ERK2-Pyruvate Kinase Axis Permits Phorbol 12-Myristate 13-Acetate-induced Megakaryocyte Differentiation in K562 Cells*

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**Background:** Pyruvate kinase plays a crucial role in tumor cell proliferation, however, its role in differentiation is relatively unelucidated.

**Results:** PKM2 and PKR dependent metabolic switch toward energy production and nuclear translocation of PKM2 was observed during megakaryocyte differentiation.

**Conclusion:** ERK2 controlled status of PK isoforms is essential for megakaryocytic differentiation.

**Significance:** Metabolic shift impact the process of differentiation.

Metabolic changes that contribute to differentiation are not well understood. Overwhelming evidence shows the critical role of glycolytic enzyme pyruvate kinase (PK) in directing metabolism of proliferating cells. However, its role in metabolism of differentiating cells is unclear. Here we studied the role of PK in phorbol 12-myristate 13-acetate (PMA)-induced megakaryocytic differentiation in human leukemia K562 cells. We observed that PMA treatment decreased cancer-type anabolic metabolism but increased ATP production, along with up-regulated expression of two PK isoforms (PKM2 and PKR) in an ERK2-dependent manner. Interestingly, silencing of PK (PKM2 and PKR) inhibited PMA-induced megakaryocytic differentiation, as revealed by decreased expression of megakaryocytic differentiation marker CD61 and cell cycle behavior. Further, PMA-induced ATP production reduced greatly upon PK silencing, suggesting that PK is required for ATP synthesis. In addition to metabolic effects, PMA treatment also translocated PKM2, but not PKR, into nucleus. ERK1/2 knockdowns independently and together suggested the role of ERK2 in the up-regulation of both the isoforms of PK, proposing a role of ERK2-PK isoform axis in differentiation. Collectively, our findings unravel ERK2 guided PK-dependent metabolic changes during PMA induction, which are important in megakaryocytic differentiation.

Cellular differentiation is a process of formation of specialized cell types. During this process, cells acquire a specific function, morphology, and metabolic behavior. The changes in energy metabolism during differentiation are critical to the cell functionality (1). Studies have revealed that proliferating cells have anabolic metabolism (macromolecule biosynthesis), whereas differentiated cells prefer catabolic metabolism (2). To study these features experimentally, an in vitro model of K562 multipotent hematopoietic myeloid precursor cells was used, where PMA3 (3) exposure initiated myeloid (megakaryocytic) differentiation (4–7), accompanied by several changes (4, 8, 9), a complete understanding of which between dividing and differentiated cells has been a challenge. Although changes in expression and isoform composition of glycolytic enzyme like PK have been implicated in erythroid differentiation (10–12) and the coexistence of PKM2 and PKR has been identified during myeloid lineage commitment, the role of each of these isoforms in the process of differentiation remains elusive.

PK is an important metabolic regulatory enzyme in glycolysis pathway. Depending upon the metabolic requirement, it is expressed in four isoforms PKM1, PKM2, PKL, and PKR (13–15). PKM2 isoform expresses in all dividing cells (16) and switches between a highly active tetrameric and relatively inactive dimeric form (17). Decreased activity of the PKM2 dimer promotes the shunting of upstream glycolytic intermediates into biosynthetic pathway (18), whereas the active tetrameric PKM2 form supports glycolysis-driven energy production (19). The dimerization-tetramerization oscillation of PKM2 is a reversible process in normally dividing cells, which is regulated by the intracellular fructose 1,6-bisphosphate concentrations (20, 21). Various studies based on post-translational modifications like tyrosine 105 phosphorylation (22), xenograft tumor model studies (23), and physical interaction of some proteins, such as Oct4 (24), PML (25), A-Raf (3), and pathogenic E7 (26, 27) etc., have confirmed that reduced PKM2 activity promotes tumor progression (28). PKM2 dimer has also been demon-

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3 The abbreviations used are: PMA, phorbol 12-myristate 13-acetate; PK, pyruvate kinase; p-, phosphorylated form.
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strated to act as a protein kinase and regulate gene transcription to affect important cellular functions (29). Diverse glycolytic and nonglycolytic functions of PKM2 make it a multifunctional molecule (29–31) with an important link between metabolic transformation and gene expression. Other isoforms of human PK: PKM1 present in skeletal muscle, heart, and brain (32); PKL in hepatocytes, kidney, small intestine, and pancreatic beta cells; and PKR in erythrocytes (33, 34) exist in inactive monomer and active tetramer form, catalyzing glucose in oxidative glycolysis and produce large amounts of energy (ATP) consumed by cells for cellular homeostasis and specialized functions. PKM2 is the only isoform known to date for both canonical and noncanonical cellular functions related to tumor development and cancer cell proliferation. However, its role along with other PK isoforms is not clearly stated in cellular differentiation. Our study explains how during differentiation, coexistence of both PKM2 and PKR isoforms is a metabolic requirement of cells, regulated by ERK1/2 pathway, during PMA-induced megakaryocytic differentiation.

Experimental Procedures

Reagents and Antibodies—PMA was procured from Sigma. U0126, SB203580 were procured from Cell Signaling Technology (Beverly, MA). PMA and U0126 were dissolved in DMSO, and aliquots of stock solutions stored at −20 °C. For metabolic assays, a glucose assay kit was purchased from Biovision. The K562 cell line was obtained from National Centre for Cell Sciences (Pune, India) and maintained in RPMI 1640 medium containing 0.1% DMSO as described (37). Glycerol gradient analysis was done as described previously (38). Briefly, 500 g of cells lysate protein was loaded on the top of an 11–25% glycerol gradient and centrifuged at 50,000 rpm for 16–18 h at 4 °C. Fractions of 75 μl each were taken from the gradient and assayed for PK activity.

Cell Culture and in Vitro Differentiation—Human chronic myeloid leukemia blast crisis K562 cell line was maintained in RPMI 1640. To induce differentiation, exponentially growing K562 cells were seeded at a density of 5 × 10^5 per ml and treated with PMA at 20 ng/ml concentration. The untreated cells were cultured in RPMI 1640 medium containing 0.1% DMSO as mock control. For MAPK signaling pathway studies, cells were pretreated with U0126 and stimulated with PMA.

Western Blotting—Protein lysate was prepared using radioimmune precipitation assay buffer, and proteins were separated on an 8% SDS-PAGE and then transferred to nitrocellulose membrane (ndi, Membrane Technologies). The membrane was blocked with 5% BSA for 1 h and incubated with required primary antibodies overnight at 4°C, followed by an appropriate secondary antibody for 1 h at room temperature. Protein bands were detected using Luminata forte (Millipore).

Flow Cytometry, Cell Surface Markers, and Cell Cycle Analysis—Experimental cells were harvested, washed twice with PBS, and resuspended in 95 μl of PBS, followed by addition of 5 μl of anti-human phycoerythrin-conjugated CD61 monoclonal antibody to the cell suspension and incubation for 30 min at room temperature. Thereafter, cells were washed three times with PBS, and at least 1 × 10^4 cells were used for flow cytometry analysis (BD Biosciences).

For cell cycle analysis, the DNA content was quantified by propidium iodide staining. Cells (1 × 10^6) were collected, centrifuged (400 × g), and washed twice with PBS. Cell pellet was resuspended in 0.5 ml of PBS, followed by dropwise addition of cell suspension to 2 ml of prechilled 75% ethanol and incubated in ice for 60 min. Cells were then centrifuged and washed with PBS and resuspended in 0.3 ml of PBS containing 100 mg/ml of RNase A and 50 mg/ml propidium iodide. After 20 min of room temperature incubation, the cells were analyzed by flow cytometry. The data were offline analyzed using ModFit LT 4.0 (Verity Software House).

PK Isoforms, ERK1/2 Knockdown by shRNA and Overexpression of PKM2—The K562 cells were infected with lentivirus containing control shRNA (pLKO.1 vector) and pLKO.1-shRNA-PKM2 (kind gift from Dr. Marta Cortés-Cros, NIBR, Basel, Switzerland) and PKR vector to knock down endogenous PKM2 and PKR isoforms, respectively (35). Similarly, pLKO.1-shRNA-ERK1and ERK2 (a kind gift from Dr. John Blenis, Harvard Medical School, Boston, MA) (36) was used to knock down endogenous ERK1 and ERK2. For overexpression of PKM2, cells were transfected with vector (pcDNA) alone or with c-Myc-tagged PKM2. Transfections were confirmed by immunoblotting.

PK Activity and Glycerol Gradient Assay—PK activity was measured spectrophotometrically (UV-1800; Shimadzu, Kyoto, Japan), using the NADH/lactate dehydrogenase-coupled assay as described (37). Glycerol gradient analysis was done as described previously (38). Briefly, 500 μg of cells lysate protein was loaded on the top of an 11–25% glycerol gradient and centrifuged at 50,000 rpm for 16–18 h at 4 °C. Fractions of 75 μl each were taken from the gradient and assayed for PK activity.

Cell Metabolism Analysis—The metabolic properties were studied by measuring lactate production, glucose uptake, NADPH synthesis, and ATP production. For lactate and glucose, cell culture media was collected and centrifuged at maximum speed to remove any cell debris, deproteinized using TCA, the pH was adjusted to between 7.0 and 7.4, and then glucose and lactate were analyzed using kits as per manufacturer’s instructions. NADPH and ATP were analyzed using kits (BioVision) as per the manufacturers’ specifications. All the measurements were normalized to the protein content of the cells.

Statistical Analysis—Statistical significance was calculated using one-way analysis of variance. p values of less than 0.05 were considered statistically significant.

Results

K562 cells were used as an in vitro model of multipotent hematopoietic precursors to study the molecular mechanisms associated with erythro/megakaryocyte differentiation process.
These cells are known to undergo megakaryocytic or erythroid lineage differentiation, depending upon the nature of chemical stimulation (39, 40).

**Features Associated with PMA-induced Megakaryocytic Differentiation in K562 Cells**

*General Features*—K562 cells treated with PMA (20 ng/ml) showed morphological changes and megakaryocytic differentiation, characterized by the expression of a known cell surface marker, CD61 (Fig. 1, A–C). Expression intensity of CD61 increased with treatment time, showing a significant ($p<0.001$) increase after 16 h, with maximum at 48 h (Fig. 1, B and C), thus confirming the induction of *in vitro* differentiation of megakaryocytes.

The increased appearance of cell surface marker CD61 after 16 h, accounting for the megakaryocytic differentiation, coincided with the presence of one-third of the total population of cells in tetraploidy at the same time (Table 1), apart from inhibited proliferation and an arrest of a large population of diploid cells in $G_1$ phase with a concomitant decline of S phase cells from 16 to 48 h (Fig. 1D).

*Metabolic Features*—PMA induction altered the biosynthetic metabolism in dividing K562 cells, supported by high glucose uptake and lactate production (aerobic glycolysis) along with NADPH synthesis (41). Glucose consumption and lactate production in the conditioned media, ATP and NADPH levels from the cellular extracts of PMA-treated K562 cells when measured showed a decreasing trend in glucose consumption, lactate production and intracellular NADPH levels, beginning at 8 h followed by a significant drop at 24–48 h (Fig. 2, A–C). In contrast, the cellular ATP levels increased remarkably between 24–48 h (Fig. 2D), demonstrating a shift in metabolism from biosynthetic to energy producing during PMA-induced megakaryocytic differentiation, suggesting the importance of metabolic changes from aerobic to oxidative glycolysis in PMA-induced differentiating K562 cells.
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TABLE 1
Percent changes in cell cycle phases and ploidy status at different time points after PMA treatment of K562 cells

|        | Diploid |                  |        | Tetraploid |                  |
|--------|---------|------------------|--------|------------|------------------|
|        | Samples | Ploidy | G<sub>1</sub> phase | G<sub>2</sub> phase | S phase | Ploidy | G<sub>1</sub> phase | G<sub>2</sub> phase | S phase |
|        | %       | %     | %                 | %                 |        | %       | %                 | %                 |        |
| 0 h    | 100     | 34.70 | 6.66              | 58.64              |        | 0.000  | 0.000             | 0.000             | 0.000  |
| 8 h    | 81.24   | 15.94 | 8.00              | 76.06              |        | 18.76  | 100               | 0.000             | 0.000  |
| 16 h   | 70.63   | 59.67 | 8.00              | 32.33              |        | 29.37  | 100               | 0.000             | 0.000  |
| 24 h   | 80.08   | 65.72 | 8.00              | 26.28              |        | 19.18  | 100               | 0.000             | 0.000  |
| 48 h   | 80.26   | 61.47 | 8.00              | 30.53              |        | 20.09  | 100               | 0.000             | 0.000  |

FIGURE 2. Metabolic behavior of PMA-treated and untreated K562 cells at 0 – 48 h. High glucose uptake, lactate production, and anabolic synthesis are hallmarks of cancer cells. To assess the metabolism, these parameters were measured in K562 cells. A, rate of glucose uptake by K562 cells at different time points. B, lactate production rate. C, NADPH is produced in pentose phosphate pathway, and it provides reducing power for biosynthesis. NADPH synthesis is linked with anabolism in cancer cells. Intracellular NADPH was measured in the extract of 5 million cells, using a kit. D, ATP production is a measure of energy metabolism. As described under “Experimental Procedures,” intracellular ATP was measured in the extract of 1 million cells, using a kit.

PK Expression and Activity Changes, Preceded by p-ERK1/2 Increase, upon PMA-induced Megakaryocyte Differentiation in K562 Cells

To unravel further the PMA-induced metabolic changes, PK status and isoform compositions were investigated. Immunoblots of the cellular extracts of PMA-treated K562 cells at different time points between 0 and 48 h showed that the expression of PKM2 isoform increased from 8 to 48 h, whereas other isoform, PKR, appeared by 8 h and increased in a time-dependent manner up to 48 h (Fig. 3A). Presence of PKM1, incidentally, was not detected in K562 cells (Fig. 3A).

Assessing the status of MAPK signaling molecules showed an increase in the phosphorylated form of ERK1/2 (p-ERK1/2) between 15 min and 16 h after PMA treatment, followed by its relative decrease at 24 and 48 h (Fig. 3A). The expression of phosphorylated p38 (p-p38) remained more or less similar up
to 16 h, the point of initiation of megakaryocytic differentiation, and declined thereafter (Fig. 3A). These events after PMA exposure coincided with CD61 expression, which showed a significant increase after 16 h (Fig. 1, B and C), the appearance of which was concomitant to the process of megakaryocytic differentiation.

The same cell lysate when used in parallel to investigate the PK enzyme activity, showed an increased enzymatic activity at 8 h, which was significantly high at 24 and 48 h (Fig. 3B). To analyze the isoforms and their oligomeric status of PK during PMA-induced megakaryocytic differentiation, glycerol density gradient centrifugation was performed to separate different oligomeric forms of the two isoforms of PK. The activity measured in fractions collected from top to bottom of the glycerol gradient showed two peaks that represented two oligomeric forms of PK (Fig. 3, C and D). The vertical shift in peak I (fractions 18–20) indicated an increase in dimer form, whereas the same shift in peak II (fractions 21–23) depicted a tetramer, in PMA-treated K562 cells when compared with control (no PMA) peaks (Fig. 3C). Subsequent Western blots of these fractions, using anti-PKM2 and anti-PKLR antibodies, confirmed the presence of dimeric and tetrameric PKM2 and only tetrameric PKR. Also, the PMA treatment contributed to a preferential increase in dimeric PKM2 and PKR tetramer.

Knockdown of PK Impairs PMA-induced Megakaryocytic Differentiation

To investigate the importance of PK in the process of megakaryocytic differentiation, PK was silenced using constructs containing shPKM2, shPKLR, and shPKM2+shPKLR to knock down the expression of the two isoforms independently and together, along with control vector (pLKO) for comparison. PMA or mock treatment for 48 h followed by Western blotting confirmed the event of silencing of PK isoforms (Fig. 4A). Upon silencing of PKM2 or PKR or both, the PK activity decreased when compared with transfected control (pLKO). The same was true when PK silenced cells were treated with PMA and compared with the PMA-treated control (p<0.001) (Fig. 4B). These changes along with changes in the morphological profile (Fig. 4C), reduction in the total tetraploid population of cells at the same time (Fig. 4H and Table 2), apart from a significant reduction in CD61-positive cells, especially under double (PKM2+PKR) knockdown condition, validated the role of both the isoforms of PK in the process of megakaryocyte differentiation (Fig. 4, D–G).

PK Silencing Impairs the PMA-induced Metabolic Shift

Because PMA-induced megakaryocyte-differentiating K562 cells shifted metabolism toward energy production (Fig. 2), it was pertinent to study the effect of PK isoform silencing on ATP production to establish whether PK mediates the PMA-
induced energy production during megakaryocyte differentiation. The status of metabolism when assessed in absence of PMA for glucose uptake, lactate production, and NADPH formation showed a significant decrease when compared with vector control (without PMA). However, a comparison between PMA-treated PK silenced and vector transfected control cells showed no significant change (Fig. 5, A–C). Interestingly, PMA-treated PK silenced cells showed a sharp decrease in ATP production, when compared with PMA-treated vector control (p<0.001) (Fig. 5D). The results of unresponsiveness toward PMA in absence of the PKM2 and PKR enzymes, independently or together, echoed the disturbed state of the cells and the changes in the metabolic adaptation with no shift toward oxidative glycolysis.

FIGURE 4. Knockdown of PK isoforms and consequent effects on PMA-induced differentiation. A, knockdown efficiency as observed in Western blot in the presence and absence of PMA treatment of 48 h. Vector is control shRNA (pLKO). B, changes in PK activity upon knockdown in presence and absence of PMA treatment of 48 h. C, morphological changes in vector and PK knockdown cells with or without PMA treatment. D–G, FACS analysis and bar diagram for changes in CD61 appearance on surface of K562 cells. H, cell cycle analysis showing changes in percent of cells in S and G1 phases and ploidy status (Table 2). Dip, diploid; MW, molecular mass.

ERK2 Pathway Regulates PK Status during Megakaryocyte Differentiation

To find out whether the increase in PK expression and activity during megakaryocyte differentiation process was related to the MAPK signaling pathway, in the initial phase standard pharmacological inhibitors, U0126 and SB203580, were used to inhibit MAPK pathways in K562 cells. SB203580 decreased p-ERK and p-P38 indicated efficient inhibition by U0126 and SB203580, respectively (Fig. 6, A and B). ERK signaling inhibition by U0126 down-regulated the expression and activity of both PK isoforms; however, p38 signaling inhibitor SB203580 did not show any effect on PK expression and activity. Therefore, this study suggests that the MAPK signaling pathway is involved in regulating PK expression and activity during megakaryocyte differentiation.
TABLE 2
Percent changes in cell cycle phases and ploidy status at after PMA treatment of PK isoform knockdown K562 cells

| Samples          | Diploid |                  |                  | Tetraploid |                  |                  |
|------------------|---------|------------------|------------------|-----------|------------------|------------------|
|                  | Ploidy  | G₁ phase ( % )   | G₂ phase ( % )   | Ploidy    | G₁ phase ( % )   | G₂ phase ( % )   |
| Vector           | 99.77   | 39.90            | 4.96             | 55.64     | 0.23             | 100              |
| Vector + PMA     | 74.30   | 70.60            | 8.00             | 21.40     | 25.70            | 14.0             |
| shPKM2           | 98.97   | 49.29            | 0.42             | 59.29     | 1.03             | 54.36            |
| shPKM2 + PMA     | 99.82   | 45.62            | 0.75             | 53.65     | 0.18             | 100              |
| shPKLR           | 99.17   | 47.86            | 3.55             | 48.59     | 0.43             | 14.62            |
| shPKLR + PMA     | 99.54   | 73.96            | 0.09             | 25.95     | 0.45             | 00.00            |
| shPKM2 + LR      | 99.64   | 41.11            | 0.34             | 58.55     | 0.36             | 00.00            |
| shPKM2 + LR + PMA| 99.18   | 75.35            | 7.73             | 20.28     | 0.82             | 20.94            |

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FIGURE 5. PK silencing and its effect on metabolic behavior of K562 cells in presence and absence of PMA. A–C, bar graph showing glucose consumption, lactate production, and NADPH formation (see text). D, Intracellular ATP production measured in relative luminescence units (see text).
activity (Fig. 6, A and B). On assessing the expression of CD61 marker, cell cycle, PK expression, and activity in U0126 pretreated cells, CD61 reduced significantly (Fig. 6, D–F), in addition to other features, such as absence of polyploidy (Table 3). No such effect was noticed upon SB203580 treatment. Cell cycle analysis indicated that the inhibition of p38 MAPK pathway shifted the S phase population of cells to the G1 phase with the appearance of polyploidy (Fig. 6G and Table 3). These results showed that expression of CD61, PK expression, activity, and tetraploidy formation depended on ERK1/2 pathway; however, the cell cycle arrest at G1 phase appeared to be independent of ERK1/2 pathway, during in vitro megakaryocytic differentiation. Further, an overexpression of PKM2 in presence of the MAPK inhibitor, U0126, treated cells did not result in a significant increase in CD61-positive cells in response to PMA (Fig. 7, A–D). We suggest two reasons for this aborted adaptive metabolism and dysregulation of differentiation process: 1) in the presence of ERK1/2 inhibitor, there is decrease observed in the endogenous expression of PKR; and 2) it is possible that there is an absence of post-translational modification of ectopically overexpressing PKM2 in the background of non-availability of ERK (30). We further delineated the involvement of either ERK1 or ERK2 in regulating the expressions of PK isoforms and the megakaryocyte differentiation. The expression status of PK iso-
forms and the cellular differentiation marker CD61 was examined in the backgrounds of ERK1 and/or ERK2 knockdown conditions. We observed that the abolishment of only ERK2 disturbed the expression status of PK isoforms (PKR, PKM2), as well as their activity (Fig. 8, A and B), and down-regulated the expression of CD61 (Fig. 8, C and D).

**PMA Induces Nuclear Translocation of PKM2 Isoform through ERK Activation**

Several studies have reported noncanonical presence of PKM2 inside the nucleus (30, 43–45); therefore, expression of PKM2 and PKR isoforms in cytoplasmic and the nuclear fractions was analyzed. Immunoblots of nuclear fraction revealed the presence of PKM2, but not PKR, upon PMA induction of K562 cells (Fig. 9A). Inhibition of ERK by U0126 abrogated the nuclear translocation of PKM2, consistent with recent studies showing EGFR-stimulated ERK-dependent recruitment of PKM2 inside nucleus (29–31, 46), which has not been reported in PMA-induced megakaryocyte differentiation earlier. PK activity assay in cytoplasmic and nuclear fractions showed almost no enzymatic activity in the nuclear fraction, suggesting that translocated PKM2 is dimeric (Fig. 9B). These results sug-
suggest a possible noncanonical role of dimeric PKM2 in megakaryocytic differentiation.

Discussion

Metabolic adaptation is important in supporting the fundamental processes required for cellular homeostasis. Glycolysis, a vital pathway in central metabolism, provides metabolic flexibility by virtue of its intermediates that act as substrates for anabolic and catabolic processes. PK acts as a glycolytic valve by regulating the channeling of glucose carbons into biosynthetic or energy producing pathways (19, 20, 47). The role of PK in switching metabolism of proliferating cells toward anabolic synthesis is well known (48). However, the role of PK in metabolic changes during differentiation is not well studied. This study examined the status and role of PK in PMA-induced differentiation of K562 cells.

The metabolic changes upon PMA induction favored ATP production over biosynthesis, which correlated with S-G1 phase shift of K562 cells on PMA treatment (Figs. 1D and 2). We demonstrated that PMA treatment in K562 cells increased the expression of isoforms of PK (PKM2 and PKR) along with megakaryocytic differentiation marker CD61 (Fig. 1B), in two distinct oligomeric states (PKM2 in dimer and PKR in tetramer form) (Fig. 3, A–D).

The conclusions drawn were validated by silencing the isoforms of PK (PKM2 and PKR) independently or together in cells, which resulted in impaired PMA-induced megakaryocytic differentiation, confirmed by the reduction of CD61 expression, caused by aborted adaptive metabolism or dysregulation of differentiation progression processes. The reversal of PMA-induced ATP production upon PK silencing suggested that PK up-regulation is important for energy metabolism (Fig. 5D). Although the decrease in ATP production was nearly same in PKM2 and PKR silenced K562 cells, it was further decreased when both PK isoforms were silenced in the cells, indicating the role of both PKM2 and PKR in ATP production. No effect of PMA on glucose, lactate, and NADPH in PK silenced cells (Fig. 5, A–C) clearly suggested the need of PK by K562 cells for being able to metabolically respond to PMA treatment. The control-vector transfected cells, however, responded to PMA treatment as reflected by decrease in glucose, lactate and NADPH (Fig. 5), which was in agreement with the effects of PMA observed (Fig. 2).

The up-regulation of two distinct oligomeric states (dimeric PKM2 and tetrameric PKR) by PMA possibly reflects a differential requirement of these isoforms. Because nuclear PKM2 is known to moonlight as a transcriptional regulator, it is possible that PKM2 nuclear translocation upon PMA induction might...
regulate genes that support megakaryocyte differentiation. It is notable that only PKM2 and not PKR translocated into nucleus by PMA, suggesting a possible functional “division of labor” among these isoforms during differentiation. Negligible PK activity in nuclear fractions showed that PKM2 dimer is almost inactive in nucleus. The drop in PMA-induced ATP production by silencing either PKM2 or PKR is same, even though PKM2 has suboptimal activity compared with PKR. This highlights the contribution of both isoforms in energy production and also points out that inactive PKM2 may affect metabolism through nuclear gene regulation (29).

Cells were not arrested in the G₁ phase in PMA-induced and PKM2 silenced cells; however, no such inhibition of G₁ arrest was observed in PKR silenced and PMA-induced cells, suggesting that nuclear translocation of PKM2 may have a role in G₁ arrest of cell cycle. Decrease in CD61 appearance, irrespective of G₁ arrest, in both PKM2 and PKR silenced cells (PMA-induced) denotes that G₁ arrest and differentiation could be independent (49). Further, this result also indicates a disturbed metabolism in absence of PK that might be inhibiting cells to differentiate, and therefore, CD61 did not appear in PKM2 or PKR knockdown cells (Fig. 4F and Table 2). Knocking down PK completely (both isoforms) further diminished CD61 marker expression, signifying the contribution of both isoforms in the PMA-induced megakaryocyte differentiation in K562 cells (Fig. 4F).

Delineating the role of ERK1/2 signaling regulated expression of both PKM2 and PKR, in the knockdown experiments of ERKs, showed that ERK2 played a predominant role. However, ERK activation was observed essential for nuclear translocation of PKM2, and not PKR isozyme, substantiating the likelihood of a crucial role of nuclear PKM2 in differentiation, which is yet to be explored. PMA induced a p38 pathway, but inhibition of this pathway did not affect the PMA-induced megakaryocyte differentiation in K562 cells, consistent with other reports (50, 51).

In summary, our findings unravel an important role of ERK2 regulated PK isoforms in PMA-induced K562 differentiation and provide insights into the metabolic changes during differentiation and their significance in such process. Inducing a shift in metabolism of tumor cells away from biosynthesis may result in their differentiation. Thus, this study also highlights the relevance of using metabolic changes in differentiation therapy.

Author Contributions—N. C. designed, carried out the experiments, interpreted data, and drafted the manuscript. M. A. I. contributed to study design, data analysis, interpretation, and drafting of manuscript. F. A. S. participated in experimental data acquisition and figure preparation. P. G. participated in experimental data acquisition. The work was carried out under the overall supervision of R. N. K. B., who conceived the study, interpreted the data, and critically reviewed the manuscript. All authors read and approved the final version of the manuscript.

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