PKM2 isoform-specific deletion reveals a differential requirement for pyruvate kinase in tumor cells

THE DAVID H. KOCH INSTITUTE FOR INTEGRATIVE CANCER RESEARCH
Massachusetts Institute of Technology

William J. Israelsen[1], Talya L. Dayton[1], Brian P. Fiske[1], Aaron M. Hosios[1], Gary Bellinger[1], Jie Li[2], Yimin Yu[1], Mika Sasaki[3], James W. Horner[4,5], Laura N. Burga[3], Jianxin Xie[6], Ronald A DePinho[4,7], Tyler Jacks[1], Gerburg M. Wulf[3], Dolores Di Vizio[8], Gordon B. Mills[2], Lewis C. Cantley[3,9], Matthew G. Vander Heiden[1,4*]

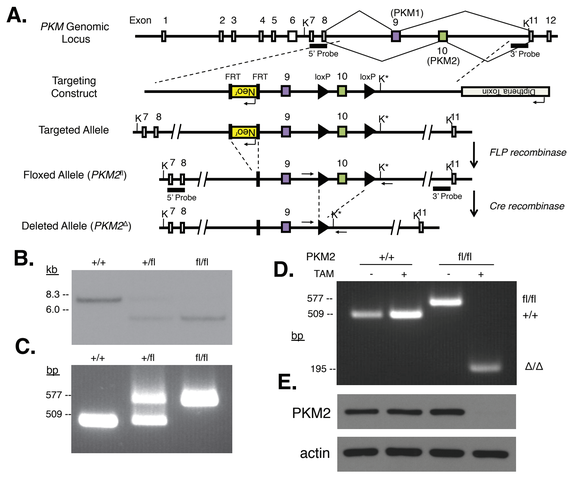
[1]Koch Institute for Integrative Cancer Research at Massachusetts Institute of Technology, Cambridge, MA 02139, USA; [2]Department of Systems Biology, University of Texas M. D. Anderson Cancer Center, Houston, TX 77030, USA; [3]Beth Israel Deaconess Medical Center, Department of Medicine-Division of Signal Transduction, Boston, MA 02115, USA; [4]Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA 02115, USA; [5]Institute for Applied Cancer Science and the Department of Genomic Medicine, University of Texas M. D. Anderson Cancer Center, Houston, TX 77030, USA. (Current address); [6]Cell Signaling Technology, Inc., Danvers, MA 01923, USA; [7]Department of Cancer Biology, University of Texas M. D. Anderson Cancer Center, Houston, TX 77030, USA. (Current address); [8]Division of Cancer Biology and Therapeutics, Cedars-Sinai Medical Center, Los Angeles, CA; the Urological Diseases Research Center, Boston Children's Hospital; and Department of Surgery, Harvard Medical School, Boston, MA 02115, USA; [9]Department of Systems Biology, Harvard Medical School, Boston, MA 02115, USA.

ABSTRACT

The M2 pyruvate kinase isoform (PKM2) is expressed in all cancers and plays a role in regulating anabolic metabolism to support cell proliferation. Non-metabolic PKM2 functions have also been implicated in cancer, but the importance of PKM2 as a glycolytic enzyme or as a modulator of gene expression is not well defined for tumors *in vivo*. To determine whether PKM2 is required for the development and growth of tumors arising from an endogenous normal tissue, we generated mice harboring a conditional allele for *PKM* exon 10. Deletion of this isoform-specific exon selectively abolishes PKM2 protein production while allowing mRNA splicing to generate PKM1 protein. We crossed these mice to a model of human breast cancer driven by Cre-mediated loss of the *Brca1* tumor suppressor gene. Contrary to prior expectation, mammary tumor formation was accelerated in PKM2 conditional mice despite robust PKM2 deletion. PKM2-null tumors displayed heterogeneous compensatory PKM1 expression, and expression of PKM1 was observed only in non-proliferating tumor cells. In contrast, proliferating cells in the tumor had no detectable pyruvate kinase expression. This suggests that PKM2 protein is not necessary for tumor cell proliferation and implies that the inactive state of PKM2 is associated with the proliferating cell population within tumors, while cells with active pyruvate kinase are involved in aspects of tumor progression that do not involve proliferation. Consistent with these findings, variable expression of PKM2 was observed across a panel of human tumors, and recurrent mutations resulting in disruption of one *PKM2* allele are found in human cancer. Together, these data support a model where regulation of PKM2 activity allows the enzyme to support the differential metabolic requirements of both proliferating and non-proliferating tumor cells.

FIGURE 1

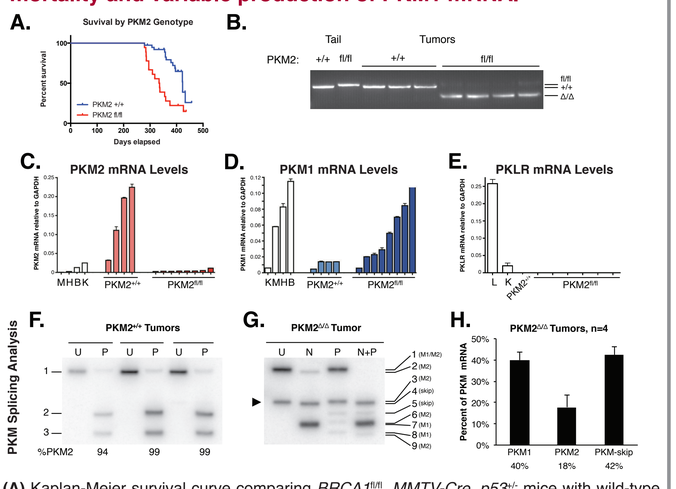
Generation and validation of PKM2 conditional mice.



(A) The mouse PKM locus, the targeting construct used to introduce loxP sites flanking exon 10, and the resulting targeted, floxed and deleted alleles are depicted. Inclusion of either exon 9 or 10 by alternative mRNA splicing produces PKM1 or PKM2 transcripts, respectively. **(B)** Southern blot analysis of KpnI-digested mouse genomic DNA using a probe that targets a region of genomic DNA 5' to the targeting construct. Digestion of the wild-type PKM allele yields an ~8.3 kb fragment while an ~6.0 kb fragment is produced by digestion of the floxed allele. **(C)** PCR genotyping of genomic DNA from $PKM2^{+/+}$, $PKM2^{+/fl}$, and $PKM2^{fl/fl}$ mice. Genotyping primers anneal outside of the loxP insertion sites as indicated by arrows in (A), and produce amplicons of 509 bp from the $PKM2^{+}$ allele and 577 bp from the $PKM2^{fl}$ allele. **(D)** PCR genotyping of $PKM2^{+/+}$ *Cre-ER* and $PKM2^{fl/fl}$ *Cre-ER* MEFs that were treated with 4-hydroxytamoxifen (TAM) or mock treated. Recombination is induced in TAM treated $PKM2^{fl/fl}$ *Cre-ER* MEFs, resulting in a $PKM2^{\Delta}$ allele producing an amplicon of 195 bp. **(E)** PKM2 protein was analyzed by western blot from $PKM2^{fl/fl}$ *Cre-ER* MEFs with and without TAM treatment. Lanes correspond to those specified in (D).

FIGURE 2

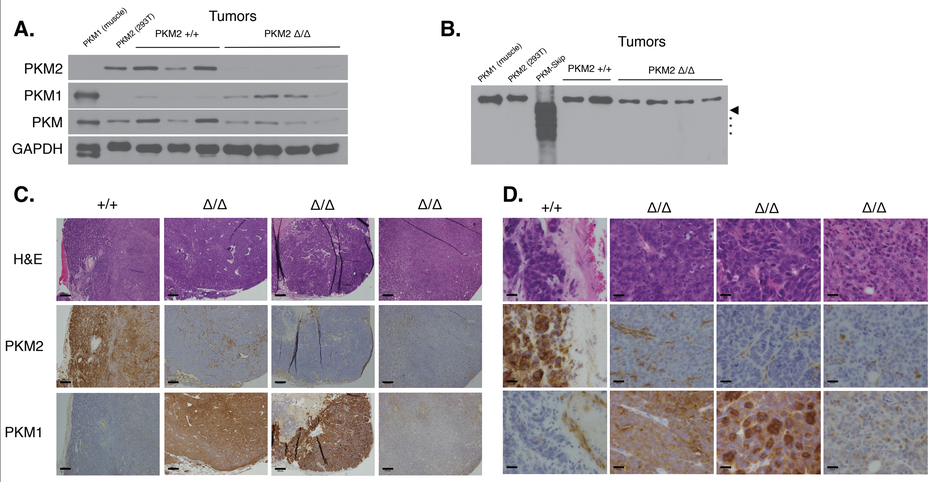
PKM exon 10 deletion in mammary tumors results in accelerated mortality and variable production of PKM1 mRNA.



(A) Kaplan-Meier survival curve comparing $BRCA1^{fl/fl}$, *MMTV-Cre*, $p53^{+/-}$ mice with wild-type ($PKM2^{+/+}$) or conditional *PKM2* alleles ($PKM2^{fl/fl}$). **(B)** PCR genotyping of the *PKM2* allele in mammary tumors derived from $PKM2^{+/+}$ and $PKM2^{fl/fl}$ mice. PCR analysis of tail DNA from $PKM2^{+/+}$ and $PKM2^{fl/fl}$ mice is shown as a control. **(C)** PKM2 mRNA transcript levels in tumors from $PKM2^{+/+}$ and $PKM2^{fl/fl}$ mice, and the indicated normal mouse tissues, were determined by qPCR. M, muscle; H, heart; B, brain; K, kidney. **(D)** PKM1 mRNA transcript levels in tumors from $PKM2^{+/+}$ and $PKM2^{fl/fl}$ mice, and the indicated normal mouse tissues, were determined by qPCR. K, kidney; M, muscle; H, heart; B, brain. **(E)** PKLR mRNA transcript levels in tumors from $PKM2^{+/+}$ and $PKM2^{fl/fl}$ mice, and the indicated normal mouse tissues, by qPCR. L, liver; K, kidney. **(F)** *PKM* splicing analysis: autoradiograph of Uncut [U] and PstI digested [P] mRNA samples obtained by RT-PCR with primers amplifying the region from *PKM* exons 8 to 11. Uncut PKM1 and PKM2 amplicons are of identical length (band 1). PstI digests only the PKM2 amplicon, producing bands 2 and 3. Results from three representative tumors from $PKM2^{+/+}$ mice are shown. Quantification of the percent PKM2 transcript as determined by densitometry normalized to 32P-dCTP incorporation is shown below each sample. **(G)** Autoradiograph of Uncut [U] and digested mRNA samples obtained by RT-PCR with primers amplifying the region from PKM exons 8 to 11. Analysis of mRNA from a representative tumor for a $PKM2^{fl/fl}$ mouse is shown. Uncut PKM1 and PKM2 amplicons are of identical length, but the PKM1 transcript is digested with NcoI while the PKM2 transcript is digested with PstI. The amplicon corresponding to the PKM-skip mis-spliced product is marked with an arrowhead (band 3). Bands corresponding to PKM1 are 1,6,7; bands corresponding to PKM2 are 1,2,5,8,9; bands corresponding to PKM-skip are 3,4. Uncut [U] and NcoI [N], PstI [P], and NcoI+PstI digested [NP] samples are shown. Figure S2 shows schematically how each band is generated. **(H)** Quantification of PKM mRNA splicing in tumors from $PKM2^{fl/fl}$ mice, as determined by densitometry of RT-PCR analysis as in (G), normalized to 32P-dCTP incorporation. Data are displayed as means ± s.e.m, n=4.

FIGURE 3

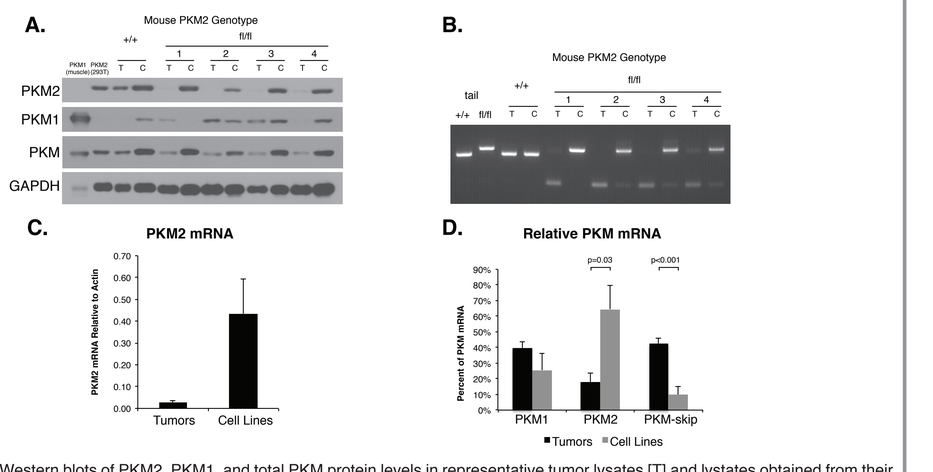
PKM exon 10 deletion in mammary tumors leads to variable expression of PKM1 protein.



(A) Western blots of PKM2, PKM1, and total PKM protein levels in tumors from $PKM2^{+/+}$ and $PKM2^{fl/fl}$ mice using isoform specific antibodies, or an antibody that recognizes an epitope that is common to PKM1 and PKM2. Muscle and 293T cell lysates serve as controls for PKM1 and PKM2 protein expression, respectively. Western blot for GAPDH is included as a loading control. **(B)** PKM protein in tumors from $PKM2^{+/+}$ and $PKM2^{fl/fl}$ mice visualized by western blot using an anti-PKM antibody (recognizes an epitope that is common to PKM1 and PKM2). This blot was intentionally over-exposed. Tumors from $PKM2^{+/+}$ and $PKM2^{fl/fl}$ mice are analyzed with muscle and 293T cell lysates serving as controls for PKM1 and PKM2 protein, respectively. Recombinant PKM-skip protein produced is E. coli is also included to determine the expected size of any truncated PKM2 present in the mouse tumors. For reference, the size of full-length PKM-skip is marked with an arrowhead and PKM-skip degradation products are marked with asterisks. **(C)** Histology of one $PKM2^{+/+}$ tumor and three $PKM2^{\Delta/\Delta}$ tumors analyzing PKM isoform expression. Tumor sections were stained with Haematoxylin and Eosin (H&E) or antibodies against PKM2 or PKM1. Scale bars represent 200 µm. **(D)** High power micrograph of the same tumors analyzed as described in (C). Scale bars represent 20 µm.

FIGURE 4

Cell lines derived from PKM2-deleted mammary tumors retain expression of PKM2.



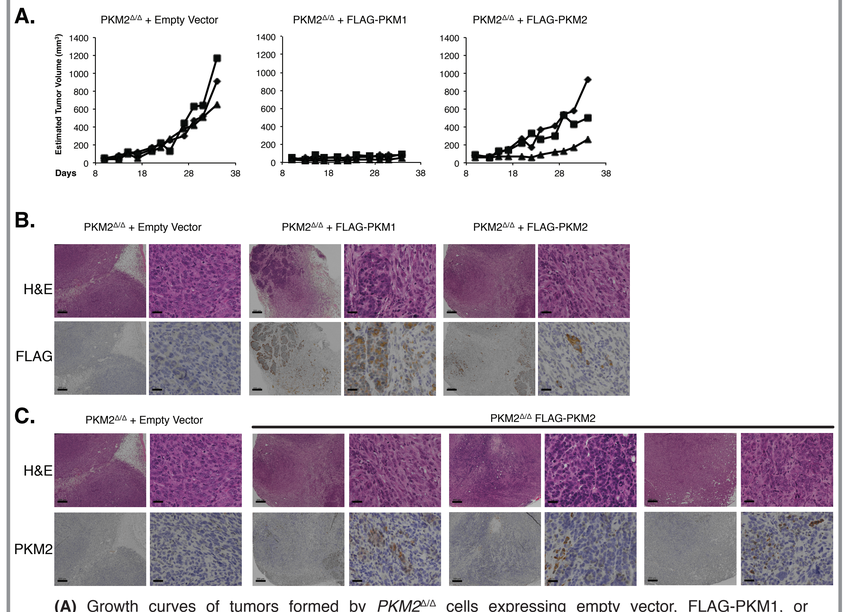
(A) Western blots of PKM2, PKM1, and total PKM protein levels in representative tumor lysates [T] and lysates obtained from their derivative cell lines [C]. Material was obtained from $PKM2^{+/+}$ and $PKM2^{fl/fl}$ mice as labeled. Muscle and 293T cell lysates serve as controls for PKM1 and PKM2 protein expression, respectively. Western blot for GAPDH is included as a loading control. **(B)** PCR genotyping of tumors [T] and their derivative cell lines [C] from $PKM2^{+/+}$ and $PKM2^{fl/fl}$ mice. PCR analysis of tail DNA from $PKM2^{+/+}$ and $PKM2^{fl/fl}$ mice is shown as a control. **(C)** PKM2 mRNA transcript levels were analyzed in $PKM2^{\Delta/\Delta}$ tumors and derivative cell lines by quantitative RT-PCR as shown. Data are shown as means ± s.e.m, n=4 tumors, n=3 cell lines. **(D)** Quantification of PKM alternative splicing in $PKM2^{\Delta/\Delta}$ tumor-derived cell lines and their parent tumors as determined by the same analysis described in Figure 2G. Data are displayed as means ± s.e.m, n=4 tumors, n=4 cell lines. P-values were obtained by Student's t-test.

CONCLUSIONS

- **PKM2 is not required by all tumor cells**
- **Pyruvate kinase is required for non-proliferating cells, but not proliferating cells in tumors**
- **PKM2 expression is variable in human cancers**
- **Recurrent point mutations disrupting pyruvate kinase are found in human cancers**

FIGURE 5

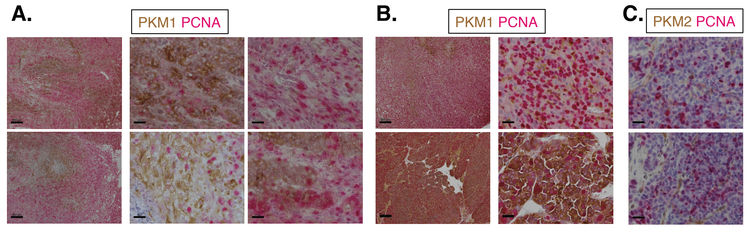
Allograft tumor growth does not select for PKM2 expression.



(A) Growth curves of tumors formed by $PKM2^{\Delta/\Delta}$ cells expressing empty vector, FLAG-PKM1, or FLAG-PKM2 following injection into nude mice. Tumor volumes over time were obtained by caliper measurement. **(B)** Representative anti-FLAG immunohistochemistry and H&E staining of tumor sections from the allograft experiment shown in (A). Scale bars represent 200 µm and 20 µm at low and high magnification, respectively. **(C)** Anti-PKM2 immunohistochemistry and H&E staining of a representative $PKM2^{\Delta/\Delta}$ tumor derived from empty vector control cells and three independent $PKM2^{\Delta/\Delta}$ tumors derived from FLAG-PKM2 cells. The top right $PKM2^{\Delta/\Delta}$ FLAG-PKM2 tumor shown is the same $PKM2^{\Delta/\Delta}$ FLAG-PKM2 tumor shown in (B). Scale bars represent 200 µm and 20 µm at low and high magnification, respectively.

FIGURE 6

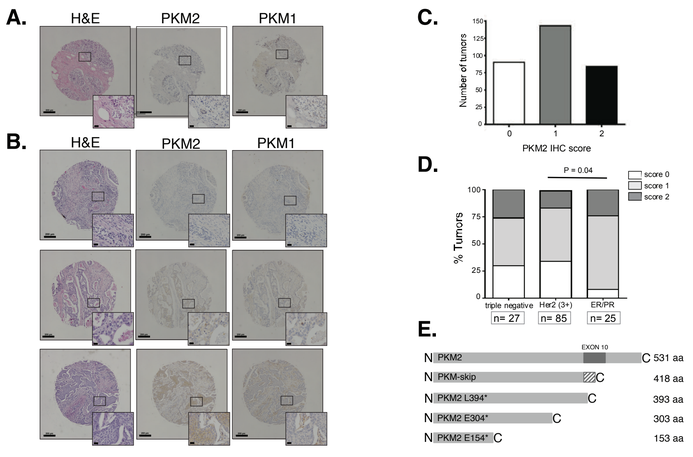
Tumor cells proliferate in the absence of PKM2, but not in the presence of PKM1.



(A) Representative immunohistochemistry of $PKM2^{\Delta/\Delta}$ allograft tumor sections dual stained for PKM1 (brown) and PCNA, a marker of proliferation (red). Scale bars represent 200 µm and 20 µm at low and high magnification, respectively. **(B)** Mammary tumors from $PKM2^{fl/fl}$ $BRCA^{fl/fl}$ *MMTV-Cre* $p53^{+/-}$ mice analyzed by immunohistochemistry. Tissue sections were dual stained for PKM1 (brown) and PCNA (red) as shown. Scale bars represent 200 µm and 20 µm at low and high magnification, respectively. **(C)** Mammary tumors from $PKM2^{fl/fl}$ $BRCA^{fl/fl}$ *MMTV-Cre* $p53^{+/-}$ mice analyzed by immunohistochemistry. Tissue sections were dual stained for PKM2 (brown) and the PCNA (red). Scale bars represent 20 µm.

FIGURE 7

Human breast tumors show variable expression of PKM2.



(A) Representative TMA cores containing normal human breast. Shown from left to right are H&E staining, PKM2 IHC, and PKM1 IHC. Insets show higher magnification. Scale bars represent 200 µm and 20 µm at low and high magnification, respectively. **(B)** Representative TMA cores containing human breast tumor samples stained with H&E, and for PKM2 and PKM1 using immunohistochemistry. Cores were scored for intensity of PK-M2 staining, and an examples of each PKM2 IHC intensity score is shown (0=negative; 1=weak; 2=strong). Insets show higher magnification. Scale bars represent 200 µm and 20 µm at low and high magnification, respectively. **(C)** Distribution of PKM2 IHC intensity scores of 317 tumors from two different TMAs containing breast tumor samples. **(D)** Quantification of PKM2 expression in human breast tumors relative to breast tumor subtype (triple negative, Her2 amplified, or ER/PR positive). The distribution of PKM2 scores is significantly different between Her2 amplified samples and ER/PR positive samples by Chi-square test (P=0.038). **(E)** Schematic showing truncations of PKM2 caused by mutations found in human cancers.