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Enhancing Mitochondrial Respiration Suppresses Tumor Promoter TPA-Induced PKM2 Expression and Cell Transformation in Skin Epidermal JB6 Cells

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Abstract

Differentiated cells primarily metabolize glucose for energy via the tricarboxylic acid cycle and oxidative phosphorylation, but cancer cells thrive on a different mechanism to produce energy, characterized as the Warburg effect, which describes the increased dependence on aerobic glycolysis. The M2 isoform of pyruvate kinase (PKM2), which is responsible for catalyzing the final step of aerobic glycolysis, is highly expressed in cancer cells and may contribute to the Warburg effect. However, whether PKM2 plays a contributing role during early cancer development is unclear. In our studies, we have made an attempt to elucidate the effects of varying mitochondrial respiration substrates on skin cell transformation and expression of PKM2. Tumorigenicity in murine skin epidermal JB6 P+ (promotable) cells was measured in a soft agar assay using 12-*O*-tetradecanoylphorbol-13-acetate (TPA) as a tumor promoter. We observed a significant reduction in cell transformation upon pretreatment with the mitochondrial respiration substrate succinate or malate/pyruvate. We observed that increased expression and activity of PKM2 in TPA-treated JB6 P+ cells and pretreatment with succinate or malate/pyruvate suppressed the effects. In addition, TPA treatment also induced PKM2 whereas PKM1 expression was

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No potential conflicts of interest were disclosed.

suppressed in mouse skin epidermal tissues *in vivo*. In comparison with JB6 P+ cells, the nonpromotable JB6 P- cells showed no increase in PKM2 expression or activity upon TPA treatment. Knockdown of PKM2 using a siRNA approach significantly reduced skin cell transformation. Thus, our results suggest that PKM2 activation could be an early event and play a contributing role in skin tumorigenesis.

Introduction

There are several ways in which cancer cells distinguish themselves from normal cells, including, but not limited to, the way they obtain energy. Cellular energy stored as ATP is produced through respiration, which is composed of a set of metabolic reactions that take place in the cytosol (glycolysis) and mitochondria (mitochondrial respiration) to convert nutrients (e.g., glucose) into ATP. Otto Warburg, who first noted a difference between the metabolism of glucose in cancer cells and normal adult tissues, observed that cancer cells take up glucose at higher rates than normal tissue but use a smaller fraction of this glucose for oxidative phosphorylation (1). This phenomenon is therefore termed the Warburg effect or "aerobic glycolysis," which is now well recognized (2–4).

Associated with this metabolic switch is up- or downregulation of important metabolic enzymes. One such enzyme is the M2 splice isoform of pyruvate kinase (PKM2). Pyruvate kinase (PK), which is an essential enzyme involved in controlling glucose metabolism as the final rate-limiting step in glycolysis, has a noteworthy role in tumors (5). Vertebrates have 4 tissue-specific isozymes of PK: an L isozyme present in the liver and kidney, an R isozyme found primarily in erythrocytes, and the M isozymes M1 and M2, the former identified in most adult tissues and the latter in embryonic tissues and adult stem cells (6–8). The M1 and M2 isoenzymes are found on the same gene but arise from alternative splicing of exons 9 and 10 of the *PKM* gene regulated by heterogeneous nuclear ribonucleoproteins (hnRNP; refs. 9–12). These isoenzymes have 23 different amino acids along a 56-amino acid stretch, which enables each isoenzyme to have a unique region for allosteric regulation (13). Not only do we observe a disparity between PKM1 and PKM2 splicing and expression in embryonic and adult tissues, but it also arises when examining cancer cells. PKM2 is primarily expressed in most human cancers, which include colorectal cancer, lung cancer, liver cancer, breast cancer, brain cancer, kidney cancer, and perhaps in cervical cancers (14–28). In contrast to PKM2, PKM1 is actively repressed in cancer cells (11, 14, 29). Because of these recent discoveries between the relationship of certain common cancers and the expression of the metabolic gene *PKM2*, a connection between abnormal mitochondrial function and cancer was identified. However, at what stage PKM2 is unregulated and whether it is important for early cancer development are unknown. Because cancer cells prefer to bypass mitochondrial oxidative phosphorylation to generate ATP, we were also interested in knowing whether boosting mitochondrial respiration can prevent tumorigenesis. Herein, we used skin epidermal cells as well as skin carcinogenesis mouse models, both well characterized to study tumor promotion, to address these questions in early skin tumorigenesis.

Materials and Methods

Cell lines, reagents, and treatment

Murine skin epidermal JB6 Cl-41 P+ (promotable) and Cl 30-7b P- (nonpromotable) cells were used to study tumor promotion (both cell lines were purchased from American Type Culture Collection). Cells were grown in Eagle's minimal essential medium (EMEM) medium containing 4% FBS, 1% glutamine, and 1% penicillin in a 37°C incubator under 5% CO₂. The levels of *Mycoplasma* were routinely (once every 3 months) monitored in these cells using a MycoAlert Mycoplasma Detection kit purchased from Lonza, and the results were negative.

The tumor initiator 7,12-dimethylbenz(α)anthracene (DMBA; Sigma) was dissolved in dimethyl sulfoxide (DMSO; Sigma); the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA; Sigma) was also prepared in DMSO. The final concentration of TPA for the cell culture studies was 100 nmol/L except for the soft agar assay, which was 5 nmol/L. Malate, pyruvate, and succinate (Sigma) were dissolved in PBS (pH 7.4) at the concentration of 15, 30, and 30 mmol/L, respectively, and sterilized by passing through a 0.22-μm filter. Malate + pyruvate or succinate alone was diluted in the ratio of 1:400 for all of the experiments. Succinate or malate plus pyruvate are the substrates often used to measure mitochondrial respiration. Malate alone is unable to drive respiration; it needs to be combined with pyruvate. The concentrations of the mitochondrial substrates match those for the mitochondrial respiration assay (30). All other chemicals were purchased from Sigma.

Animal treatments

Twelve C57BL/6, 6 DBA/2 female mice (6- to 8-week old, purchased from the Jackson Laboratory), 6 manganese superoxide dismutase (MnSOD) transgenic (31), and 6 nontransgenic littermates (both in C57BL/6 background) were housed in the LSU Health Sciences Center–Shreveport (LSUHSC-S) Animal Resource Facility under standard regulations. The LSUHSC-S Animal Facility is AAALAC approved and maintains a consultation team of 2 veterinarians. The program is also monitored by the NIH Office for Protection from Research Risk and the U.S. Department of Agriculture. All animals were provided food and water *ad libitum*. Animals were euthanized under general anesthesia (overdose of pentobarbital) at the termination of the study. This method is consistent with the recommendation by the Panel on Euthanasia of the American Veterinary Medical Association.

Mice in each strain were separated into the control and DMBA/TPA-treated groups ($n = 3$ per group), and hair on the back was shaved. Two days later, for the DMBA/TPA group, a single dose of 100 nmol/L DMBA was painted on the backs of mice. After 2 weeks, 4 μg of TPA was applied to the same area for 24 hours. The control group received DMSO treatment at each time. Mice were then euthanized, skin tissues were removed, and skin epidermal cells were collected as we described previously (32). The skin tissues from 1 group of the C57BL/6 mice were used for oxygen consumption assays, and the remaining groups were used for the biochemical assays.

Measurements of oxygen consumption of JB6 cells and skin cells stripped from mouse skin tissues

JB6 P+ cells (2×10^6 /mL) resuspended in growth medium or stripped skin cells resuspended in mitochondrial isolation buffer (0.225 mol/L mannitol, 0.075 mol/L sucrose, 1 mmol/L EGTA, pH 7.4) were suspended in a thermostated closed vessel at 37°C. Oxygen consumption was measured polarographically using a Clark-type O₂ electrode (Yellow Spring Instruments). The rate of mitochondrial O₂ consumption was determined as the cyanide-sensitive rate after the addition of antimycin A to the final concentration of 1 μmol/L (33). The percentages of the oxygen consumption of each treatment versus that of the vehicle control were collected from each experiment, and the data from at least 3 experiments were combined and plotted.

PKM2 siRNA transfection

Cells were seeded at 2×10^5 cells per well in 6-well tissue culture plates. The cells were incubated at 37°C in a 5% CO₂ incubator until they became 70% to 80% confluent. For each transfection, 2 μL of the PKM2 siRNA duplex (sc-62821; Santa Cruz Biotechnology) was diluted into 100 μL of siRNA transfection medium (sc-36868; Santa Cruz Biotechnology) and labeled as solution A. Solution B consisted of 2 μL of transfection reagent (sc-29528; Santa Cruz Biotechnology) diluted into 100 μL of siRNA transfection medium. Solutions A and B were mixed gently and incubated for 30 minutes at room temperature. The cells were washed once with 2 mL of siRNA transfection medium. For each transfection, 0.8 mL of siRNA transfection medium was added to each tube containing the solution A/B mixture, mixed, and directly added to the washed cells. Cells were incubated for 5 to 7 hours at 37°C in a 5% CO₂ incubator. Immediately, 2 mL of 1× normal growth medium was added to the cells containing the transfection mixture. The cells were incubated for an additional 24 hours and assayed via Western blot analysis. Fluorescein-conjugated control siRNA (sc-36869; Santa Cruz Biotechnology) was used to monitor transfection efficiency.

Preparation of whole-cell lysate

Collected skin cells were suspended in 250 μL of PBS containing a proteinase inhibitor cocktail (Calbiochem). Cells were sonicated on ice for 2 strokes (10 s per stroke) with a Fisher Sonic dismembrator (Model 100, Scale 4). After incubating on ice for 30 minutes, cell lysates was centrifuged at $18,000 \times g$ for 20 minutes, and the supernatant was collected and designated as whole-cell lysate.

Western blot analysis

Antibodies against PKM2 (ab38237) and PKM (ab6191) were purchased from Abcam. An antibody against PKM1 (AP7476b) was purchased from Abgent. Antibodies against glyceraldehyde 3-phosphate dehydrogenase (GAPDH; sc-32233) and succinate dehydrogenase complex subunit B (SDHB; sc-25851) were purchased from Santa Cruz Biotechnology.

PKM activity assay

PKM activities were analyzed using the lactate dehydrogenase (LDH)-coupled assay as described previously (34). The standard assay mixture contains in a final volume of 400 μ L: 10 mmol/L Tris-acetate, pH 7.5; 10 mmol/L $MgCl_2$; 50 mmol/L KCl; 2 mmol/L ADP; 10 mmol/L phosphoenolpyruvate; 4.4 units of LDH; and 0.12 mmol/L NADH. The reaction was started by adding 5 μ L of whole-cell lysate and 4 μ L of 5'-AMP (1 mmol/L), and the decrease in absorbance at 340 nm (NADH) was recorded for 1 minute. 5'-AMP brings PKM2 to its maximal velocity. The baseline was measured without the addition of phosphoenolpyruvate and 5'-AMP. For measuring PKM1 activity, 5 μ L of whole-cell lysate and 4 μ L of fructose 1,6-bisphosphate (FBP, 1mmol/L) were added to the assay mixture, and the decrease in absorbance at 340 nm (NADH) was recorded for 1 minute. FBP brings PKM1 to its peak velocity.

Determination of pyruvate, malate, and succinate by high-performance liquid chromatography

The Shimadzu high-performance liquid chromatography (HPLC) system consisted of a binary, high-pressure gradient solvent delivery pump (model LC 20AB), an autosampler equipped with a cooling sample device (model SIL-20AC HT), a UV-visible absorbance detector (model SPD-20A), and data processing software (LCsolution Version 1.23). The tray compartment containing sample vials was cooled at 4°C. Separation of pyruvic acid, malic acid, and succinic acid was carried out with a XDB-C18 column (4.6 $\times$ 250 mm, 80 Å) and a Guard column (4.6 $\times$ 12.5 mm, 80 Å). The mobile phase was 10 mmol/L phosphate buffer (pH 2.4, adjusted with phosphoric acid) containing 4% methanol (0.0–15.0 minutes), followed sequentially by a linear gradient of 4% to 80% methanol (15.0–16.0 minutes), 80% methanol (16.0–17.0 minutes), a linear gradient of 80% to 4% methanol (17.0–18.0 minutes), and 4% methanol (18.0–20.0 minutes). A constant flow rate of 0.5 mL/min was applied. The detection was monitored at 215-nm wavelength, and the run time was 20 minutes. Whole-cell lysate was prepared from the stripped skin cells isolated from the C57BL/6 mice. It was then adjusted to 0.5 μ g/ μ L in the same volume and passed through 10-kD molecular weight cutoff spin columns. The elutes were collected for HPLC [retention time: pyruvate (6.176 minutes); malate (6.759 minutes); and succinate (14.055 minutes)].

Anchorage-independent growth assay in soft agar

Soft agar-based cell transformation assay was carried out in 6-well plates. The bottom agar was made using 1.25% agar, 2 $\times$ EMEM medium, 10% FBS, PBS, glutamine, and penicillin and was incubated in a 50°C water bath for 15 minutes. The mixture was then divided and treated with various treatments. In each well, 3.5 mL of the agar mix was added and allowed to harden for 30 minutes. The top agar mix contained 0.33% agar and 2×10^5 cells. The cell treatments were added at 2 $\times$ concentration to the top agar and equally mixed with 2×10^5 cells resulting in a final concentration of 0.33% agar for the top layer. Each well received 1 mL of the top agar mix containing cells. The agar was allowed to solidify and incubated in a 37°C incubator under 5% CO₂ for 7 days and stained with Neutral Red for 24 hours.

Statistical analysis

All of the studies have been repeated at least 3 times. Student's *t* test was used for two-group comparison, and one-way ANOVA followed by the Newman–Keuls posttest was used for multigroup comparisons. Data were reported as mean $\pm$ SEM. $P < 0.05$ was considered significant.

Results

Mitochondrial respiration substrates suppressed transformation of JB6 P+ cells induced by the tumor promoter TPA

To determine how enhancing mitochondrial respiration effects skin cell transformation, we used 2 sets of respiration substrates, malate/pyruvate and succinate, both of which are often used to drive mitochondrial respiration. JB6 P+ cells were utilized because of their ability to transform upon treatment with the tumor promoter TPA. We treated JB6 P+ cells with malate/pyruvate or succinate, both at a 1:400 dilution. Concurrently, the cells were treated with 5 nmol/L TPA and utilized in the soft agar assay. As shown in Figure 1A, we observed a significant decrease in the number of colonies in the presence of malate/pyruvate after TPA treatment, which complementarily functions to transport pyruvate back to the mitochondria, whereas the number of colonies in the DMSO and malate/pyruvate controls stayed at lower levels. The addition of this mitochondrial substrate thus showed its ability to interfere with tumor promotion. Succinate, the resulting product of the conversion of succinyl coenzyme A (succinyl-CoA) by succinyl-CoA synthetase, produced similar results as that seen with malate/pyruvate, and the number of transformed colonies also declined (Fig. 1B). Thus, it seems that enhancing mitochondrial respiration can prevent the transformation of JB6 P+ cells.

PKM2 was upregulated upon TPA treatment, which was suppressed by mitochondrial respiration substrates

Notably, the aforementioned experiment shows the effect of increased respiration substrates on tumorigenesis promoted by TPA. Herein, to examine the specific action of how these respiration substrates regulate tumor promotion, we studied PKM2 expression and its activity in tumorigenesis in JB6 P+ cells. Figure 2 shows that TPA (24-hour treatment) induced only the expression of PKM2, not the whole PKM, in JB6 P+ cells. However, in the presence of the respiration substrates malate/pyruvate and succinate, the increased levels of PKM2 expression were reduced. These respiration substrates alone had no obvious effect on the expression of PKM2 or PKM. A similar trend was observed with the analysis of PKM2 activities using an LDH-coupled assay in the presence of respiration substrates. We detected a reduction in the levels of PKM2 activity when malate/pyruvate was present in conjunction with TPA, as compared with the level of activity of PKM2 upon treatment with TPA alone (Fig. 2B, left). Similarly, the increases in PKM2 activity upon TPA treatment were suppressed in the presence of succinate (Fig. 2B, right). To reveal the effects of these treatments on mitochondrial respiration, the level of oxygen consumption of intact cells after each treatment was measured. As shown in Figure 2C, TPA decreased mitochondrial respiration by approximately 30%, and both sets of respiration substrates suppressed this

decrease. These novel results show that in the presence of respiration substrates, PKM2 protein expression and activity are repressed.

PKM2 was upregulated in mouse skin epidermal tissues during the early stage of skin carcinogenesis

To complement the aforementioned experiment and further delineate the role of PKM2 in early carcinogenesis, we treated mouse skin with DMBA and TPA and isolated skin cells. Figure 3A shows that in C57BL/6 mice, the expression levels of PKM2 were increased whereas that of PKM1 were decreased in skin epidermal tissues treated with DMBA/TPA compared with the vehicle control and a constant expression of the PKM protein (Fig. 3A, left). The changes in the expression levels of PKM2 and PKM1 were correlated with the changes in their enzymatic activities (Fig. 3A, right). This outcome of increased PKM2 activity/decreased PKM1 activity displays the possibility of an important role of the PKM1/PKM2 switch in early carcinogenesis. We have repeated the studies using DBA/2 mice, which are relatively sensitive to skin carcinogenesis in comparison with C57BL/6 mice. As shown in Figure 3B, a similar magnitude of this metabolic shift was observed.

To detect whether mitochondrial respiration is also affected, oxygen consumption of the intact skin cells was measured. Indeed, as shown in Figure 3C (left), the levels of mitochondrial respiration were reduced by approximately 30% in the DMBA/TPA-treated skin cells. The levels of the respiration substrates in the skin tissue homogenate were then determined using the reverse-phase HPLC method. As shown in Figure 3C (right), the concentrations of pyruvate, malate, and succinate remained at similar levels after DMBA/TPA treatment.

PKM2 was upregulated only in promotable JB6 P+ cells, not in nonpromotable JB6 P- cells, upon TPA treatment

After examining the changes of PKM2 in tumor promotion in TPA-induced early carcinogenesis models, we wanted to ensure that our results were consistent only with cell transformation and not present in nonpromotable skin cells. As shown in Figure 4A, the expression levels of PKM2 at 1 and 24 hours post-TPA treatment were compared between promotable JB6 P+ and nonpromotable JB6 P- cells. Both promotable (P+) and nonpromotable (P-) cells displayed low levels of PKM2 expression at the 1-hour time point. However, the expression in the promotable cells was induced after 24 hours substantially whereas expression stayed low and constant in the nonpromotable P- cells. In comparing the relative activity of PKM2 in each cell type, a significant increase in PKM2 activity was revealed only in the promotable line, consistent with the previous results. These results show that PKM2 function is important, distinctively for TPA-induced skin cell transformation.

Knockdown of PKM2 suppressed TPA-induced skin cell transformation

Given the strong correlation between PKM2- and TPA-induced skin cell transformation, we further examined whether the increase of PKM2 was necessary for cellular transformation. JB6 P+ cells were transfected with siRNA to PKM2 and whole-cell lysate was prepared. Figure 5A shows the effectiveness of the siRNA transfection. Subsequently following the observation of successful knockdown, TPA-induced skin cell transformation was measured

using the soft agar assay with PKM2 knockdown cells (Fig. 5B). Our results showed a significant decrease in transformed colonies among PKM2 knockdown cells, similar to the effect of the respiration substrates, malate/pyruvate, and succinate. These results reiterate our previous experimental conclusions and strengthen the evidence that PKM2 is important in early carcinogenesis.

PKM2 activation was suppressed by overexpression of MnSOD

Our previous studies have shown that MnSOD is highly expressed and oxidative stress remains at lower levels in nonpromotable JB6 P⁻ cells than in promotable P⁺ cells (35). To study whether oxidative stress generated by tumor promoters can activate PKM2, MnSOD transgenic mice and their nontransgenic littermates were treated with DMBA and TPA and the mouse skin epidermal tissues were collected. As shown in Figure 6, DMBA/TPA induced both the expression and activity of PKM2 in the nontransgenic mice. These increases were clearly reduced in the MnSOD transgenic mice, suggesting that antioxidant enzymes, especially MnSOD, can modulate metabolism via inhibiting PKM2 activation.

Discussion

As previously observed in the Warburg effect, cancer cells develop an appetite for glucose as their source of energy production and therefore mitochondrial respiration is often compromised. In the case of skin tumorigenesis, previous studies revealed decreased mitochondrial complex activities and membrane potential during early skin tumorigenesis (36, 37). In addition, uncoupling mitochondrial electron transportation from ATP synthesis has been suggested to function as a tumor-promoting event (37). However, the cause of cancer metabolism is not clear until the recent discoveries of "oncometabolic enzymes" (38).

The identification of PKM2, the alternative isozyme of PKM, that is reliably present in common cancers suggests that the transition to decreased dependence of cells on oxidative phosphorylation may contribute to early carcinogenesis as the loss of tissue-specific PKM1 and PK-L isozymes transitions to the expression of PKM2 (39). The PKM1/M2 isoforms are generated through alternative splicing, which is controlled by the hnRNP family members. Overexpression of the 3 hnRNP proteins has been observed in cancer cells that are mediated by proliferation-associated transcription factors, such as c-Myc (10). In addition, redox signaling may also play a role in regulating PKM2 activation. As shown in Figure 4, PKM2 is activated only in promotable JB6 P⁺ cells and not in nonpromotable P⁻ cells. Our previous studies have shown that MnSOD is higher and TPA-induced oxidative stress is lower in P⁻ cells than in P⁺ cells (35).

Furthermore, delineating this inherent ability of cancer formation, the replacement of PKM2 with PKM1 reduces the capacity of human tumor cells lines to develop into a tumor, allowing for the notion that by a change in the splicing of PK, tumorigenesis can be induced through shifting cellular metabolism to aerobic glycolysis (40). Even though PKM2-expressing cells rely on the less efficient glycolytic generation of ATP, ATP levels in PKM1- and PKM2-expressing cells are the same (41), either resulting from alternative fuel sources from lipids or amino acids or escalating the rate at which glycolysis proceeds may explain the ability of PKM2-expressing cells to produce sufficient ATP levels (42, 43). Nevertheless,

the advantages observed in PKM2-expressing tumors and the selective pressure for PKM2 expression in tumors for a propensity to grow may be because of better sustainability under hypoxic conditions resulting from a decreased need for oxidative metabolism. Therefore, aerobic glycolysis resulting from PKM2 mediation may waste glucose consumption but could provide many advantages for cancer cells.

To mediate isoform M2, it needs to be allosterically activated by α -FBP, which ultimately regulates PKM2 tetrameric-dimeric oscillation (19, 34). The dimeric form of PKM2 leads to a decrease in the ATP/ADP ratio and an upregulation of FBP levels (26). On the other hand, the activity of PKM1 is inhibited by ATP but not by FBP (44). These alterations and mediation mechanisms of PKM2 bring to notice as to how the tumor PKM2 increases in cells that produce more lactate and consume less oxygen than cells expressing PKM1, which ultimately is advantageous for rapid growth in cancer cells (45). Other metabolic sources apart from FBP, such as serine, can activate PKM2 whereas alanine, proline, and fatty acids inhibit PKM2 (46–48). Our current study suggests that mitochondrial respiration substrates malate/pyruvate and succinate may also confer PKM2 inhibition and, in turn, reduce TPA-induced skin cell transformation. The identification of various mediators of PKM2, and the conclusion that its expression and activity have a significant effect on tumorigenesis, opens up the possibility to exploit PKM2 as a target for cancer detection, prevention, and control. Although tumor PKM2 is not an organ-specific marker, it is a strong marker of metastasis (16). Tumor PKM2 is released into the blood and stool of tumor patients and thus could be utilized for detection, as it plays a role in early carcinogenesis as we have shown earlier (9). Research has already begun to make progress in developing small-molecule inhibitors of PKM2 (49).

In summary, our studies revealed a number of interesting findings. First, the results help to define at what stage of cancer development this PKM1 to PKM2 shift occurs. As observed in mouse skin tissues (Fig. 3), PKM2 is upregulated and PKM1 is downregulated within 24 hours after the first tumor promoter TPA treatment, suggesting that this metabolic shift is an early event during skin carcinogenesis. Second, this shift is accompanied by a decrease in mitochondrial respiration, indicating that downregulation of mitochondrial respiration occurs early in carcinogenesis. This decrease is confirmed by our previous studies that tumor promoters cause decreases in mitochondrial membrane potential and complex activities. Thus, by disrupting early activation of PKM2, the results we have obtained, along with the extensive work of others, may provide help in developing diagnostic, preventative, and anticancer therapies.

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Figure 1.

Mitochondrial respiration substrates suppressed TPA-induced skin cell transformation. JB6 P+ (2×10^5) cells were suspended in soft agar, which was supplemented with the vehicle control (DMSO), tumor promoter TPA (5 nmol/L), mitochondrial respiration substrates, or the combination of the 2 and incubated for 7 days. The numbers of colonies were counted ($n = 3$ per treatment group, 3 separate experiments were carried out, and a representative result was shown). A, malate and pyruvate were used as the mitochondrial respiration substrate. M/P, malate plus pyruvate (37.5 and 75 $\mu\text{mol/L}$, respectively). B, succinate was used as the mitochondrial respiration substrate. S, succinate (75 $\mu\text{mol/L}$). *, $P < 0.05$ compared with the DMSO group; #, $P < 0.05$ compared with the TPA group.

Figure 2.

Mitochondrial respiration substrates suppressed TPA-induced PKM2 activation. JB6 P+ cells were pretreated with malate plus pyruvate (37.5 and 75 $\mu\text{mol/L}$, respectively) or succinate (75 $\mu\text{mol/L}$) for 30 minutes followed by TPA treatment (100 nmol/L) for 24 hours. Whole-cell lysate was prepared and used for Western blot analysis (A; GAPDH served as the loading control) and PKM2 activity assays (B). The levels of oxygen consumption were measured as an indicator of mitochondrial respiration (C). M/P, malate plus pyruvate; S,

succinate; control, DMSO (0.01%). *, $P < 0.05$ compared with the DMSO group; #, $P < 0.05$ compared with the TPA group.

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Figure 3.

PKM2 was also activated during early skin carcinogenesis. Mouse skin epidermal cells were collected from C57BL/6 (A) and DBA/2 (B) mice, and whole-cell lysate was used for Western blot analysis and PKM2 activity assay. In a separate study, skin cells were stripped after DMBA/TPA or vehicle treatment and the levels of mitochondrial consumption were measured C, whole-cell lysate was prepared, and the concentrations of pyruvate, malate, and succinate were determined by reverse-phase HPLC. Ctrl, control (DMSO) treatment group. *, $P < 0.05$ compared with the control group.

Figure 4.

PKM2 was activated only in promotable JB6 P+ cells. Both JB6 P+ and P- cells were treated with TPA (100 nmol/L) for 1 or 24 hours, and whole-cell lysate was prepared for the experiments. A, Western blot analysis of PKM2. B, PKM2 activity assays. Ctrl, control (0.01% DMSO for 24 hours). *, $P < 0.05$ compared with its own control group.

Figure 5.

Knockdown of PKM2 suppressed TPA-induced skin cell transformation. JB6 P+ cells were used for the experiments. A, the expression levels of PKM2 were detected in siRNA-transfected cells by Western blot analysis. SDHB served as the loading control. B, soft agar colony formation assay was conducted using siRNA-transfected cells. Ctrl/D, control siRNA plus DMSO treatment; PKM2/D, PKM2 siRNA plus DMSO treatment; Ctrl/T, control siRNA plus TPA (5 nmol/L) treatment; PKM2/T, PKM2 siRNA plus TPA treatment. *, $P < 0.05$ compared with the control siRNA/DMSO group; #, $P < 0.05$ compared with the control siRNA/TPA group.

Figure 6.

PKM2 activation was suppressed by overexpression of MnSOD during early skin carcinogenesis. Mouse skin epidermal cells were collected and whole-cell lysate was used for Western blot analysis (A) and PKM2 activity assay (B). nTg, skin epidermal samples from nontransgenic (wild-type) mice; TgH, skin epidermal samples from MnSOD transgenic mice; Ctrl, control (DMSO) treatment group. *, $P < 0.05$ compared with the control group.