Loss of PKM2 in Lgr5\(^+\) intestinal stem cells promotes colitis-associated colorectal cancer

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The regulatory properties of pyruvate kinase M2 isoform (PKM2), the key glycolytic enzyme, influence altered energy metabolism including glycolysis in cancer. In this study, we found that PKM2 was highly expressed in patients with ulcerative colitis or colorectal cancer (CRC). We then investigated the effectiveness of conditionally ablating PKM2 in Lgr5\(^+\) intestinal stem cells (ISC) using a mouse model of colitis-associated CRC (AOM plus DSS). Tamoxifen-inducible Lgr5-driven deletion of PKM2 in ISC (PKM2\(\Delta\)Lgr5-Tx) significantly promoted tumor incidence and size in the colon and lower body weight compared with findings in vehicle-treated mice (PKM2\(\Delta\)Lgr5-Veh). Histopathologic analysis revealed considerable high-grade dysplasia and adenocarcinoma in the colon of PKM2\(\Delta\)Lgr5-Tx mice while PKM2\(\Delta\)Lgr5-Veh mice had low- and high-grade dysplasia. Loss of PKM2 was associated with dominant expression of PKM1 in Lgr5\(^+\) ISC and their progeny cells. Further, the organoid-forming efficiency of whole cancer cells or Lgr5\(^+\) cells obtained from colon polyps of PKM2\(\Delta\)Lgr5-Tx mice was significantly increased when compared with PKM2\(\Delta\)Lgr5-Veh mice. Cancer organoids from PKM2\(\Delta\)Lgr5-Tx mice exhibited increased mitochondrial oxygen consumption and a shift of metabolites involved in energy metabolism. These findings suggest that loss of PKM2 function in ISC promotes colitis-associated CRC.

Pyruvate kinase (PK) is involved in the final step of glycolysis by transferring the phosphate group from phosphoenolpyruvate to ADP and producing pyruvate and ATP. It has four isozymes (L, R, M1, and M2), which exhibit different kinetic properties at the expressing tissues\(^1\). Among them, the M2 isoform of PK (PKM2) has received the most attention due to its involvement in the Warburg effect in cancer. Most cancer cells utilize aerobic glycolysis, known as the Warburg effect, to facilitate the synthesis of cellular building blocks (amino acids, nucleotides, and lipids) needed to produce a new cell\(^2\). Previous studies suggested that PKM2 is a key glycolytic enzyme that is involved in aerobic glycolysis and anabolic metabolism in cancer cells\(^3,4\). Lower activity of PKM2 in tumor cells, which exists in a dimeric form, could allow the accumulation of glycolytic intermediates to enter the glycolysis branch pathway\(^5\). In addition to its metabolic function, PKM2 can promote transcriptional activities through interactions with Oct-4\(^6\), HIF-1\(\alpha\)\(^7\), STAT3\(^8\), and \(\beta\)-catenin\(^9\). These PKM2 functions might support metabolic reprogramming and progression of cancer.

Although the critical role of PKM2 in cancer development is well known, recent studies have yielded conflicting results about the requirement for PKM2. Loss of PKM2 in a mouse model of breast cancer resulted in accelerated tumor growth and mortality\(^10\). In another study, knockdown of PKM2 led to impairment of tumor cell proliferation in vitro and had no effect on in vivo tumor xenograft growth\(^11\). In addition, a recent study showed that depletion of PKM2 did not affect c-MYC-induced liver tumor formation\(^11\). These results challenge the idea that PKM2 is essential in tumorigenesis.

PKM2 is the dominant isoform in normal colon tissues and is overexpressed in tumor-induced colon tissues\(^12,13\). Also, PKM2 levels in serum and feces were elevated in inflammatory bowel disease (IBD) patients\(^14,15\). Patients with IBD, such as Crohn’s disease and ulcerative colitis (UC), are at high risk of developing colorectal cancer (CRC). More than 20% of patients with IBD develop colon cancer within 30 years\(^17\). Genetic mutations in
PKM1 in PKM2 addressed PKM2 expression in patients with UC or CRC. Mice with specific deletion of PKM2 in Lgr5

Lgr5 acts as a marker for cancer stem cells (CSC) involved in glycolysis such as PFKFB3, PKM, and PFKP were highly increased in UC patients (Fig. 1b). Previous CXCLs, STATs, and IL-8) associated with inflammation were significantly up-modulated in UC patients. Genes potentially UC correlated (Fig. 1a). As expected, since UC is highly correlated with inflammation, genes (MMPs, CXCLs, STATs, and IL-8) associated with inflammation were significantly up-modulated in UC patients. Genes involved in glycolysis such as PFKFB3, PKM, and PFKP were highly increased in UC patients (Fig. 1b). Previous reports demonstrate that PFKFB3 is associated with UC and CRC. However, there has been little characterization of the function of PKM in inflammation-induced CRC. We thus focused on PKM function in UC and CRC. First, we examined PKM expression in the colon tissues of CRC patients. As shown in Fig. 1c, PKM expression in those tissues was significantly higher than in normal tissue. We next investigated the clinical relevance of PKM and CRC. Patient cohorts from the GEO were dichotomized according to PKM expression. Patients with higher PKM levels had poor clinical outcomes and vice versa across multiple sample sets (Fig. 1d), consistent with an oncogenic function of PKM in CRC. Others have reported that PKM2 is the predominant isoform in normal colon epithelial cells and colon cancers. We also found that mRNA levels of PKM2, but not PKM1, were highly expressed in both human normal and cancer colon tissues (data not shown). Thus, we can speculate that PKM from gene expression profile data represent PKM2 rather than PKM1. To determine whether PKM2 expression is changed during inflammation and colon oncogenesis, we used immunohistochemistry (IHC) to validate expression of PKM2 in UC and CRC. Indeed, PKM2 expression was increased in UC tissues and greater yet in dysplastic tumors (Fig. 1e). Moreover, in CRC patients, the expression of Lgr5, the cancer stem cell marker, was positively correlated with PKM2 expression (Fig. 1f). These results suggest that PKM2 expression is increased during inflammation-induced CRC development, either as a consequence of oncogenic transformation or as a metabolic oncogene.

Results
PKM2 as an oncogenic factor in inflammation-induced CRC. Ulcerative colitis (UC) is a contributing factor to CRC. To elucidate which factors are involved in UC, we performed genomic analysis of previously reported gene expression data (GSE4380, GSE3680, and GSE47908; Gene Expression Omnibus (GEO) in the National Center for Biotechnology Information) to explore for genes differentially expressed in UC, we compared normal colon to UC samples by applying class comparison analysis. We found 893 genes that were potentially UC correlated (Fig. 1a). As expected, since UC is highly correlated with inflammation, genes (MMPs, CXCLs, STATs, and IL-8) associated with inflammation were significantly up-modulated in UC patients. Genes involved in glycolysis such as PFKFB3, PKM, and PFKP were highly increased in UC patients (Fig. 1b). Previous reports demonstrate that PFKFB3 is associated with UC and CRC. However, there has been little characterization of the function of PKM in inflammation-induced CRC. We thus focused on PKM function in UC and CRC. First, we examined PKM expression in the colon tissues of CRC patients. As shown in Fig. 1c, PKM expression in those tissues was significantly higher than in normal tissue. We next investigated the clinical relevance of PKM and CRC. Patient cohorts from the GEO were dichotomized according to PKM expression. Patients with higher PKM levels had poor clinical outcomes and vice versa across multiple sample sets (Fig. 1d), consistent with an oncogenic function of PKM in CRC. Others have reported that PKM2 is the predominant isoform in normal colon epithelial cells and colon cancers. We also found that mRNA levels of PKM2, but not PKM1, were highly expressed in both human normal and cancer colon tissues (data not shown). Thus, we can speculate that PKM from gene expression profile data represent PKM2 rather than PKM1. To determine whether PKM2 expression is changed during inflammation and colon oncogenesis, we used immunohistochemistry (IHC) to validate expression of PKM2 in UC and CRC. Indeed, PKM2 expression was increased in UC tissues and greater yet in dysplastic tumors (Fig. 1e). Moreover, in CRC patients, the expression of Lgr5, the cancer stem cell marker, was positively correlated with PKM2 expression (Fig. 1f). These results suggest that PKM2 expression is increased during inflammation-induced CRC development, either as a consequence of oncogenic transformation or as a metabolic oncogene.

PKM2 deletion in Lgr5+ ISC or Villin+ epithelial cells aggravates inflammation-induced CRC. To investigate the regulatory role of PKM2 in ISC, PKM2f/f mice were crossed with Lgr5CreERT2 mice. We used tamoxifen treatment to show specific deletion of PKM2 in GFP-expressing Lgr5+ ISC and their progeny (Fig. S1) and a combination of AOM and DSS treatment to induce inflammation-derived CRC (Fig. 2a). PKM2f/fLgr5CreERT2-Tamoxifen (PKM2ΔLgr5-Tx) mice had more significant body weight loss (Fig. 2b) and increased morbidity (87.23% vs. 71.43%, data not shown) when compared to PKM2f/fLgr5CreERT2-Vehicle (PKM2ΔLgr5-veh) mice. The tumor load was significantly higher in the colon of PKM2ΔLgr5-Tx mice than in PKM2ΔLgr5-veh mice while tumor size distribution was comparable in PKM2ΔLgr5-Tx and -veh mice (Fig. 2c,d). About half of the PKM2ΔLgr5-Tx mice developed adenocarcinoma while PKM2ΔLgr5-veh mice had low- and high-grade dysplasia. Tumors progressed to more severe dysplasia and areas affected by dysplasia were larger in the colons of PKM2ΔLgr5-Tx mice than in PKM2ΔLgr5-veh mice (Fig. 2e). The acceleration of tumor growth associated with PKM2 loss was further confirmed in mice lacking PKM2 in intestinal epithelial cells including ISC (PKM2ΔIEC mice), which were generated by crossbreeding with PKM2f/f and VillinCre mice (Fig. S2A–D). These results suggest that the deletion of PKM2 in Lgr5+ ISC or whole epithelial cells accelerates inflammation-induced colon tumor growth.

Cancer cells with PKM2 depletion express high levels of PKM1. As reported by others, we found high levels of PKM2 but not PKM1 expression in the colon epithelium of PKM2f/f mice in the steady state (Fig. S3A,B). Interestingly, highly activated PKM1 but not PKM2 expression was found in the epithelium of PKM2ΔIEC mice in the steady state (Fig. S3A,B). After tamoxifen treatment of PKM2f/fLgr5CreERT2 mice, PKM2 expression was depleted in the epithelium of Lgr5-GFP+ cells but there were no changes in Lgr5-GFP− cells (Fig. S1A). PKM2-depleted Lgr5-GFP+ cells expressed PKM1 in the steady-state condition (Fig. S1A). We assessed expression patterns of PKM1 and PKM2 in the colon polyps after treatment with AOM plus DSS. PKM2-intact Lgr5-GFP− or Lgr5-GFP+ ISC-derived cancer cells in colon polyps did not express PKM1 in PKM2ΔLgr5-Tx or PKM2ΔLgr5-veh mice (Fig. 3a). In contrast, PKM2-null cancer cells that originated from Lgr5-GFP+ cells in colon polyps of PKM2ΔLgr5-Tx mice expressed mainly PKM1 (Fig. 3a). Western blot
analysis showed higher levels of PKM1 in normal colon epithelial cells (Fig. S1B) and polyp tissues (Fig. 3b) in PKM2ΔLgr5-Tx mice than in PKM2ΔLgr5-Veh mice. Total levels of PKM2 protein in normal colon epithelium (Fig. S1B) and polyp tissues (Fig. 3b) were slightly lower in PKM2ΔLgr5-Tx mice than in PKM2ΔLgr5-Veh mice as...
PKM2 was partially depleted in the intestines (i.e., Lgr5⁺ cells only). The levels of total PKM, including PKM1 and PKM2, were identical in the two groups (Figs 3b and S1B). Because PKM2 is involved in epidermal growth factor receptor-promoted β-catenin transactivation 9 and PKM1 causes proliferation arrest 29, we next assessed β-catenin activation and cell proliferation. We found no significant differences in expression of nuclear translocation of β-catenin or numbers of Ki67-positive proliferating cells in PKM2- and PKM1-positive cells in colon

Figure 2. Lgr5⁺ specific-deletion of PKM2 accelerates development of inflammation-induced CRC. (a) Treatment scheme for AOM/DSS colon cancer model. PKM2ΔLgr5CreERT2 mice were injected with vehicle (Veh) or tamoxifen (Tx) following induction of colon cancer by treatment of AOM and DSS. (b) Weight loss (n = 32) and (c) representative images of colons from AOM/DSS-treated PKM2ΔLgr5 mice. (d) Tumor load (n = 17 for Veh, n = 18 for Tx) and tumor size (n = 15 for Veh, n = 17 for Tx) in AOM/DSS-treated mice. (e) Colon histology of AOM/DSS-treated mice. Arrowheads indicate colon polyps. Representative images of low- and high-grade dysplasia and adenocarcinoma. Scale bar = 100μm. H&E stained sections were scored for severity and area of dysplasia (n = 4 for Veh, n = 6 for Tx). All data are mean ± s.e.m. Statistical analyses were done by Student's t-test or two-way ANOVA with Bonferroni post-hoc test. *p < 0.05, **p < 0.01.

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polyp tissues (Fig. 3c). These results indicate that cancer cells originating from PKM2-depleted Lgr5+ ISC activate PKM1 expression and depletion of PKM2 does not attenuate β-catenin activation and cell proliferation.

**Loss of PKM2 in Lgr5+ ISC increases cancer stem cell-like function.** To further examine the effects of PKM2 loss on tumor growth, we isolated cells from colon polyps of AOM/DSS-treated PKM2ΔLgr5–Veh and –Tx mice. The blots were cropped. Each target gene and the control gene were run on the same gel. The full-length blots are presented in Fig. S5B. (c) Confocal analysis of β-catenin and Ki67 expression in colon polyps from AOM/DSS-treated PKM2ΔLgr5+ mice. Scale bar = 50 μm (a) and 100 μm (c). Data are representative of three independent experiments.

![Figure 3](https://www.nature.com/scientificreports/)

**Figure 3.** Cancer cells with PKM2 deletion express PKM1. (a) Immunofluorescence images of PKM2 and PKM1 expression and (b) Western blot analysis of colon polyp tissues from AOM/DSS-treated PKM2ΔLgr5–Veh and –Tx mice. The blots were cropped. Each target gene and the control gene were run on the same gel. The full-length blots are presented in Fig. S5B. (c) Confocal analysis of β-catenin and Ki67 expression in colon polyps from AOM/DSS-treated PKM2ΔLgr5+ mice. Scale bar = 50 μm (a) and 100 μm (c). Data are representative of three independent experiments.
Metabolites involved in energy metabolism are altered in cancer tissues and organoids of PKM2-deficient mice. Since PKM2 is an essential enzyme for the metabolic reprogramming of cancer cells, we investigated the metabolites of colon polyps (Fig. 5a) and of organoids from colon polyps in the absence of PKM2. While there were no significant changes within the glycolytic pathway, intermediates of the pentose phosphate pathway such as 6-phosphogluconate (6PG), sedoheptulose-7-phosphate (S7P), and tricarboxylic acid (TCA) cycle intermediates (e.g., fumarate) were higher in colon polyps of AOM/DSS-treated PKM2ΔLgr5–Veh and –Tx mice than in PKM2ΔLgr5–Veh and –Tx mice. We further found that in the glycolytic pathway, glucose, glucose-6-phosphate (G6P), fructose-1, 6-bisphosphate (FBP), and lactate were significantly higher in cancer organoids of PKM2ΔLgr5–Veh and –Tx mice than in PKM2ΔLgr5–Veh mice. In addition, high levels of intermediates of the pentose phosphate pathway, such as S7P and the TCA cycle (e.g., malate), were found in cancer organoids from PKM2-deficient mice but not in PKM2-intact mice (Fig. 5b). Overall, PKM2-deficiency in Lgr5+ cells accelerated intermediates related to glycolytic, pentose phosphate, and TCA cycle pathways in colon polyps.
PKM2 loss increases mitochondrial ATP production. Since PKM2 contributes to the metabolic switch from mitochondrial oxidative phosphorylation (OXPHOS) to aerobic glycolysis in cancer cells, we next addressed oxygen consumption rate (OCR), an indicator of mitochondrial respiration, in the absence of PKM2. After treatment with the ATP synthase inhibitor oligomycin, average OCR of cancer organoids from colon polyps of PKM2ΔLgr5-Tx mice was lower than in those from PKM2ΔLgr5-Veh mice (Fig. 6a). Of note, addition of the uncoupling agent carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP) led to a considerable increase in maximal respiration of cancer organoids from colon polyps of PKM2ΔLgr5-Tx mice when compared to those of PKM2ΔLgr5-Veh mice (Fig. 6a,b). ATP-linked respiration and coupling efficiency \[100 \times \text{ATP-linked respiration}/\text{basal respiration}\], both defined as the respiration that is used to drive mitochondrial ATP synthesis, was higher in cancer organoids from colon polyps of PKM2ΔLgr5-Tx mice than in PKM2ΔLgr5-Veh mice (Fig. 6b,c). In addition, the cell respiratory control ratio (maximal respiration/proton leak), the general indicator for mitochondrial function, was higher in cancer organoids from PKM2ΔLgr5-Tx mice than from PKM2ΔLgr5-Veh mice (Fig. 6c). Colon crypts of naïve PKM2ΔLgr5-Tx mice also exhibited increased maximal respiration and cell respiratory control ratio, suggesting that PKM2 loss led to enhanced mitochondrial oxidation capacity (Fig. S4). These results indicate that PKM2 deficiency in ISC results in activation of mitochondrial ATP production in colon polyps of CRC-induced mice.

Genes associated with activation of the Wnt pathway and tumor progression are increased in cancer tissues and organoids of PKM2-deficient mice. We performed RNA-sequencing (RNA-seq) experiments using colon polyps from PKM2ΔLgr5-Tx and -Veh mice after treatment with AOM/DSS. Among 37 genes significantly differentially expressed between the two groups, 19 genes showed significant up-regulation (fold-change, ≥1.5; \(p < 0.05\)) in colon polyps from PKM2ΔLgr5-Tx when compared with PKM2ΔLgr5-Veh mice (Fig. 7a). Spp1, Dkk2, and Apcc1, Wnt target genes and well-known genes that enhance tumor growth and metastasis, were highly expressed in colon polyps from PKM2ΔLgr5-Tx mice but not from PKM2ΔLgr5-Veh.
mice. In contrast, 18 genes were down-regulated in colon polyps from PKM2ΔLgr5-Tx mice including cytochrome P450 (CYP) 2 family (Fig. 7a). Gene expression patterns were further addressed in colon polyp tissues (Fig. 7b) and cancer organoids from colon polyps (Fig. 7c) by real-time PCR. Of note, mRNA levels of PKM1, Hbb-b2, and Spp1 were significantly elevated in both colon polyp tissues and cancer organoids from colon polyps from PKM2ΔLgr5-Tx mice. Taken together, these results further support the conclusion that PKM2-deficiency in ISC accelerates progression of CRC in the murine model.

**Discussion**

In this study, we demonstrated that despite high PKM2 expression in patients with UC or CRC and its correlation with poor clinical outcomes in patient-derived genomic data, development of inflammation-induced CRC was accelerated in mice with the deletion of PKM2 in Lgr5+ISC or in intestinal epithelial cells. We similarly observed enhanced formation of cancer organoids obtained from colon polyps (Fig. 7b) and cancer organoids from colon polyps (Fig. 7c) by real-time PCR. Of note, mRNA levels of PKM1, Hbb-b2, and Spp1 were significantly elevated in both colon polyp tissues and cancer organoids from colon polyps from PKM2ΔLgr5-Tx mice. Taken together, these results further support the conclusion that PKM2-deficiency in ISC accelerates progression of CRC in the murine model.

PKM2 expression has been considered a hallmark of cancer; however, recent studies have yielded contradictory results regarding the requirement for PKM2 in tumor growth. Mice deficient in PKM2 have showed enhanced tumorigenesis in several experimental models. In our current study, inflammation-induced CRC was more severe when there was PKM2 loss in ISC, suggesting that PKM2 is not absolutely required for tumor maintenance and growth in the colon. Similar results were observed in an APC-driven colon cancer model. We found significant levels of PKM1 were activated when PKM2 was deleted in both colon tissues and organoids from colon polyps (Figs 3 and 4). It has been reported that the expression of PKM1, which resulted from PKM2 loss, was found only in non-proliferating cells in breast cancer. However, we observed that proliferation...
of PKM1-expressed cancer cells was similar to that of cells expressing PKM2 in inflammation-induced CRC (Fig. 3c), consistent with other studies. The most recent study revealed that PKM1 promotes tumor growth in mouse lines expressing PKM1 or PKM2. PKM1-activated glucose catabolism and PKM1-dependent autophagy contributed to malignancy (e.g., small-cell lung cancer). Although we need to explore this further, we speculate that compensatory expression of PKM1 by deletion of PKM2 provides metabolic advantage to support the expansion of cancer cells in different ways than PKM2.

We observed increased formation of organoids in PKM2-deficient colon polyps in the absence of Wnt activators (i.e., Wnt3a and R-spondin1) (Fig. 4). As reported previously, the majority of adenoma organoids proliferate and propagate in the absence of exogenous Wnt activators. The fact that Wnt activators-independent CRC organoids had mutations in the Wnt signaling pathway (e.g., Apc, Ctnnb1, and Tcf7l2) suggests that cancer cells from PKM2-deficient mice might carry those mutations. However, we could not find changes of oncogenes such as Apc, Ctnnb1, and Tcf7l2; instead we found up-regulated levels of genes such as Spp1, Dkk2, and Apodd1, which are associated with aberrant Wnt/β-catenin signaling (Fig. 7). For instance, osteopontin, encoded by Spp1, led to β-catenin stabilization and nuclear translocation via Akt-mediated GSK-3β inhibition. Moreover, overexpression of osteopontin was detected in mice with activation of the Wnt pathway via mutation of Apc. One recent study demonstrated elevation of Dkk2 expression in CRC of mice with Apc mutation and found that treatment of small interfering RNAs for β-catenin suppressed the Dkk2 upregulation. Another study showed that Apodd1 is a direct target gene of the β-catenin/Tcf complex. A potential mechanism suggested by others is that PKM2 negatively regulates β-catenin via miR-200a, inhibiting β-catenin translation. When taken together, our findings imply that PKM2 deficiency enhances Wnt signaling pathway-related genes and accelerates the tumorigenic process of inflammation-induced CRC.

Our results indicate an increase in the forming efficiency of cancer organoids from PKM2-deficient mice after passage of cancer organoids (Fig. 4b). In addition, the population of Lgr5+ CSC was elevated in cancer tissues from PKM2ΔLgr5-Tx mice (Fig. 4c). Single Lgr5+ CSC with PKM2 depletion formed organoids at a higher frequency than CSC with PKM2 (Fig. 4d). We speculate that CSC-like cells are enriched in cancer tissues of mice with PKM2 loss. Moreover, cancer organoids from PKM2ΔLgr5-Tx mice showed an elevation of ATP-linked respiration and mitochondrial function (Fig. 6), which might result from compensatory expression of PKM1. Although there
is no consensus on whether CSC rely on aerobic glycolysis or OXPHOS, there is increasing evidence that CSC adopt mitochondrial oxidative metabolism. Colon CSC have increased mitochondrial function, and their stemness is regulated by the maintenance of mitochondrial function. Previous studies reported that cancer cell lines resistant to anticancer drugs (i.e., fluorouracil and oxaliplatin) exhibited an up-regulation of PKM1 expression, OXPHOS, and stem-like traits including the expression of CD133 and formation of anchorage-independent spheres. Considering these findings, we speculate that increased self-renewal of CSC-like cells in cancer tissues from PKM2ΔLgr5 Tx mice might be due to PKM1-induced shifts of cancer metabolism.

Although cancer tissues from PKM2-deficient and -intact mice contained nearly equal amounts of glycolytic intermediates, several metabolites in glycolysis were elevated in cancer organoids from colon polyps with PKM2 loss (Fig. 5). This discrepancy might be related to the fact that cancer organoids were in relatively higher glucose culture conditions than the cancer tissues, which were in low-glucose tumor microenvironment conditions. A recent study revealed that cancer cells expressing only PKM1 increased the flux of glucose through glycolysis and the TCA cycle. We speculate that PKM1 expression in PKM2-deficient cancer organoids contributes to the increase of glycolysis in a glucose-sufficient condition and to entry of glucose into the TCA cycle. In addition, cancer tissues elevate some intermediates in the pentose phosphate pathway. In another recent study, downregulated PKM2 resulted in an increase of the NADPH/NADP ratio in pancreatic cancer cells during hypo-glucose conditions, suggestive of activation of the pentose phosphate pathway. When all of these findings are taken together, it appears that depletion of PKM2 might be involved in a shift of glucose flux to the pentose phosphate pathway under hypo-glucose conditions.

Overall, our study demonstrates that loss of PKM2 accelerates the progression of colitis-induced CRC by AOM/DSS treatment. We found the enhanced Wnt/β-catenin pathway and CSC-like function in cancer organoids in the absence of PKM2. Recent studies on targeting PKM2 function in cancer cells showed that the depletion of PKM2 might be involved in a shift of glucose flux to the pentose phosphate pathway under hypo-glucose conditions.

Materials and Methods

Ethics statement. All animal experiments were approved by the Institutional Animal Care and Use Committee of Asan Medical Center (Seoul, Korea) (Approval No. 2016-12-131). Animal experiments were performed under anesthesia with a mixture of ketamine (100 mg/kg) and xylazine (20 mg/kg), and all efforts were made to minimize suffering. All experiments were performed in accordance with relevant guidelines and regulations.

Mice. PKM2fl/fl, Lgr5-EGFP-IRESCreERT2, and Villin-cre mice were purchased from Jackson Laboratory (Bar Harbor, ME). All mice were maintained under specific-pathogen-free conditions in the animal facility at Asan Medical Center, where they received sterilized food and water ad libitum. For Lgr5+ cell-specific deletion of PKM2, PKM2fl/flxLgr5CreERT2 mice at age 6–8 weeks were injected intraperitoneally with 1 mg of tamoxifen (MP Biomedicals, Aurora, OH) in sunflower seed oil (Sigma Aldrich, St. Louis, MO) once a day for 5 consecutive days. PKM2fl/flxLgr5CreERT2 mice were injected with sunflower seed oil alone (vehicle) for control.

Experimental colitis-associated colorectal cancer. Male mice were given a single intraperitoneal injection of AOM (Sigma Aldrich) (12.5 mg/kg body weight) in combination with three cycles of DSS (molecular weight 36,000–50,000; MP Biomedicals) treatment as illustrated in Fig. 2a. Body weight was monitored twice a week.

Histological scoring. The entire colon was removed, opened longitudinally, and scored for polyp numbers. Colon tissues were then formed into Swiss rolls, fixed in 4% paraformaldehyde (PFA), and embedded in paraffin. Tissue sections were stained with hematoxylin-eosin (H&E). Histological scoring was performed blindly by pathologists. Tumors were graded and scored as 1 = low-grade dysplasia, 2 = high-grade dysplasia, 3 = intramucosal adenocarcinoma, 4 = invasive adenocarcinoma. Area affected by dysplasia was scored as 1, <10%; 2, 10–25%; 3, 25–50%; 4, >50% of colon.

Culture of organoid using colon polyps and sorted Lgr5+ cells. Colon polyps were removed and washed with PBS containing gentamicin (50 μg/ml, Thermo Fisher, Waltham, MA). Tissues were minced with a scissor and incubated for 1 h in RPMI 1640 medium (Thermo Fisher) containing collagenase IV (2.5 mg/ml, Thermo Fisher, Waltham, MA). Tissues were minced with a scissor and incubated for 1 h in RPMI 1640 medium (Thermo Fisher) containing collagenase IV (2.5 mg/ml, Sigma Aldrich), DNase (0.2 mg/ml, Sigma Aldrich) at 37 °C with stirring. Cell suspensions were filtered and centrifuged at 500 × g for 5 min. For construction of organoids, the dissociated cells were seeded at concentrations of 2 × 10^7 cells per well with Matrigel (Corning, Corning, NY) in a 24-well plate. For isolation of Lgr5-GFP cells, cell pellets were incubated with purified anti-mouse CD16/32 antibody (BD Biosciences, Franklin Lakes, NJ), followed by staining with Live/Dead Cell Stain Kit (Thermo Fisher) and EpCAM (clone G8.8, Thermo Fisher). Cell sorting was performed using a FACS AriaII cell sorter (BD Biosciences). Sorted cells were seeded at 1 × 10^5 cells per well with Matrigel. All organoids were cultured in EN medium containing B27 supplement (Thermo Fisher), N2 supplement (Thermo Fisher), EGF (R&D Systems, Minneapolis, MN), Noggin (R&D Systems), N-acetyl cysteine (Sigma Aldrich), and penicillin-streptomycin (Thermo Fisher) in advanced DMEM/F12 (Thermo Fisher). EN medium was replaced every 2–3 days. For subculture, dissociation of organoids was carried out by resuspending organoids in TrypLE Express (Thermo Fisher) for 10 min at 37 °C.

Immunofluorescence staining. Colon tissues were fixed with 4% PFA and dehydrated with 15% and 30% sucrose in PBS. Dehydrated tissues were then embedded in frozen section compound, frozen, and sliced into 6-μm sections. Tissue sections were fixed with −20 °C acetone for 5 min, blocked with PBS containing 5% BSA for
1 h at room temperature (RT), and stained with primary antibodies overnight at 4 °C. Tissues were washed in PBS, incubated with secondary antibodies at RT for 1 h, stained with 4’,6-diamidino-2-phenylindole (DAPI; Thermo Fisher) for 2 min at RT, and mounted with PermaFluor mountant (Thermo Fisher). For staining, organoids seeded in an eight-well chamber (Thermo Fisher) were fixed with 4% PFA in PBS for 10 min at RT. After organoids were washed in PBS and subsequently permeabilized in PBS containing 0.5% Triton X-100 for 20 min at RT, they were blocked with 0.5% BSA in PBS for 1 h at RT. After staining, images were captured on an LSM 710 confocal microscope (Carl Zeiss, Oberkochen, Germany). Primary antibodies were rabbit anti-PKM1 (clone D30G6), rabbit anti-PKM2 (clone D78A4), rat anti-Ki67 (clone 11F6), and mouse anti-β-catenin (clone 14). Secondary antibodies were Alexa Fluor 546 donkey anti-rabbit IgG, Alexa Fluor 647 donkey anti-rabbit IgG, Alexa Fluor 488 goat anti-mouse IgG, Alexa fluoro 594 goat anti-rat IgG. Antibodies for analysis were from Cell Signaling Technology (Danvers, MA), BD Biosciences, Abcam (Cambridge, UK), and BioLegend (San Diego, CA).

**Metabolomics.** Colon polyp tissues (22 ± 1 mg) were homogenized using a Tissuelyser (Qiagen, Valencia, CA) with chloroform/methanol (2:1, v/v). The homogenate was incubated at 4 °C for 20 min. The internal standard (13C5 Glutamine-d4) was added to the sample, followed by centrifugation. After collection of supernatant, H2O was added and centrifuged. For metabolomics analysis, organoids were washed and lysed using cold methanol/H2O (80/20, v/v) by vigorous vortexing, and then centrifuged. Aqueous phase samples were dried by vacuum centrifuge and reconstituted with 50% methanol. Metabolites were analyzed with liquid chromatography-tandem mass spectrometry (LC-MS/MS) system equipped with a 1290 high-performance liquid chromatography system (Agilent Technologies, Santa Clara, CA), Qtrap 5500 (AB Sciex, Framingham, MA), and reverse-phase column (Synergi fusion RP 50 × 2 mm; Phenomenex, Torrance, CA). Multiple reaction monitoring was used in negative-ion mode and the extracted ion chromatogram (EIC) corresponding to the specific transition for each metabolite was used for quantitation. The area under the curve of the EIC was normalized to that of the EIC of the internal standard. The peak area ratio of each metabolite to internal standard was normalized using the protein concentration or the weight of each sample.

**Measurement of OCR by XF24 Flux Analyzer.** Organoids were dissociated with TrypLE Express for 10 min at 37 °C. Dissociated single cells were seeded at 5 × 105 cells per well with Matrigel in XF24 cell culture microplates (Agilent Technologies) and cultured in EN medium for 5–6 days. One hour before measurement, culture medium was replaced with OCR assay media [minimal DMEM (Sigma Aldrich) supplemented with GlutaMAX (2 mM, Thermo Fisher), pyruvate (5 mM, Thermo Fisher), glucose (20 mM, Junsei Chemical, Tokyo, Japan)] in the 37 °C non-CO2 incubator for 1 h. We used a Seahorse Bioscience XF24 analyzer (Agilent Technologies) to measure OCR. Oligomycin (1 μM), FCCP (1 μM), and rotenone and antimycin (1 μM) were injected for OCR measurements. All reagents were purchased from Sigma Aldrich. After the measurements, cells were lysed with RIPA buffer (Thermo Fisher) and isolated proteins were quantified with Pierce BCA Protein Assay Kit (Thermo Fisher) for normalization.

**RNA-seq analysis.** RNA from colon polyps of AOM/DSS-treated mice was isolated using mirVana miRNA isolation kit (Thermo Fisher). A library was prepared with 1 μg of total RNA for each sample by TruSeq mRNA Sample Prep kit (Illumina, San Diego, CA). The protocol consisted of polyA-selected RNA extraction, RNA fragmentation, random hexamer-primed reverse transcription, and 100 nt paired-end sequencing by the HiSeq4000 platform (Illumina). The libraries were quantified using qPCR according to the qPCR Quantification Protocol Guide (KAPA Library Quantification kits for Illumina Sequencing platforms) and qualified using the 2100 Bioanalyzer (Agilent Technologies). RNA-seq experiments and statistical analysis were performed by Macrogen, Inc. (Seoul, Korea).

**Western blot.** The colonic epithelial cells from naïve mice or colon polyps from AOM/DSS-treated mice were lysed in RIPA buffer (Thermo Fisher) with protease inhibitor (Sigma Aldrich). Supernatants were collected by centrifugation at 11,000 × g for 10 min. Concentrations of proteins in the supernatant were determined using Pierce™ BCA Protein Assay Kit (Thermo Fisher). Proteins were boiled with Laemmli sample buffer and separated with 10% SDS-PAGE. Proteins were blotted onto a PVDF membrane (Merck Millipore, Burlington, MA). After membranes were blocked with 5% skim milk in TBST for 1 h, they were incubated overnight at 4 °C with the respective primary antibodies against PKM1, PKM2, PKM (clone G103A3, Cell Signaling Technology) or β-actin (Cell Signaling Technology), diluted in blocking buffer. Membranes were washed with TBST buffer and incubated with an appropriate HRP-conjugated secondary antibody (Cell Signaling Technology) for 2 h at RT. Signals were developed with enhanced chemiluminescence (DoGEN, Seoul, Korea) and visualized using ImageQuant LAS 4000 (GE Healthcare, Buckinghamshire, UK). Relative band intensity was normalized to β-actin and quantified by ImageJ 1.48 open source software (https://imagej.nih.gov/ij/).

**Statistics.** GraphPad Prism software (GraphPad, La Jolla, CA) was used for statistical analysis. Significant differences between two groups were analyzed with two-tailed unpaired t-test. Multiple groups were analyzed by one- or two-way ANOVA followed by Bonferroni’s post hoc test (*p < 0.05; **p < 0.01).

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Author Contributions
Y.K. performed experiments and data analysis and wrote the manuscript; Y.-S.L. helped with experiment design and with data analysis; S.W.K., S.K., T.-Y.K., S.-H.L. and J.-S.I. performed data acquisition; S.W.H., J.K. and E.N.K. did histological evaluation; Y.-Y.P. and M.-N.K. contributed to the conception and design of the study and supervised the study.

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