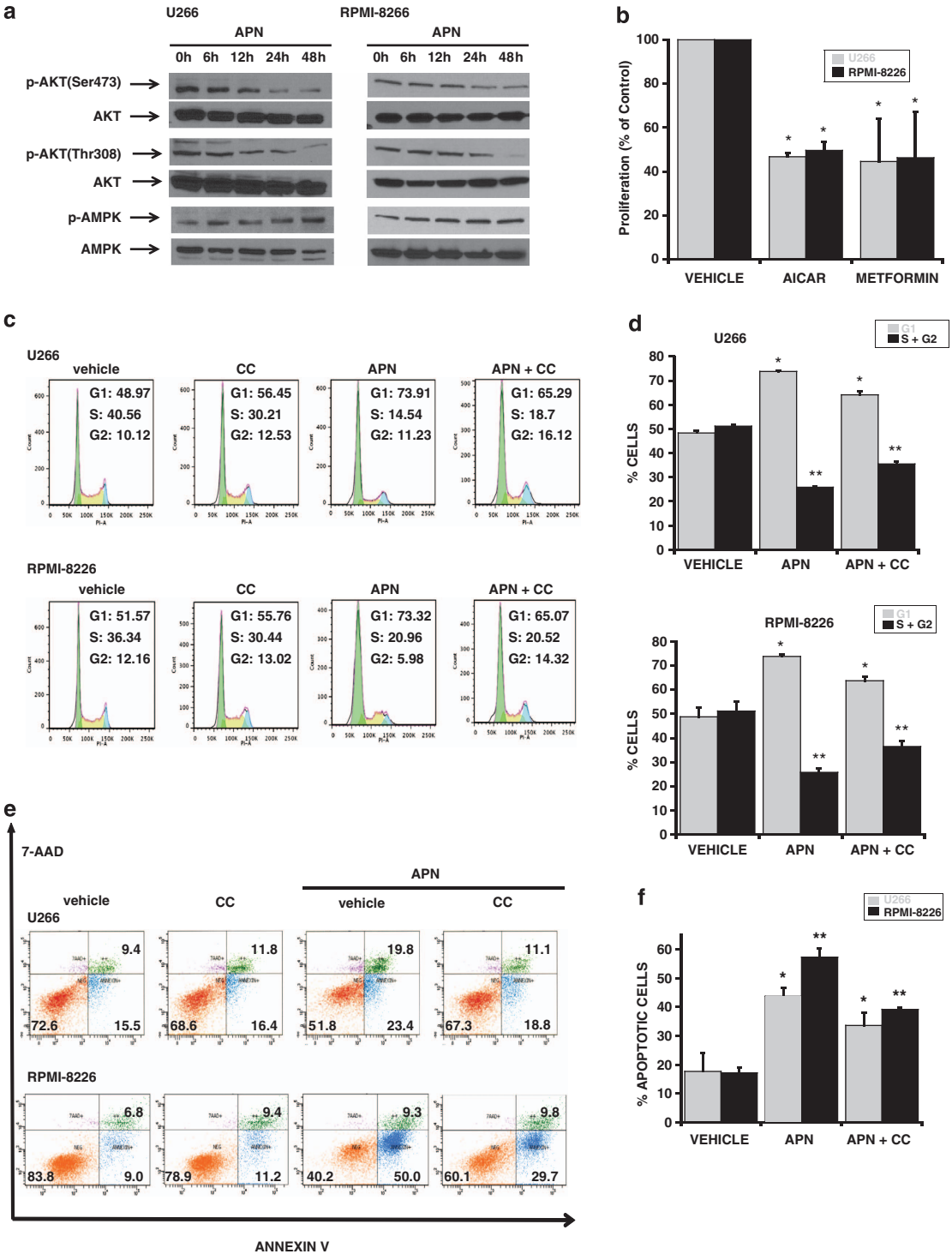

Figure 2. Adiponectin inhibits basal AKT activation and activates AMPK to induce cell cycle arrest and apoptosis of MM cells. (**a**) Western blot of AKT inhibition and AMPK activation after exposure of U266 and RPMI-8226 cells to 20 μg/ml of recombinant full-length adiponectin (APN) for 0–48 h. (**b**) Proliferation of U266 and RPMI-8226 cells exposed to 1 mM AICAR or 20 mM metformin for 0–96 h. Proliferation was assessed by MTS assay. Data are expressed as mean ± s.d. of the percentage of control from at least two independent experiments. $*P<0.05$ compared with vehicle. Representative histogram (**c**) and quantification (**d**) of cell cycle analysis by flow cytometry of DNA content of U266 and RPMI-8226 cells pretreated with the AMPK inhibitor compound C (CC) (0.5 μM) for 1 h followed by a 48-h exposure to 20 μg/ml of APN (two independent experiments performed). Representative plot (**e**) and quantification (**f**) of flow cytometric analysis of the percentage of apoptotic cells by annexin V and 7-AAD staining after pretreatment of U266 and RPMI-8226 cells with 0.5 μM CC and subsequent exposure to 20 μg/ml of APN for 72 h (three and two independent experiments performed for U266 and RPMI-8226 cells, respectively). For panels **d** and **f**, $*P<0.05$ (U266 cells) and $**P<0.05$ (RPMI-8226 cells) for APN versus APN + CC (Student's *t*-test).

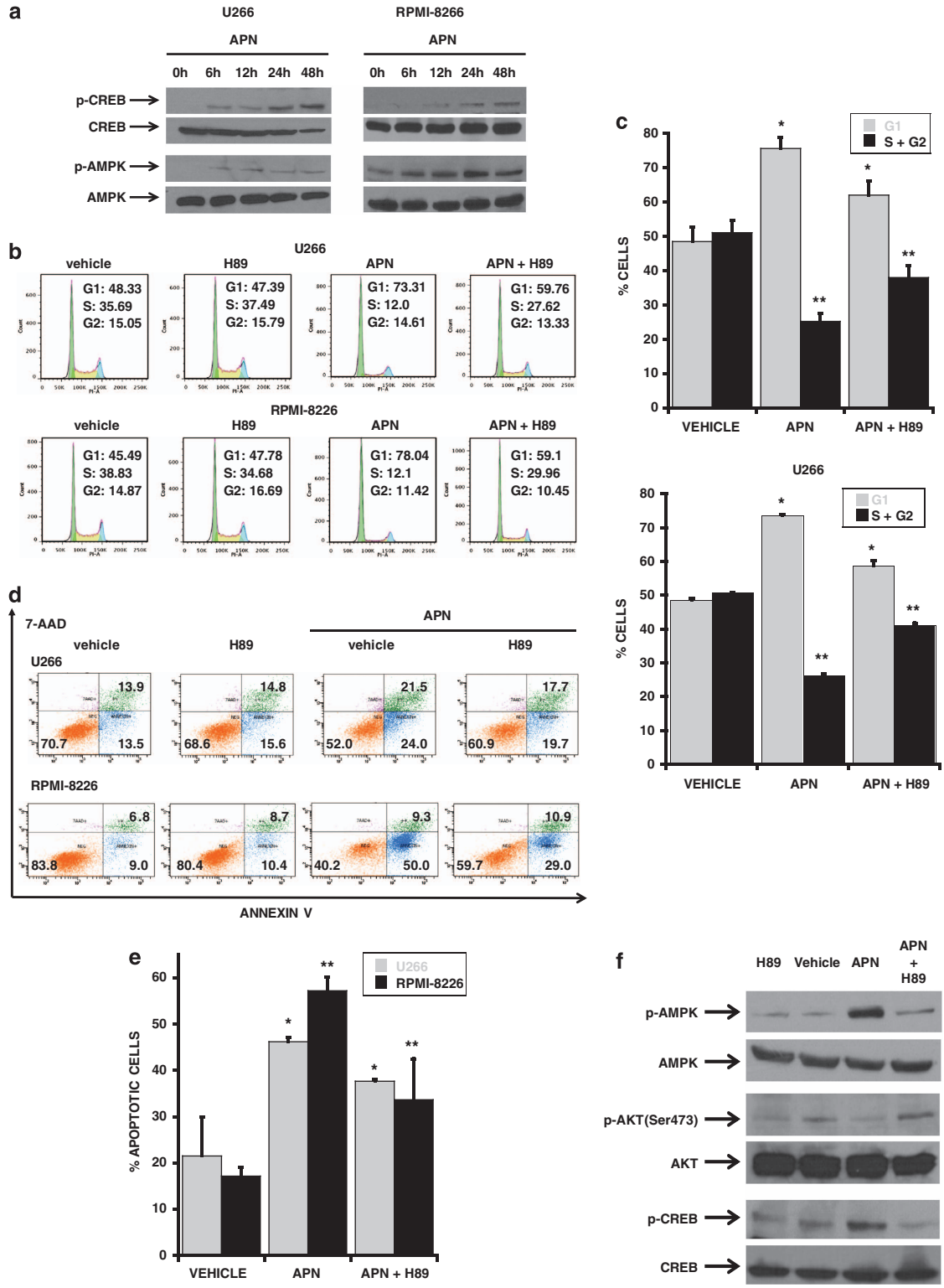

Figure 3. PKA mediates adiponectin-induced AKT inhibition, AMPK activation, cell cycle arrest and apoptosis in MM cells. (**a**) Western blot of CREB and AMPK activation after exposure of U266 and RPMI-8226 cells to 20 μg/ml of recombinant full-length adiponectin (APN) for 0–48 h (representative example of at least two independent experiments). Representative histogram (**b**) and quantification (**c**) of cell cycle analysis by flow cytometry of DNA content of U266 and RPMI-8226 cells pretreated with the PKA inhibitor H89 (5 μM) for 1 h followed by a 48-h exposure to 20 μg/ml of APN (two independent experiments performed). Representative plot (**d**) and quantification (**e**) of flow cytometric analysis of the percentage of apoptotic cells by annexin V and 7-AAD staining after pretreatment of U266 and RPMI-8226 cells with 5 μM H89 and subsequent exposure to 20 μg/ml of APN for 72 h (three and two independent experiments performed for U266 and RPMI-8226 cells, respectively). For panels **c** and **e**, $*P<0.05$ (U266 cells) and $**P<0.05$ (RPMI-8226 cells) for APN versus APN + H89 (Student's *t*-test). (**f**) Western blot of AMPK, AKT and CREB activation after pretreatment of RPMI-8226 cells with 5 μM H89 and subsequent exposure to 20 μg/ml of APN for 48 h (representative example of at least two independent experiments).

Adiponectin downregulation of ACC expression and activity through a PKA/AMPK signaling pathway

Enhanced fatty acid synthesis is a crucial metabolic adaptation that helps drive the proliferation of cancer cells, including B-cell lymphomas,[29] by providing fatty acids for the synthesis of cell membranes, precursors for lipid second messengers and substrates for energy metabolism.[30] Several lipogenic enzymes, such as fatty acid synthase and ACC, are upregulated in tumors, and evidence exists that targeting such enzymes has therapeutic potential.[29–32] Adiponectin, through activation of AMPK, can negatively regulate ACC activity by inducing its phosphorylation.[33,34] In addition, adiponectin decreased expression of lipogenic enzymes such as ACC in colon cancer cells.[35] Thus, we sought to evaluate whether adiponectin, through the PKA/AMPK pathway, downregulates the expression or activity of ACC in MM cells. We exposed RPMI-8226 and U266 cells to adiponectin and used immunoblotting to assess levels of phosphorylation on Ser79 of ACC and total levels of the enzyme. Adiponectin did not induce phosphorylation of ACC at any time points studied but instead appeared to decrease the basal levels of phosphorylation at later time points, which corresponded to a decline in total levels of the enzyme (Figure 4a). Thus, adiponectin probably inhibits ACC activity in MM cells by downregulating its expression. To evaluate whether adiponectin-induced signaling through the PKA/AMPK pathway was mediating the effects of the adipokine on ACC expression, we again used the pharmacological inhibitors of AMPK and PKA, and pretreated the MM cells before adiponectin exposure. Figure 4b shows that pretreating RPMI-8226 cells with compound C or H89 before adiponectin exposure markedly suppressed the ability of the adipokine to decrease ACC expression; as previously shown, pretreatment with compound C or H89 markedly inhibited AMPK phosphorylation (Figure 4b). Thus, adiponectin-induced signaling through the PKA/AMPK pathway mediates downregulation of ACC expression. To test whether ACC activity is crucial to MM cell survival, we treated RPMI-8226 and U266 cells with the ACC inhibitor TOFA[36,37] and assessed effects on MM cell proliferation. TOFA dose-dependently inhibited proliferation of MM cells (Figure 5a); this agent also inhibited proliferation of 5TGM1, MM1R and MM1S cells (Figure 5b). Treatment with TOFA induced apoptosis, and supplementing MM cells with palmitic acid, which provides the end products of the fatty acid synthesis pathway that are depleted by the ACC inhibitor, partially inhibited apoptosis in response to TOFA, adiponectin, forskolin and AICAR (Figures 5c and d, and Supplementary Figures S4A and B, respectively). Fatty acid supplementation also partially rescued MM cells from the antiproliferative effects of adiponectin and forskolin (Figure 5e). Taken together, these data indicate that PKA activation at least partly mediates adiponectin's antiproliferative effect. PKA activates AMPK, resulting in a subsequent downregulation of ACC activity due to decreased ACC expression.

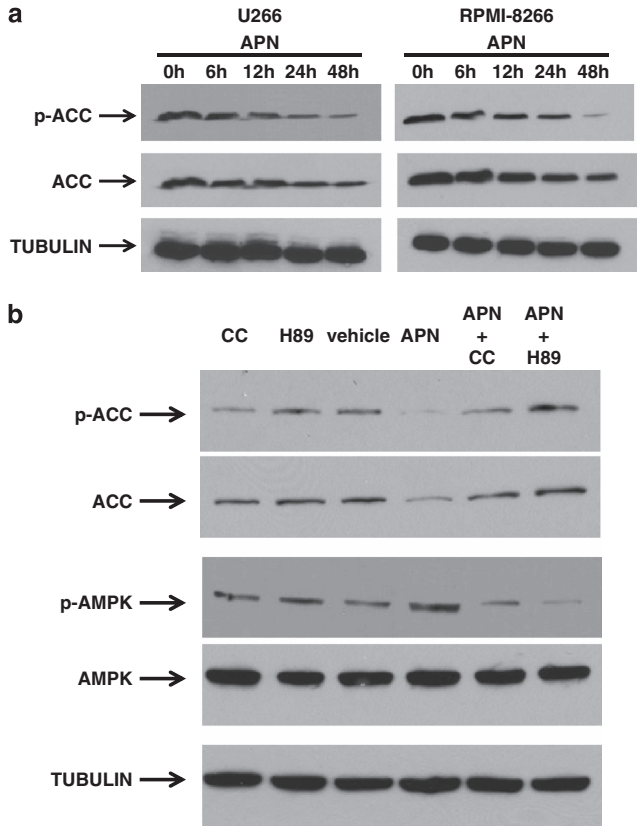

Figure 4. Adiponectin decreases expression of ACC through the PKA/AMPK signaling pathway. (**a**) Western blot of phosphorylated (Ser79) and total ACC and tubulin after exposure of U266 and RPMI-8226 cells to 20 μg/ml of recombinant full-length adiponectin (APN) for 0–48 h (representative example of at least two independent experiments). (**b**) Western blot of AMPK and phosphorylated and total ACC after pretreatment of RPMI-8226 cells with 0.5 μM CC or 5 μM H89 and subsequent exposure to 20 μg/ml of APN for 48 h (representative example of at least two independent experiments).

DISCUSSION

Our study aimed to elucidate signaling pathways mediating the antiproliferative effect of adiponectin on MM cells. Herein, we show that prolonged exposure to adiponectin inhibits the proliferation and survival of primary human MM cells, and human and mouse MM cell lines. Activation of PKA mediates this effect, at least in part, which concomitantly triggers dual pathways: one leading to the downregulation of AKT activity and another that activates AMPK activation and downregulates ACC expression. Both AKT and ACC are enzymes crucial to MM cell proliferation and survival (Figure 6). These findings strongly suggest that the adipokine suppresses MM cell proliferation *in vivo* and supports the concept that hypoadiponectinemia, as occurs in obesity, promotes proliferation and growth of MM. Thus, modulating circulating adiponectin levels or administering adiponectin or agents that stimulate pathways that the adipokine uses to mediate its antiproliferative effects are potential adjuvant therapeutic strategies for MM. Indeed, a prime candidate for testing in animal models of MM is the novel synthetic small molecule AdipoRon, an orally active agonist of AdipoR1 and AdipoR2 that closely mimics adiponectin's signaling and physiologic effects.[38]

We showed that PKA is an important signaling mediator of the antiproliferative effects of adiponectin on MM cells. PKA can mediate apoptosis by modulating phosphorylation of a variety of proteins. PKA inhibits the activity/phosphorylation of proteins that are pro-proliferative in cancer cells, such as AKT,[27] extracellular signal-regulated kinase (ERK)[39,40] and STAT3.[40] In this study, we focused on adiponectin's regulation of AMPK and AKT in MM cells. We showed that AMPK acts downstream of PKA to mediate apoptosis in MM cells in response to adiponectin or forskolin. Inhibiting PKA with a low dose of the PKA inhibitor H89 abolished the ability of both adiponectin and forskolin to induce AMPK phosphorylation without affecting baseline phosphorylation levels of the kinase. Recent studies also showing that AMPK can function as a kinase downstream of PKA support our results.[23,26] We also showed that inhibiting AMPK activity in MM cells suppressed the ability of both adiponectin and forskolin to induce apoptosis. Although the role of AMPK as a mediator of cell death in cancer cells, including MM cells, is well documented, the signaling mechanism by which PKA activation leads to AMPK activation is

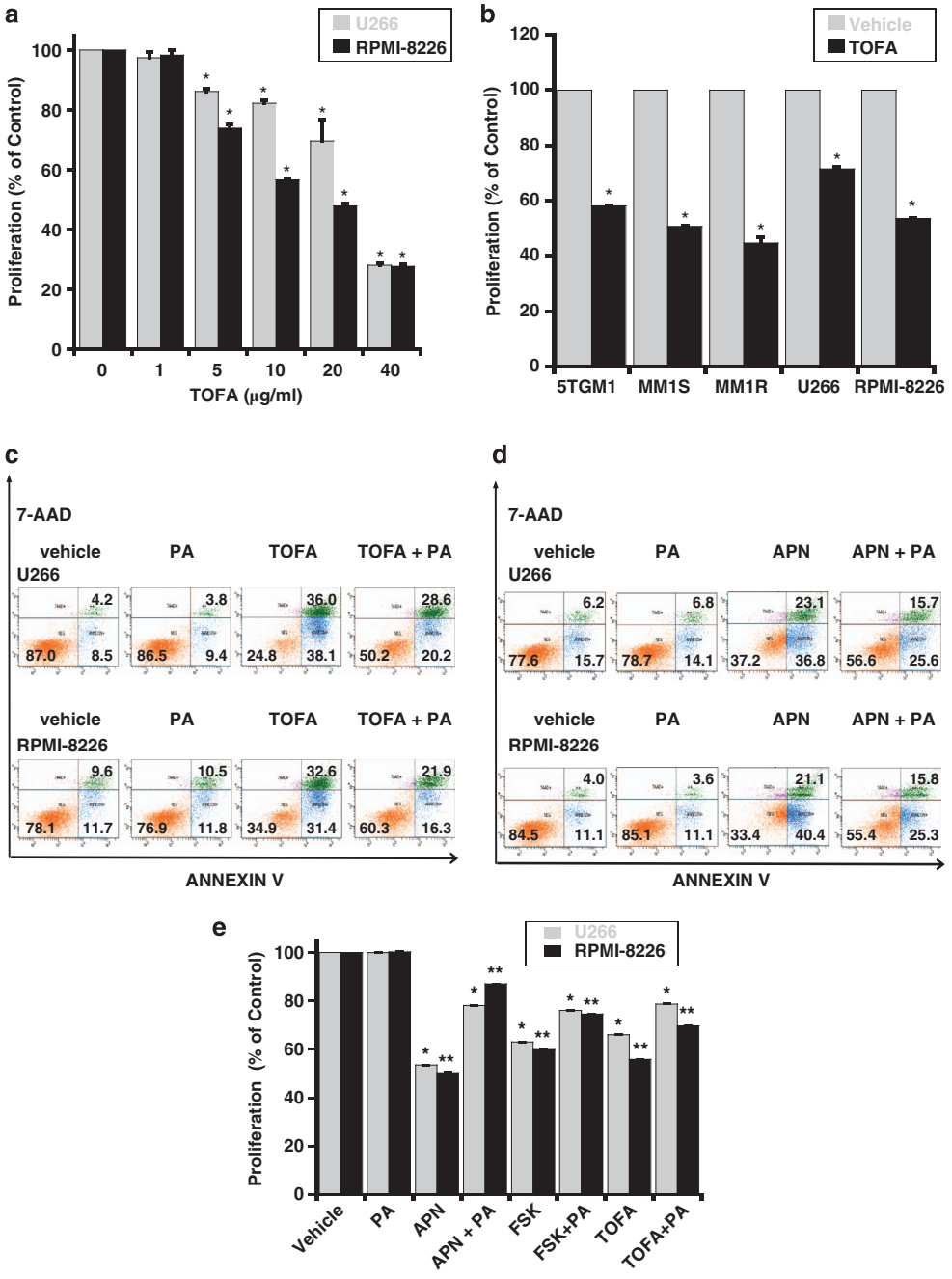

Figure 5. TOFA inhibits proliferation and induces apoptosis of MM cells, and palmitic acid supplementation rescues them from cell death. (**a**) Proliferation of U266 and RPMI-8226 cells exposed to increasing concentrations of TOFA (0–40 μg/ml) for 96 h. (**b**) Proliferation of mouse (5TGM1) and human (MM1S, MM1R, U266 and RPMI-8226) MM cells treated with 20 μg/ml of TOFA for 96 h. Proliferation in panels **a** and **b** was assessed by MTS assay. Data are expressed as mean ± s.d. of the percentage of control from two independent experiments. *$P < 0.05$ compared with untreated cells (Student's *t*-test). (**c**) Flow cytometric analysis of the percentage of apoptotic cells by annexin V and 7-AAD staining after exposure of U266 and RPMI-8226 cells to 100 μM palmitic acid with (**c**) 20 μg/ml of TOFA or (**d**) 20 μg/ml of recombinant full-length adiponectin (APN) for 72 h (representative example of two independent experiments). (**e**) Proliferation of U266 and RPMI-8226 cells exposed to 100 μM palmitic and 20 μg/ml of APN or 1 μM forskolin or 20 μg/ml of TOFA for 96 h. Proliferation was assessed by MTS assay. Data are expressed as mean ± s.d. of the percentage of control for a representative assay performed in triplicate (two independent experiments performed). *$P < 0.05$ (U266 cells); **$P < 0.05$ (RPMI-8226 cells) for treatments versus vehicle (Student's *t*-test).

less well understood. One possibility is that PKA phosphorylates LKB1 at Ser428,[41] which has been shown to enhance AMPK activation.[42] We show here that adiponectin and forskolin decrease baseline AKT activity in a PKA-dependent but AMPK-independent manner. Although PKA attenuates AKT phosphorylation,[27] the mechanism remains unclear. The PKA-dependent inhibition of AKT may have required phosphorylation of the Ras-related protein 1 by PKA, as has been recently shown in the thyrotropin (thyroid-stimulating hormone)-treated PCCL3 thyroid cell line.[27] Ras-related protein 1 has a vital role downstream of PKA in the antiproliferative effects of forskolin in NIH3T3 cells,[39] and Ras-related protein 1 serves as a mediator in cyclic AMP-induced AMPK activation in bile acid-exposed hepatocytes.[43] Thus, Ras-related protein 1 may be a downstream mediator for both

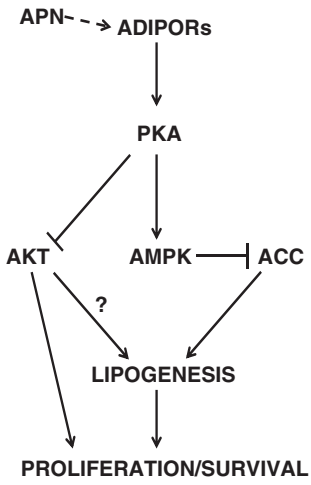

Figure 6. Proposed scheme for the antiproliferative and proapoptotic effects of adiponectin on MM cells. Adiponectin (APN) binds its receptors (ADIPORs) and activates PKA. PKA activation inhibits AKT activity and activates AMPK. AMPK activation in turn downregulates ACC expression, which is essential for lipogenesis. AKT is an enzyme vital to MM cell proliferation and survival.

AMPK activation and the inhibition of baseline AKT activity in MM cells in response to both adiponectin and forskolin.

Enhanced lipogenic activity is essential for maintaining cell membrane integrity in rapidly proliferating cancer cells.[44] ACC is a lipogenic enzyme that appears to be overexpressed in cancers;[32] it catalyzes the synthesis of malonyl coenzyme A, which is the rate-limiting step in long-chain fatty acid biosynthesis.[45] Its expression is regulated in several physiologic states to modulate lipogenesis.[46] We show here that adiponectin decreases expression of ACC in MM cells, which decreases ACC activity and fatty acid synthesis. We propose that adiponectin's suppression of lipogenesis mediates the antiproliferative effect of adiponectin in MM cells. This notion is bolstered by our observation that supplementing culture media with palmitic acid, a downstream metabolite of ACC, rescued MM cells from adiponectin-induced cell death. Moreover, we observed that pharmacologic inhibition of ACC with TOFA inhibited proliferation and induced apoptosis in MM cells, which were also suppressed by palmitic acid supplementation. Our finding that lipogenesis is important to MM cell survival is supported by a recent study showing that inhibiting fatty acid synthesis by using pharmacologic inhibitors that target fatty acid synthase, a lipogenic multienzyme also vital to fatty acid synthesis, inhibited proliferation of MM cell lines.[47] Herein, we further show that the PKA/AMPK pathway mediates adiponectin-induced downregulation of ACC expression. The involvement of AMPK in suppressing lipogenesis through regulating ACC is not unexpected, having been previously shown to inhibit ACC activity by inducing its phosphorylation.[33,34] AMPK activation's role in inhibiting fatty acid synthesis/lipogenesis in response to the AMPK activator AICAR and adiponectin has also been shown in glioblastoma[48] and colon cancer[35] cells, respectively. Surprisingly, we did not observe an increase in ACC phosphorylation in response to adiponectin but rather a steady decline in phosphorylation over time compared with baseline that correlated closely with a decline in total levels of ACC. Although we cannot exclude the possibility that an increase in ACC phosphorylation occurred earlier in response to adiponectin (<6 h), this seems unlikely because AMPK phosphorylation increased steadily over time, and we would expect that both ACC and AMPK phosphorylation would closely parallel each other, as previous studies have shown with prolonged exposure to activators of AMPK.[48,49] Nevertheless, regulation of ACC expression is a well-documented physiologic mechanism for modulating lipogenesis,[45] and according to our findings appears to be the mechanism by which AMPK downregulates ACC activity. Indeed, activators of AMPK have been shown to decrease expression of fatty acid synthase[49,50] and sterol regulatory element-binding protein 1,[19] a transcription factor that regulates expression of several enzymes required for fatty acid biosynthesis.[51] Also, the PI3K/AKT pathway positively regulates lipogenesis.[52,53] Thus, adiponectin's PKA-dependent but AMPK-independent downregulation of AKT activity probably also contributes to decreased lipogenesis in MM cells. Our findings point to another possible mechanistic link between obesity and MM. Free fatty acid levels, including palmitic acid, are elevated in obesity[54] because of increased adipose tissue and may be counteracting the antilipogenic effects of adiponectin.

We have shown that prolonged exposure to adiponectin induces cell cycle arrest and apoptosis in MM cells through a PKA/AMPK signaling pathway. We offer evidence that these antiproliferative effects are mediated, at least in part, by a PKA-dependent inhibition of AKT activity and PKA/AMPK-dependent downregulation of the critical lipogenic enzyme ACC, which may, at least partly, lead to decreased lipogenesis. Overall, our findings suggest that agents that activate the PKA/AMPK pathway, or that inhibit ACC, may be useful adjuvants in treating MM. Moreover, the observed antimyeloma effect of adiponectin supports the concept that decreased circulating levels of the adipokine, as occurs in obesity, promotes MM progression.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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