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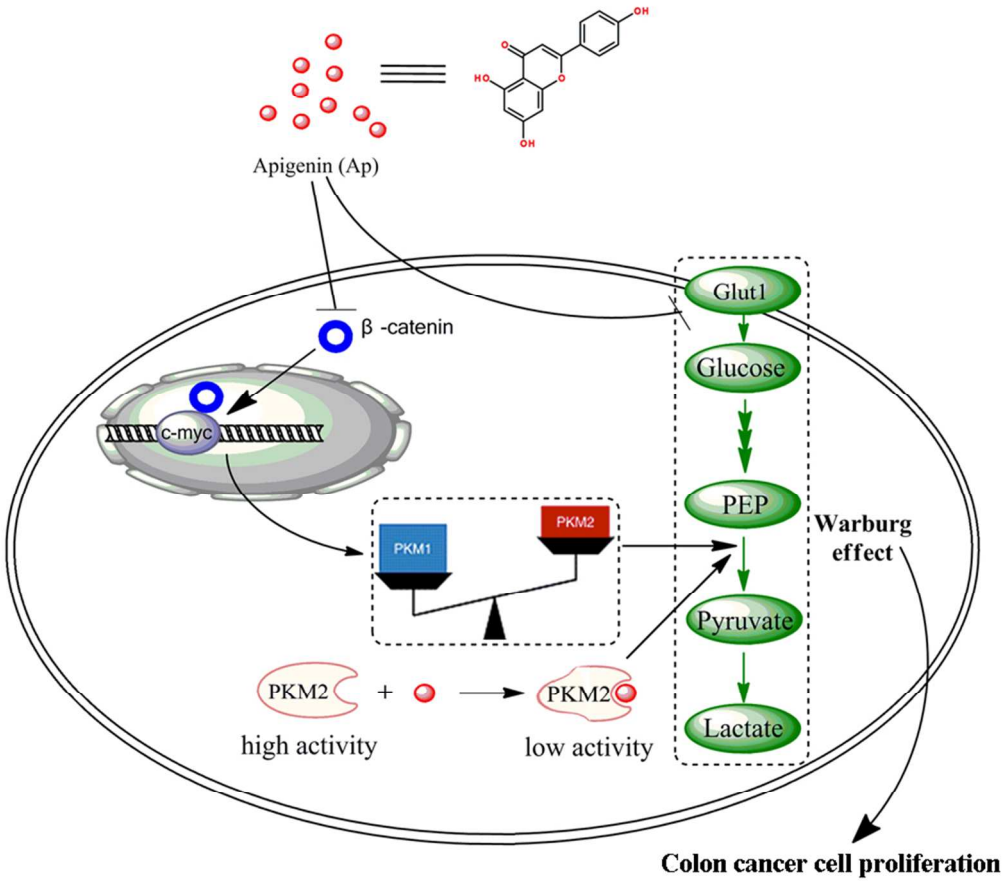

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Apigenin Restrains Colon Cancer Cell Proliferation via Targetedly Blocking the PKM2-dependent Glycolysis

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ABSTRACT:

Apigenin (AP), as an anti-cancer agent, has been widely explored. However, the molecular targets of apigenin on tumor metabolism are unclear. Currently, we found that AP could block the cellular glycolysis through restraining the tumor-specific pyruvate kinase M2 (PKM2) activity and expression, and further significantly induce the anti-colon cancer effects. The IC_{50} values of AP against HCT116, HT29 and DLD1 cells respectively were 27.9 ± 2.45, 48.2 ± 3.01 and 89.5 ± 4.89 μM. Fluorescence spectra and solid-phase AP extraction assays proved that AP could directly bind to PKM2 and markedly inhibit PKM2 activity in vitro and in HCT116 cells. Interestedly, in the present of D-fructose-1, 6-diphosphate (FBP), the inhibitory effect of AP on PKM2 had not been reversed, which suggests AP is a new allosteric inhibitor of PKM2. Meantime, RT-PCR and western blot assays showed that AP could ensure a low PKM2/PKM1 ratio in HCT116 cells via blocking β-catenin/c-Myc/PTBP1 signal pathway. Hence, PKM2 represents a novel potential target of AP against colon cancer.

KEY WORDS: Apigenin (AP); Colon cancer cells; Pyruvate kinase M2 (PKM2); Glycolysis; Cell proliferation

1. Introduction

Apigenin (AP), 4′, 5, 7-trihydroxyflavone, a well-known flavonoid compound, is generally found in various dietary plant foods, such as celery, thyme, celeriac, chamomile, onions, orange and tea et al (Zheng et al. 2005). Apigenin has significantly exhibited anti-tumor activity in various kinds of cancer cells, including colon cancer [1, 2], lung cancer [3], prostate cancer cells [4], pancreatic cancer [5], hepatocarcinogenesis and breast cancer [6]. AP inhibits the proliferation of tumor cells through coordinating multiple survival-related pathways, including PI3K/Akt/NF-κB [7], Wnt/β-catenin [8], insulin-like growth factor [9], mTOR signaling pathway et al [10]. Nonetheless, the potential targets of AP-induced anti-tumor effects and its impact on tumor metabolism have not been investigated.

As everyone knows that Warburg effect is a metabolic characteristic of cancer cells. Even in the case of adequate oxygen, tumor cells prefer to convert glucose into lactate instead of complete oxidation via Krebs cycle [11]. Hence, glycolysis is the major pathway for energy generation of tumor cells, and is crucial for the cancer cell proliferation and survival [12]. Pyruvate kinase (PK)-catalyzed reaction is the final rate-limiting step of glycolysis [13]. Mammalian cells have two PK genes: PKM1 and PKM2 are encoded by one PK gene, while PKL and PKR are encoded by another gene [14]. Most tumor cells uniformly express PKM2, thereby PKM2 plays a pivotal role in the cancer progression as a key regulator of the Warburg effect [15]. Hence, screening drugs which targeting inhibit PKM2 activity and expression is a potentially novel strategy for anticancer therapy. In previous studies, some inhibitors of PKM2 have been found, but few of them have selective.

In this study, we firstly found that AP exhibited anti-colon cancer effects through blocking the glycolysis pathway of PKM2-mediated. Further, fluorescence spectra and solid-phase AP extraction assays showed that AP could selectively bind to PKM2 and further suppress PKM2 activity in vitro and in HCT116 cells, but not PKM1. Fructose 1, 6-bisphosphate (FBP), an intermediate metabolite of glycolysis, is an allosteric effector of PKM2, and can allosterically activate the PKM2 activity [16]. Interestedly, the restraining effect of AP on PKM2 had not been

reversed by FBP, which hints AP is a new allosteric inhibitor of PKM2. Meantime, AP also could negatively regulate the PKM2/PKM1 ratio via blocking the β-catenin/c-Myc/PTBP1 signal pathway. Hence, our results suggest that PKM2 represents a novel potential target of AP against colon cancer.

2. Materials and methods

2.1 Chemicals and antibodies

RPMI-1640, DMEM/F12 (1:1) medium and fetal bovine serum (FBS) were purchased from GIBCO, Inc (GrandIsland, NY, USA). Apigenin (AP), D-fructose-1, 6-diphosphate (FBP), Glucose, oligomycin, 2-deoxy-D-glucose (2-DG) and Lithium (LiCl) were purchased from Sigma-Aldrich, Inc (St. Louis, MO, USA). Antibodies for PKM1 and PKM2 were obtained from Cell Signaling Technology, Inc (Danvers, MA, USA). Antibodies for β-catenin, Glu1 and c-Myc were obtained from Bioworld Technology, Inc (Minneapolis, MN, USA); Glucose Test Kit was obtained from Biovision, Inc (Mountain View, CA). Lactate Assay Kit, ATP Assay Kit and Glutaminase Test Kit were obtained from Nanjing Jiancheng Biotechnology Co., Ltd (Nanjing, China). GAPDH antibody was purchased from Abmart, Inc (Arlington, MA, USA); PTBP1 antibody was purchased from Sangon Biotechnology (Shanghai, China);Human Recombinant PKM2 (Re-PKM2) and PKM1 (Re-PKM1) were purified by our laboratory.

2.2 Cell culture

Human colon cancer cell lines (HCT116, HT29 and DLD1) were purchased from the Chinese Type Culture Collection (Shanghai China). The cell culture method was described in our previous study [17].

2.3 Cell survival assay

MTT assay was performed according to our previously described method with some modifications [17]. Apigenin was dissolved in dimethylsulfoxide (DMSO). HCT116, HT29 and DLD1 cells were incubated with the different concentration of AP (0, 10, 20, 40, 60 μM) for 24 h. Next, cells were incubated for 4 h by 5 mg/ml MTT (Sigma), and then 150 μl DMSO

(Sigma) were added to cell medium. The absorbance at 570 nm was measured. Cell survival rate was calculated with the formula: cell survival rate (%) = OD_{570} AP-treated/OD_{570} control×100 %.

2.4 Colony-formation assay

HCT116, HT29 and DLD1 cells were treated with 0, 15, 30 μM AP for 7 d respectively, and next the cell colonies were fixed, stained and photographed as the previously described method [18]. Colony formation rate was counted with the formula: colony formation rate % = (Clones/Seeded Cells) × 100 %.

2.5 Measurement of Glucose consumption, lactate production and ATP contents

HCT116 cells were incubated in 6-well plates (1×10^5 cells/ per well) overnight. Then these cells were subsequently treated with the different concentration of AP (0, 10, 20, 40 μM) for 24h. The contents of glucose and lactate in culture medium were determined with Glucose Test Kit (Biovision) and Lactate Assay Kit (Nanjing Jiancheng Bioengineering, Nanjing China) individually. The cells were broken by the ultrasound. Then cell supernatant was obtained by centrifuging at 6,000 rpm for 10 min and used to determine the ATP contents with ATP Assay Kit (Jiancheng Bioengineering).

2.6 Determination of extracellular acidification rates (ECAR)

HCT116 cells were inoculated into XFe24-well culture plate which was obtained from Seahorse Bioscience (North Billerica, MA, USA) at density1×10^4 cells/well and were subsequently intervened for 24 h with the different doses of AP (0, 20, 40 μM) after cells attachment. Cells were washed for two times and incubated for 1 h in the 37 °C non-CO_2 incubator in 500 μl XF Base Medium DMEM (356mg/l L-glutamine, pH 7.45). ECAR was determined after the successive injection of 10 mM glucose, 1μM oligomycin and 50 mM 2-DG at the indicated times. Seahorse XF24 Extracellular Analyzer was run using 3 cycles of

3 min mixing, 2 min waiting, and 3 min measurement. The data of ECAR (mpH/min) was recorded simultaneously.

2.7 Determination of Pyruvate Kinase Activity

PKM2 activity was measured as the previous method with some modifications [13, 14]. Enzymatic reactive mixture contained 50 mM Tris-HCl buffer (pH 7.5), 0.5 mM phosphoenolpyruvic acid (PEP), 100 mM KCl, 180 μM NADH, 2 mM adenosine 5-diphosphate (ADP), 10 mM $MgCl_2$, 8 U/ml of LDH and 20–100 ng re-PKM2. AP was dissolved in DMSO. FBP was dissolved in ddH_2O to the proper concentration. In the vitro inhibition experiment, the suitable AP or DMSO solution and 10 ng/μl of re-PKM2 were mixed and pre-incubated for 30 min at 37 °C. Then the suitable FBP or DMSO solution was added to the above mixture and sequentially incubated for 30 min. Re-PKM2 activity was measured and counted in the absence or presence of 125 μM FBP according to the previously described method [14]. PKM2 activity was defined as the quantity of β-nicotinamide adenine dinucleotide (NADH) oxidized by 1 mg of re-PKM2 per minute. Re-PKM1 activity was determined as above described method.

HCT116 cells were inoculated into 6-well culture plate (1×10^5 cells/well) overnight and were subsequently treated with the different concentration of AP (0, 10, 20, 40 μM) for 24 h. Then, these cells were broken and centrifuged at 6,000 rpm for 10 min and the supernatant was used to determine the PKM2 activity as above described method.

2.8 Fluorescence Spectra Measurements

Pyruvate Kinase was dissolved in phosphate buffer saline (PBS), and the final concentration was 4.3×10^{-7} M. AP was dissolved in DMSO. A solution of 1.0 ml of Pyruvate Kinase was titrated with AP of different concentration and the reaction mixture incubated for 3 min at different temperature (25 °C, 30 °C, 37 °C, 42 °C) respectively before the measurements. Fluorescence spectra of Pyruvate Kinase and Pyruvate Kinase-AP mixture were recorded from 315 to 400 nm. Excitation and emission slits were set as 10 nm. Fluorescence spectra were recorded with an excitation of 295 nm.

2.9 Solid-phase extraction assay

Solid-phase extraction assay was performed as described previously with some modifications [14]. HCT116 cell lysate (2 mg protein) was mixed with AP (6 mg), and the mixture revolved overnight at 4 °C and removed the supernatant by centrifugation. The precipitate of crystal AP with bound cellular protein was washed for three times with chilled buffer (50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 150 mM NaCl, 1 mM NaF, 10 % glycerol) followed by dissolving and boiling in SDS-PAGE loading buffer for 5 min. The samples were separated by 10 % SDS-polyacrylamide gel and next transferred to polyvinylidene fluoride (PVDF) membrane, followed by incubation with PKM2 primary and secondary antibodies. The protein band of PKM2 was visualized by enhanced chemoluminescence.

2.10 Real-time PCR assay

Real-time PCR **assay** was carried out according to described previously method with some modifications [19]. Total RNA of HCT116 cells by control or AP-treated was extracted with Trizol reagent and then reversely transcribed to cDNA by PrimeScript RT Master Mix. The relative expression of each targeted gene in RNA level was determined by Real-time PCR assay.

2.11 Western blot assay

Western blot assay was executed according to our previously described method [17]. HCT116 cells were treated as above 2.5 described for 24 h. Cells were degraded using the cell lysis buffer and incubated for 30 min on ice, followed by centrifuging for 15 min at 13,000 rpm. The equal protein was separated by SDS-PAGE assay and next transferred to PVDF membrane, followed by incubation with primary and secondary antibodies. The relative expression of each target protein was visualized with enhanced chemiluminescence.

2.12 Small interfering RNA (siRNA) transfection assay

A siRNA against β-catenin (sense 5′-GGAUGUGGAUACCUCCCAATT-3′, antisense 5′-UUGGGAGGUAUCCACAUCCTT-3′) and a negative control siRNA (sense 5′-UUCUCCGAACGUGUCACGUTT-3′, anti-sense, 5′-ACGUGACACGUUC GGA GAA

TT-3′) were obtained from Genepharma (Shanghai China), HCT116 cells were inoculated into 6-well culture plate overnight and next were transiently transfected with 10 nM of siRNA using HiPerFect Transfection Reagent (QIAGEN) as the manufacturer's instructions. The β-catenin knockdown was determined by qRT-PCR and western blot assays 48 h post-transfection.

2.13 Statistical Analysis

Statistical significance was measured with SPSS 17.0 software. Data were statistically analyzed using one-way analysis of variance, followed by Tukey's post-hoc test. Results were represented as the mean±standard deviation (SD). The P-values of less than 0.05 ($p<0.05$) and 0.01 ($p<0.01$) were indicated that the difference was significant and extremely significant compared with the control.

3 Results

3.1 AP significantly restrains colon cancer cell survival and colony formation

To explore AP-induced anti-colon cancer effects, we determined the effects of AP on survival and colony-formation ability of HCT116, HT29 and DLD1 cells. The results manifested that the survival ratios of three colon cancer cell lines (HCT116, HT29 and DLD1) showed a remarkably dose-dependent reduction with AP treatment, and further the viability rates of HCT116, HT29 and DLD1 cells were 35 %, 44 % and 66 % with 60 μM AP for 24 h. (Figure 1C). The IC_{50} values of AP against HCT116, HT29, and DLD1 cells respectively were 27.9±2.45, 48.2±3.01, and 89.5±4.89 μM. Furthermore, as shown in Figure 1 A and B, AP treatment also could significantly decrease the colony sizes and numbers of HCT116, HT29 and DLD1 cells compared to the control group. These results suggest that AP inhibits markedly colon cancer cell proliferation, and further prove that the inhibition rate of AP on HCT116 cell is highest compared with HT29 and DLD1 cells. Therefore, HCT116 cell line was selected for further experiments.

3.2 AP induces the blockage of glycolysis in HCT116 cells

The glycolysis and glutamine metabolism are the major metabolic pathways for cancer

cell survival and proliferation. To investigate whether AP affected the metabolic rates of HCT116 cells, the relevant key indicators were measured. The results showed that AP significantly inhibited the glycolytic rate in HCT116 cells, as manifested by cellular lactate production and glucose consumption (Figure 2C and D), while the glutaminase activity had no effect after AP treatment (Figure 2G). Extracellular acidification rate (ECAR) from proton production rate represents a direct indicator of glycolytic capacity. SeaHorse XF24 Extracellular Analyzers revealed that AP treatment could significantly reduce the glycolysis, glycolytic capacity and glycolytic reserve of HCT116 cell (Figure 2 E and F). Meantime, AP treatment also could seal off the expression of the glucose transporter-1(Glu1) in mRNA and protein levels (Figure 2A and B). As expected, the blockage of glycolysis by AP-induced led to the decrease of ATP contents in HCT116 cells (Figure 2H). These results indicate that AP can display the anti-proliferative effects in colon cancer through reducing the rate of cellular glycolysis.

3.3 AP exists the interaction With Re-PKM2 in vitro

To investigate whether the interaction exists between AP and re-PKM2, the effect of AP on fluorescence spectra of PKM2 were measured. As shown in Figure 3, fluorescence intensity of PKM2 was quenched obviously (near 340 nm) in the presence of AP at room temperature. The types of fluorescence quenching contain static and dynamic quenching. Dynamic quenching gives the credit to the collisions between two fluorescent luminophors, whereas static quenching results from the formation of a new non fluorescent complex [20, 21]., The dynamic quenching constant (Ksv) increases along with the rise of temperature and rate constant (Kq) is lower than 2.0×10^{10} ($L\cdot mol^{-1}\cdot S^{-1}$) in the process of dynamic quenching, but the case is the opposite in the process of static quenching in accordance with the fluorescence quenching theory [21]. As shown in table 1, Ksv values were reduced gradually with the elevation of temperature (25 °C, 30 °C, 37 °C, 42 °C) and Kq was greater than 2.0×10^{10} ($L\cdot mol^{-1}\cdot S^{-1}$), which indicates the fluorescence quenching of PKM2 by AP-induced is a static quenching through forming a new complex.

Further, we confirmed the interaction manner between AP and PKM2 by the values of binding constants (K_A) and binding sites (n). As shown in table 2, the number of binding site (n) was approximately one at 25 °C, 30 °C, 37 °C, 42 °C, which suggested that the temperature changes had little effect on the binding of AP and PKM2. The main interaction manners between a small molecule and a protein molecule have several types, including hydrogen bonding, hydrophobic interaction, electrostatic attraction, vander waals force et al [22]. The relative changes of entropy (ΔS) and enthalpy (ΔH) values can evaluate the types of the main interaction. Specifically, when ΔH > 0and ΔS > 0, hydrophobic force is the main force [22]. According to our results, the value of ΔG was negative for the AP, while their ΔH and ΔS were positive, which means that the direct interaction between PKM2 and AP is a spontaneous process along with entropy increasing, and further is driven mainly by hydrophobic force (Table. 3).

3.4 AP can allosterically inhibit the PKM2 activity in vitro

The PKM1 and PKM2 are originated from a PKM gene transcript through alternative splicing, in which 21 residues are different [23]. PKM1 exhibits high constitutive enzymatic activity, while PKM2 is less active and has allosteric effect [24]. Our results showed that AP could inhibit PKM2 activity (Figure 4A) and the inhibitory effect of AP on re-PKM2 was dose-dependent and selective, whereas AP could did not affect re-PKM1 activity in vitro (Figure 4C). According to the literature, the glycolytic metabolite FBP is an allosteric activator of PKM2 [25]. To confirm whether AP could allosterically regulate PKM2 activity, re-PKM2 activity was measured in the presence or absence of FBP. The results showed that re-PKM2 had not been activated in the presence of AP by FBP, which suggests that there is the same site for AP and FBP binding to PKM2 (Figure 4B). In other words, PKM2 activity can be allosterically inhibited by AP in vitro.

3.5 PKM2 is a potential target protein of AP in HCT116 cells

To confirm whether AP could bind to intracellular PKM2 in HCT116, we extracted cellular proteins binding to AP in HCT116 lysates by solid-phase assay. SDS-PAGE

combined with western blotting analysis revealed that PKM2 was a target protein of AP (Figure 5A and B). Further, AP also could inhibit PKM2 activity in a dose-dependent manner in HCT116 cells (Figure 5C). Subsequently, the effect of AP on PKM2 activity was determined in the presence of FBP. As expected, the inhibitory effect of AP on PKM2 activity had not been reversed by FBP (Figure 5D), which further confirms that AP is a new allosteric inhibitor of PKM2.

3.6 AP decreases the expression of PKM2 in HCT116 by blocking the β-catenin/c-Myc /PTBP1 pathway

To investigate whether AP could affect the expression of PKM2, the RT-PCR and western blot assays were performed. The results showed that AP treatment could significantly decrease the RNA and protein levels of PKM2 in a dose-dependent manner, whereas the PKM1 expression in mRNA level was markedly increased and had not change in protein level (Figure 6 A and B). Polypyrimidine Tract Binding Protein (PTBP1), a pivotal regulator of PKM splicing, which selectively promotes PKM2 splicing [26]. Minami et al study [27] showed that c-Myc could positively regulate PTBP1 expression in bladder cancer cells. Our results found that AP treatment effectively reduced the PTBP1, β-catenin and c-Myc expression in a dose-dependent manner in mRNA and protein levels in HCT116 cells (Figure 6 C and D).

Lithium (LiCl), an inhibitor of GSK-3β, is known to activate the wnt/β-catenin pathway and its downstream target gene c-Myc expression by preventing β-catenin degradation [28]. Therefore, to further confirm whether the inhibitory effect of AP on PKM2 expression was mediated by wnt/β-catenin pathway, the addition of LiCl mimicked the β-catenin over-expression. The cells were pretreated for 24 h with 10 mM LiCl, and then treated with 40 μM AP for 24 h. As shown in Figure 6E, LiCl treatment markedly increased β-catenin expression, and further the inhibitory effects of AP on β-catenin, c-Myc, PTBP1 and PKM2 expression were obviously reversed after LiCl pretreatment, and whereas the knockdown of β-catenin by siRNA had the opposite effect (Figure 6F and G). These results indicate that the inhibitory effect of AP on PKM2 expression is realized via blocking the

β-catenin/c-Myc/PTBP1 pathway in HCT116 cells.

4 Discussion

Previous studies have demonstrated that apigenin, as an edible plant-derived flavonoid, can induce markedly anti-colon cancer effects via modulating several signaling pathways, including inhibition of the AKT and ERK [1], supersession of autophagy formation [2], activation of caspase cascade pathway [29], induction of G2/M cell-cycle arrest [30]. In present, we also found that AP significantly suppressed HCT116, HT29 and DLD1 cells survival in a dose-dependent manner and the inhibitory effects of AP on three cell lines had obviously difference (Figure 1).

As shown in Figure 1 C, HCT116 displayed the highest responsive rate to AP compared to other two colon cancer cell lines (HT29 and DLD1). Duke's classification for colorectal cancer is a widely applied classification system and represents the malignancy of colon cancer [31]. It is reported that HCT116, HT29 and DLD1 cell lines are typical representative of Duke's type A，B and C in human colon cancer cell lines [31, 32], indicates that the mallgnant degree of HCT116 cell is the lowest compared with HT29 and DLD1 cell lines. Furthermore, it is generally known that the wild type p53, as a tumor suppressor gene, has a profound effect on inhibiting cancer development, whereas mutant p53 plays an opposite role. The studies showed that HCT116 cell line harbored the wild type p53, while DLD1 and HT29 cell lines were mutant p53[33, 34]. Therefore, the highest inhibitory effect of AP on HCT116 cell proliferation may owe to its lower mallgnant degree and P53 gene types.

Further, we found that AP could induce a switch of glycolytic cancer cells, and result in the decrease of colon cancer cells survival (Figure 2). Previous studies proved that different from normal cells, cancer cells preferentially take glycolysis for supplying the distinct metabolic needs of cell proliferation, even in the abundance of oxygen. Therefore, aerobic glycolysis or the Warburg effects is the main way in which tumor cells get energy and is pivotal for the survival and growth of cancer cells. Our results firstly demonstrated that in HCT116 cells, AP not only blocked the cellular intake of glucose, but also decreased glucose

consumption and lactate production, which further led to the decrease of ATP contents (Figure 2). The results prove that the anti-proliferative effects of AP on colon cancer cells partly give the credit to the blockage of glycolysis.

PKM2 is the last rate-limiting enzyme in glycolysis, which catalyzed from phosphoenolpyruvate (PEP) to pyruvate [35, 36]. Lots of evidences showed that PKM2 was a key regulator for glucose metabolism and played an important role in colon cancer progression[37, 38, 39]. Therefore, development of selectively targeting PKM2 inhibitors for cancer therapy remains an optimal strategy. PKM2 is highly homologous to PKM1 and they have the same catalytic site. However, PKM2 contains a unique allosteric regulatory site, making it possible to screen selectively inhibitor of targeting the allosteric site of PKM2. Previous study showed that PKM2 enzyme activity was strongly inhibited by apigenin in vitro [40]. In present study, we found that apigenin not only in vitro could significantly inhibit the activity of PKM2 (Figure 4A), and importantly it also could play the same effect in HCT116 cell (Figure 5C), but not the PKM1 (Figure 4C). Further, our results manifested that apigenin displayed the inhibitory effects of PKM2 through directly binding to PKM2 (Figure 3 and Figure 4). FBP is an allosteric activator of PKM2 [41]. Our results showed that PKM2 inhibition by AP-induced in vitro and in lysates of HCT116 cell is not reversed by FBP, and further speculates that apigenin specifically targets at the allosteric FBP binding site of PKM2 (Figure 4B and Figure 5D). Hence, these results indicate apigenin probably is a new allosteric inhibitor of PKM2.

Furthermore, our results also demonstrated that apigenin selectively blocked the expression of PKM2 in mRNA level, but induced the PKM1 expression in HCT116 cell, which indicates that apigenin maybe involves in the alternative splicing of PKM pre-Mrna (Figure 6 A and B). Previous studies demonstrated that c-Myc, an oncogenic transcription factor, is one of the target genes in the downstream of β-catenin [42], which can ensure a high PKM2/PKM1 ratio by up-regulating transcription of PTBP, hnRNPA1 and hnRNPA2[43]. As expectedly, we found that apigenin significantly reduced the expression of β-catenin and its downstream target gene c-Myc and PTBP1, and the β-catenin overexpression by LiCl-mimicked could partly reverse

the effects (Figure 6). Therefore, we speculate that the inhibitory effects of PKM2 expression by apigenin-induced partly attribute to β-catenin/c-Myc/PTBP1-mediated alternative splicing event.

In conclusion, apigenin targeting at PKM2-mediated glycolysis pathway plays a crucial role in inhibition of colon cancer cell proliferation (Figure 7). Further, apigenin mainly induces a switch of glycolysis in colon cells through blocking the activity and expression of PKM2. Mechanically, apigenin can bind to the allosteric site of PKM2 to inhibit the PKM2 activity; Interestedly, apigenin also selectively inhibits the PKM2 expression by blocking the β-catenin/c-Myc/PTBP1 signal pathway. The study shed light on the key role of apigenin in cancer metabolic reprogramming.

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Notes

The authors declare no conflict of interest.

Figure legends

Figure 1. Effects of AP on colon cancer cell survival. (A) Cell colony-formation ability was observed using Stereoscopic microscope. Cells were treated with 0, 15, 30 μM AP for 7 d. (B) Clone-formation rate was determined by crystal violet. (C) Cell viability ratio was measured using MTT assay. HCT116, HT29 and DLD1 cells were treated with the increasing concentration of AP (0, 10, 20, 40, 60 μM) for 24 h. *p<0.05, ** p<0.01 versus control.

Figure 2. AP induces the blockage of glycolysis in HCT116 cells. (A, B) Effect of AP on Glu-1 expression in mRNA (A) and protein (B) levels. HCT-116 cells were treated with 0, 10, 20, 40 μM AP for 24 h. (C, D, G) Effects of AP on glucose consumption (C), lactase production (D) and glutaminase activity (G). Cells were treated as Figure. 2 A described. (E, F) Effects of AP on extracellular acidification rate (ECAR). HCT-116 cells were intervened with 0, 20, 40 μM AP for 24 h. ECAR was determined after the sequential injection of 10 mM glucose, 1 μM oligomycin and 50 mM 2-DG at the indicated times. (H) Effect of AP on ATP contents in HCT116 cells. Cells were intervened as Figure. 2 A described. *p<0.05 and ** p<0.01 vs. control group.

Figure 3. Effect of AP on fluorescence spectra of PKM2. λex = 295 nm, [Apigenin]=7.5×10^{-4} M, curves1–11 pH 7.4, at room temperate. Apigenin increased as the direction of the arrow. PKM2, pyruvate kinase-2.

Figure 4. Effects of AP on the re-PKM2 and re-PKM1 activities in vitro. (A) Effect of AP on re-PKM2 activity. (B) The inhibitory effect of AP on re-PKM2 activity in the presence of 125 μM FBP. The mixture of re-PKM2 and AP pre-incubated at 37 °C for 30 min, and then the mixture which was added FBP persistently incubated at same tempreture for 30 min. PK activity was calculated by measuring the change of absorbance at 340 nm from 0 to 8 min. *p<0.05, **p<0.01 vs. control. FBP, D-fructose-1, 6-bisphosphate. (C) Effect of AP on the re-PKM1 activity.

Figure 5. The target protein bound to AP is identified by solid-phase and western blotting assays. (A) Cellular proteins by solid-phase AP-extracted were showed by SDS-PAGE in HCT116 cells. (B) The target protein bound to AP was identified by western blotting assay. (C) Effect of AP on PKM2 activity in HCT116 cells. Cells were treated as Figure. 2 A described and used the cell lysates to determine the activity of PKM2. (D) Effect of AP on PKM2 activity in HCT116 cells in the presence or absence of FBP. After cells were treated by 40 μM AP, the cell lysate incubated with 125 μM FBP for 30 min at 37 °C to determine the activity of PKM2. ** p<0.01 vs. control.

Figure 6. AP inhibits PKM2 expression by blocking the β-catenin/c-Myc/PTBP1 pathway. (A) Effects of AP treatment on the PKM1 and PKM2 expressions in mRNA (A) and protein (B) levels were measured by RT-PCR and western blotting assays. Cells were treated as Figure. 2 (A) described. (C, D) Effects of AP on the β-catenin, c-Myc and PTBP1 expressions in mRNA (C) and protein (D) levels. (E, F) Effects of β-catenin siRNA on the expression levels of β-catenin, c-Myc, PKM2 and PKM1. HCT116 cells were transfected using β-catenin siRNA or negative control (NC) siRNA for 48 h. (G) Effects of LiCl on the inhibitory levels of β-catenin, c-Myc, PKM2 and PTBP1 by AP-induced. HCT116 cells were pretreated with 10 mM LiCl for 24 h and next intervened with 40 μM AP for 24 h. *p<0.05, **p<0.01 vs. control.

Figure 7. AP restrains colon cancer cell proliferation via targetedly blocking the PKM2-dependent glycolysis.

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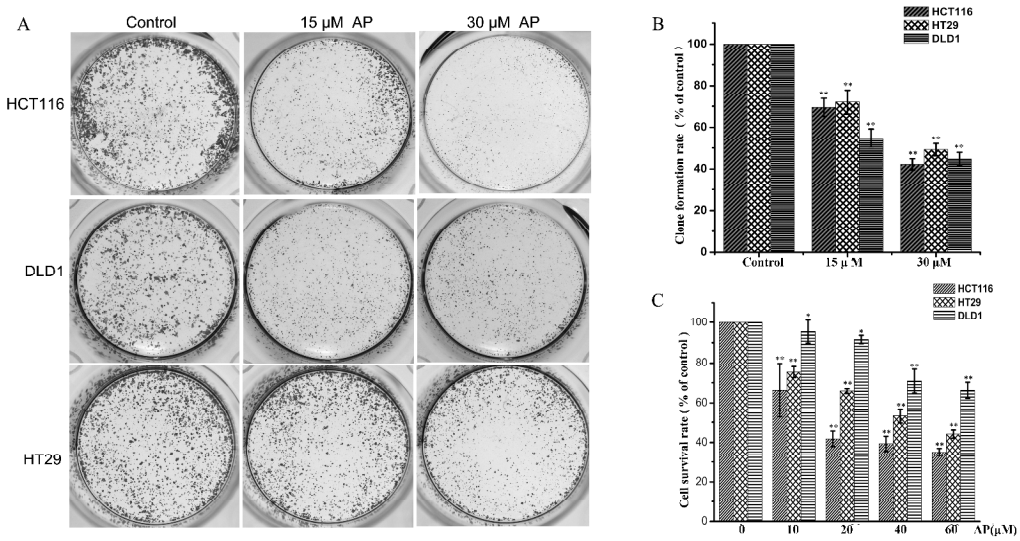
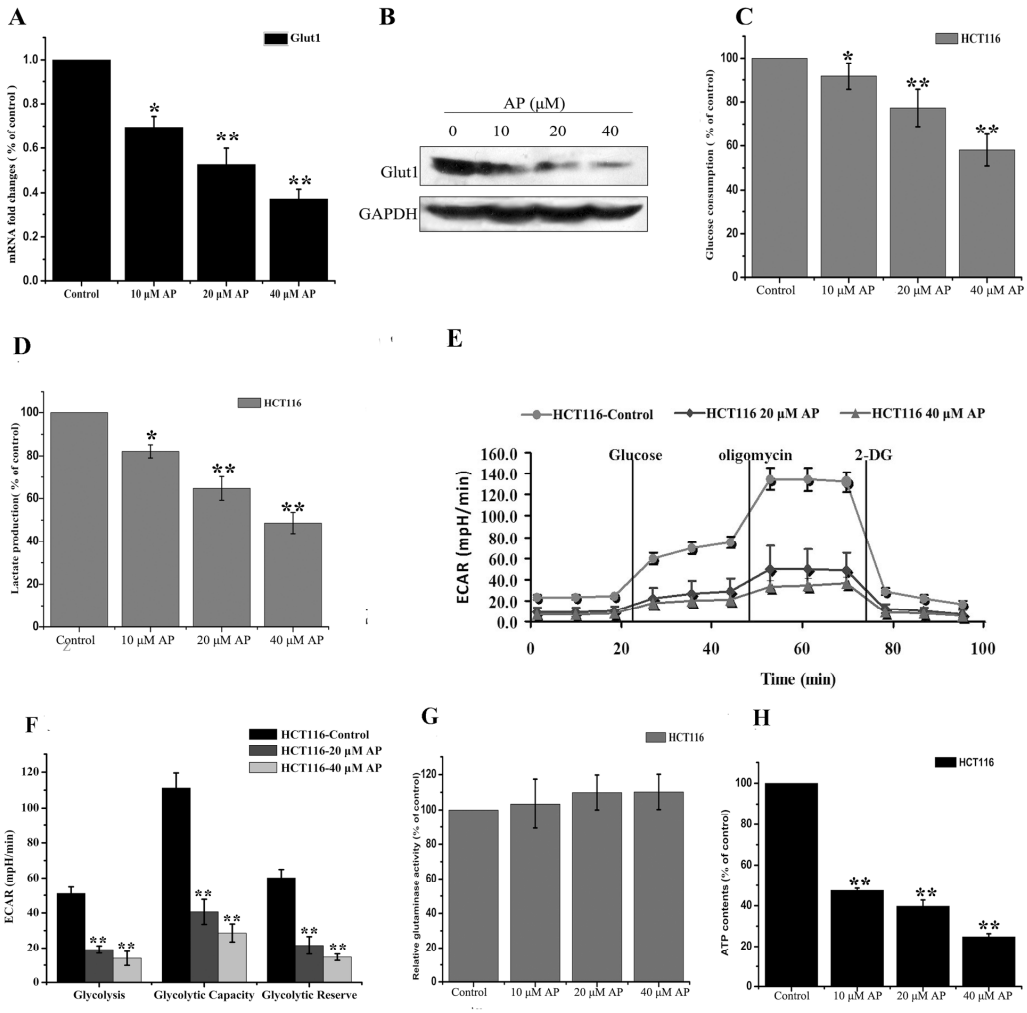

A
Control
15 μM AP
30 μM AP
HCT116
DLD1
HT29
B
HCT116
HT29
DLD1
Clone formation rate (% of control)
Control
15 μ M
30 μM
C
HCT116
HT29
DLD1
Cell survival rate (% of control)
AP(μM)


A
Glut1
mRNA fold changes (% of control)
Control
10 μM AP
20 μM AP
40 μM AP
B
AP (μM)
0 10 20 40
Glut1
GAPDH
C
HCT116
Glucose consumption (% of control)
D
HCT116
Lactate production(% of control)
E
HCT116-Control
HCT116 20 μM AP
HCT116 40 μM AP
Glucose
oligomycin
2-DG
ECAR (mpH/min)
Time (min)
F
HCT116-Control
HCT116-20 μM AP
HCT116-40 μM AP
ECAR (mpH/min)
Glycolysis
Glycolytic Capacity
Glycolytic Reserve
G
HCT116
Relative glutaminase activity (% of control)
H
HCT116
ATP contents (% of control)

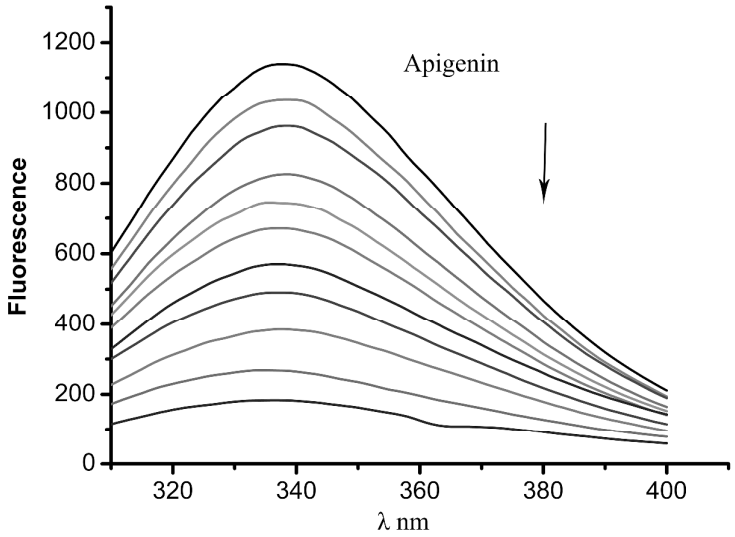

Apigenin
Fluorescence
1200
1000
800
600
400
200
0
320
340
360
380
400
λ nm

Table 1 Stern–Volmer quenching constant values of the interactions between AP and PKM2

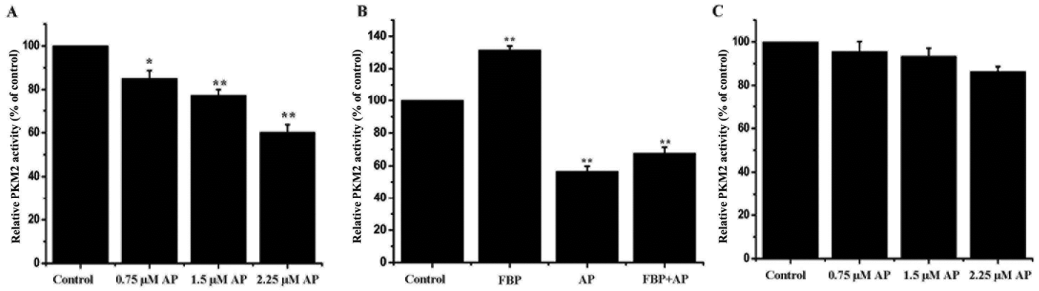
T(°C)	Ksv/10^5 (L·mol^{-1})	Kq/10^{13}(L·mol^{-1}·S^{-1})	R^2
25	2.41	2.41	0.99
30	2.30	2.30	0.99
37	2.29	2.29	0.99
42	1.87	1.87	0.99

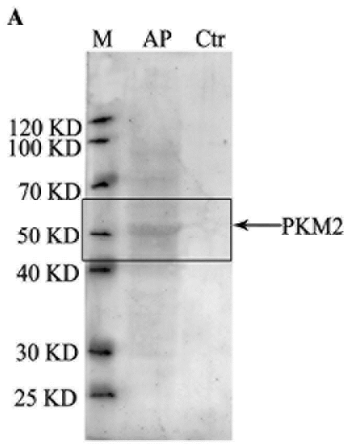
Table 2 Values of K_A and n of the interaction between AP and PKM2

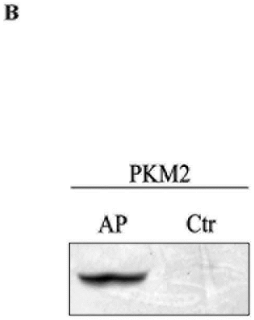
T(°C)	K_A/10^5 (L·mol^{-1})	n	R^2
25	2.80	1.02	0.99
30	3.30	1.05	0.99
37	7.26	1.11	0.99
42	9.95	1.12	0.99

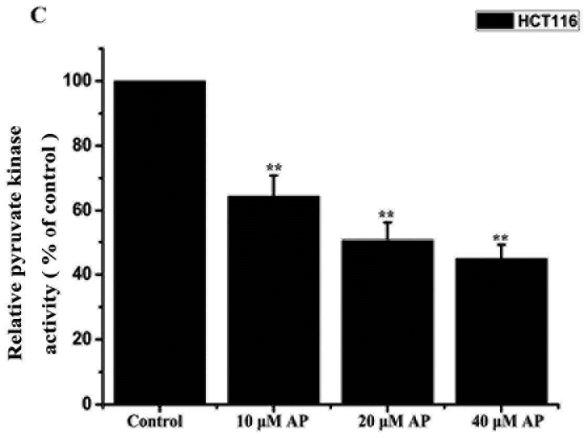
Table 3 Thermodynamic of interaction between AP and PKM2

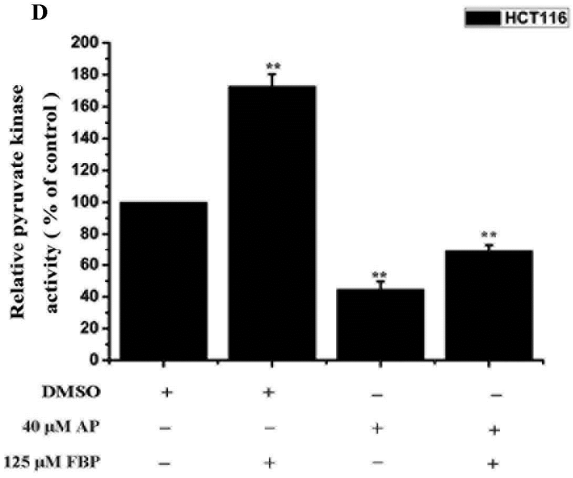
T(°C)	Δ G (kJ·mol^{-1})	Δ H (kJ·mol^{-1})	Δ S (J·mol^{-1}K^{-1})
25	-31.1	62.7	314
30	-32.0		
37	-34.8		
42	-36.2		

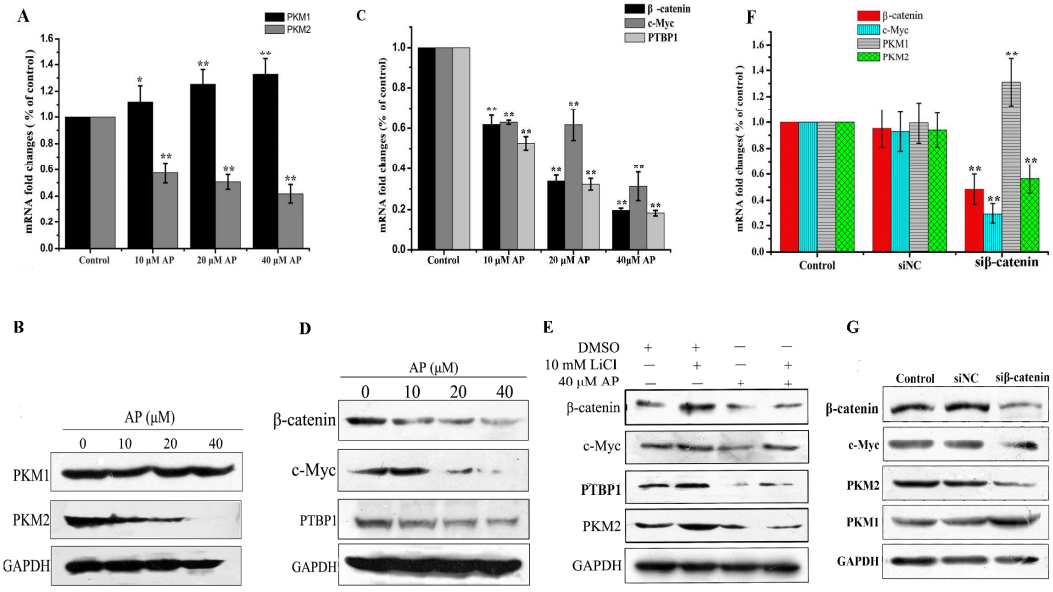

A
Relative PKM2 activity (% of control)
0
20
40
60
80
100
*
**
**
Control
0.75 μM AP
1.5 μM AP
2.25 μM AP
B
Relative PKM2 activity (% of control)
0
20
40
60
80
100
120
140
**
**
**
Control
FBP
AP
FBP+AP
C
Relative PKM2 activity (% of control)
0
20
40
60
80
100
Control
0.75 μM AP
1.5 μM AP
2.25 μM AP

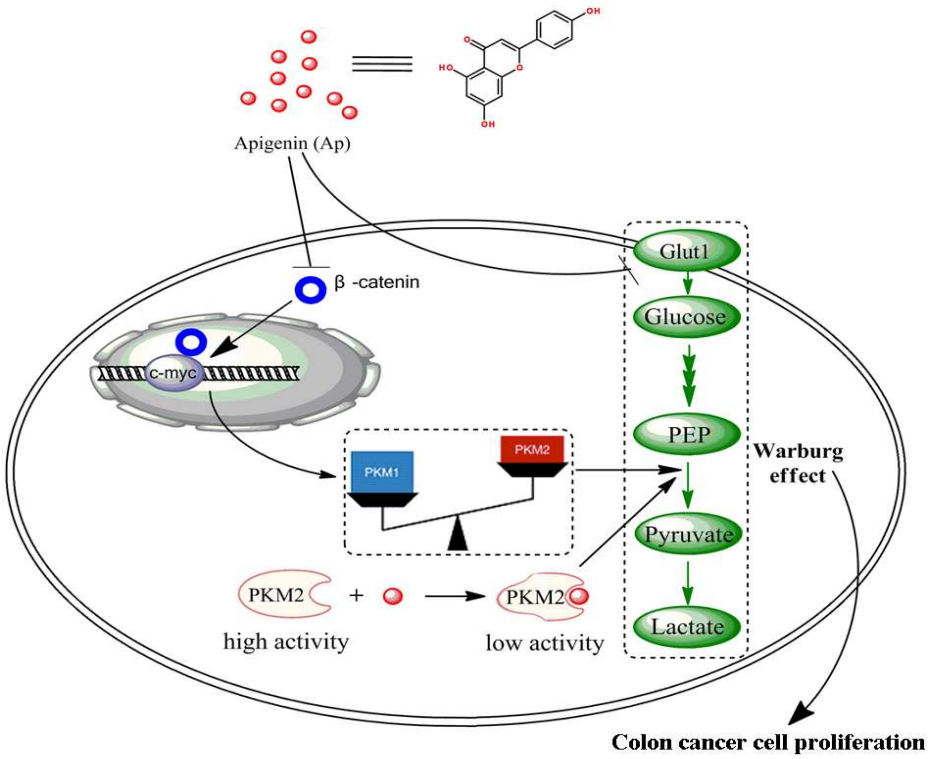

A
M
AP
Ctr
120 KD
100 KD
70 KD
50 KD
40 KD
30 KD
25 KD
PKM2


B
PKM2
AP
Ctr


C
HCT116
Relative pyruvate kinase activity (% of control)
100
80
60
40
20
0
**
**
**
Control
10 μM AP
20 μM AP
40 μM AP


D
HCT116
Relative pyruvate kinase activity (% of control)
200
180
160
140
120
100
80
60
40
20
0
**
**
**
DMSO + + – –
40 μM AP – – + +
125 μM FBP – + – +


A
PKM1
PKM2
mRNA fold changes (% of control)
Control
10 μM AP
20 μM AP
40 μM AP
C
β -catenin
c-Myc
PTBP1
mRNA fold changes (% of control)
Control
10 μM AP
20 μM AP
40μM AP
F
β-catenin
c-Myc
PKM1
PKM2
mRNA fold changes (% of control)
Control
siNC
siβ-catenin
B
AP (μM)
0 10 20 40
PKM1
PKM2
GAPDH
D
AP (μM)
0 10 20 40
β-catenin
c-Myc
PTBP1
GAPDH
E
DMSO + + — —
10 mM LiCl — + — +
40 μM AP — — + +
β-catenin
c-Myc
PTBP1
PKM2
GAPDH
G
Control siNC siβ-catenin
β-catenin
c-Myc
PKM2
PKM1
GAPDH


Apigenin (Ap)
β -catenin
c-myc
PKM1
PKM2
PKM2
high activity
PKM2
low activity
Glut1
Glucose
PEP
Pyruvate
Lactate
Warburg effect
Colon cancer cell proliferation