

AMPK promotes the survival of colorectal cancer stem cells

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Abstract

Background: Colorectal cancer (CRC) is the third most commonly diagnosed cancer in males and the second in females worldwide in 2012. In the past 20 years, strong evidence suggests that cancer stem cells are the main culprit of cancer metastasis, chemotherapy resistance, and relapse.

Methods: To further understand the unique biological properties of cancer stem cells and uncover novel molecular targets to eradicate them, we first established a panel of patient-derived xenograft (PDX) tumor models using tumors surgically removed from human colorectal cancer patients. We then isolated CRC cancer stem cells based on their ALDH activity using fluorescent-activated cell sorting (FACS) and characterized their metabolic properties.

Results: Interestingly, we found that the CRC cancer stem cells (ie, CRC cells with higher ALDH activity, or ALDH+) express higher level of antioxidant genes and have lower level of reactive oxygen species (ROS) than non-CRC cancer stem cells (ie, CRC cells with lower ALDH activity, or ALDH-). The CRC cancer stem cells also possess more mitochondria mass and show higher mitochondrial activity. More intriguingly, we observed higher AMP-activated protein kinase (AMPK) activities in these CRC cancer stem cells. Inhibition of the AMPK activity using 2 AMPK inhibitors, Compound C and Iodotubercidin, preferentially induces cell death in CRC cancer stem cells.

Conclusion: We propose that AMPK inhibitors may help to eradicate the CRC cancer stem cells and prevent the relapse of CRCs.

KEYWORDS

AMP-activated protein kinase, cancer metabolism, colorectal cancer stem cells, patient-derived xenograft

1 | INTRODUCTION

Colorectal cancer (CRC, or carcinoma of the colon and rectum) is one of the most commonly diagnosed and deadly cancers worldwide.¹ In the US, there are estimated more than 135 000 new cases of CRCs and more than 50 000 deaths due to CRCs in 2017.² The large majority of CRCs are carcinomas and 90% of the carcinomas are adenocarcinomas. Early diagnosis and surgery have dramatically improved the survival of low grade Stage I and II CRCs; however,

the 5-year survival rate for distal metastatic CRCs (at or beyond Stage III) is around 10%, which are often treated only by palliative chemotherapy.³ Therefore, there are still urgent needs to find new diagnostic and therapeutic strategies for distal metastatic CRCs.

Application and connection of stem cell concept to cancers were proposed many decades ago.⁴ In recent years, research on cancer stem cell (CSC) was re-energized first by Dick's group in leukemia and later by Clarke's group in breast cancers.^{5,6} The hypothesis of CSC states that, similar to a normal stem cell, a single stem cell-like cancer cell is

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capable of regenerating a tumor. The CSC concept is very helpful in explaining a variety of biological and clinical observations. For example, even though there are often genetic variations among the cancer cells within the same patient,^{7,8} the large majority of genetic changes are maintained among them,⁹ suggesting that most tumors may be originated from one or a few CSCs. After initial shrinkage of tumors through chemotherapy or radiotherapy, chemo-resistant or radio-resistant tumors almost inevitably appear sometime later on, suggesting that there is a small population of cells likely resistant to most of current therapies. Therefore, a better understanding of the biology of CSC will uncover new strategies to diagnose tumors earlier, treat them more efficaciously, and prevent them from relapses.

It was noted by Otto Warburg nearly a century ago that tumors showed abnormal features of energy metabolism.¹⁰ In the past decade or so, cancer metabolism has attracted renewed interests due to the development of novel biochemical and molecular biological tools and becomes one of the emerging hallmarks of all cancers.^{11,12} Cancer cells are under constant metabolic stress due to their rapid proliferation and often residing in a poorly regulated microenvironment with aberrant blood vesicles.¹² One of the key regulators of metabolic stress is AMP-activated protein kinase (AMPK), which coordinates a variety of cellular pathways to balance the energy and nutrient homeostasis.^{13,14}

Here, we investigated the metabolic properties of cancer stem cells in human colorectal cancers (CRC-CSCs). We first established 6 patient-derived xenograft (PDX) colorectal cancer models using surgically removed human colorectal tumors. We then isolated CRC-CSCs based on their higher aldehyde dehydrogenase (ALDH) activities using fluorescent-activated cell sorting (FACS). We showed that CRC-CSCs express higher level of antioxidant genes and contain less reactive oxygen species (ROS). The CRC-CSCs also possess more mitochondria and higher levels of ATP. Furthermore, we demonstrated that CRC-CSCs manifested higher AMPK activity and inhibition of AMPK preferentially kills the CRC-CSCs, suggesting that targeting AMPK may be an effective way to eradicate CRC-CSCs, thus preventing the relapse of CRCs.

2 | MATERIALS AND METHODS

2.1 | Antibodies and chemicals

ABCG2 (AbCam, ab3380); CD133 (Miltenyl Biotec, W6B3C1); ALDH1A1 (AbCam, ab52492); Acetyl-CoA Carboxylase (Cell Signaling, 3676); Phospho-Acetyl-CoA Carboxylase (Ser79) (Cell Signaling, 3661); AMPK (Cell Signaling, 2532); Phospho-AMPK α (Thr172)(Cell Signaling, 2531); EZH2 (Bethyl, A304-197A); GAPDH (Bethyl, A300-641A); Actin (Santa Cruz, sc-1616); Complex II (MitoScience, MS204); Complex IV (MitoScience, MS404); Compound C (Calbiochem, 171260); Iodotubercin (Sigma, I100); MitoSox (Invitrogen, M36008); Mitotracker Red (Invitrogen, M7512).

2.2 | Digestion of tumors and FACS

Finely minced tumors were resuspended in 100 mM phosphate buffer (pH7.0) with 8 mM DTT for 15 minutes at room

temperature. Tumor fragments were washed once with ice-cold Hank's balanced salt solution (HBSS) without Ca²⁺/Mg²⁺ and digested with collagenase III (Worthington: 58E10543), DNase I and hyaluronidase (Worthington: 58M10759) in serum-free RPMI 1640 medium (with L-glutamine) containing 1X penicillin/streptomycin/amphotericin-B, 20 mM HEPES at 37°C for 1 hour, or at 4°C overnight. Tumor cells were then filtered twice through a 40 μ M Steriflip filter (Millipore: SCNY00040) and then twice with a 20 μ M Steriflip filter (Millipore: SCNY00020). Cells were pelleted and resuspended in HBSS and Red Blood Cell Lysis Buffer (Roche: 11-814-389-001) (1:2) and incubated on ice for 5 minutes. Cells were pelleted, resuspended in cold HBSS/2% heat-inactivated fetal calf serum (HIFCS) and then separated using Ficoll-Paque Premium (GE Healthcare: 17-5442-02). Purified cells were pelleted and then resuspended in cold HBSS/3% HIFCS.

Right before ALDH FACS, cells were first washed twice with ALDEFLOUR buffer and then resuspended in ALDEFLOUR buffer in a concentration of 0.5-1 \times 10⁶ cells/mL. Cells were subsequently labeled and processed using the ALDEFLOUR kit (STEMCELL technologies, 01700). Mouse cells were labeled and excluded during FACS with mouse antibody mixtures, which are made up of equal parts of biotin mouse anti-mouse H-2K SF1-1.1 (BD Bioscience: 553564), PE-CyTM5 streptavidin (BD Bioscience: 554062), and PE-CyTM5 rat anti-mouse CD45 (BD Bioscience: 553082). 7-AAD staining (BD Bioscience: 559925) was used to exclude dead and apoptotic cells. Tumor cells were finally sorted and collected on a FACSAria II with a 100-micron nozzle.

2.3 | Gene expression profiling

RNA was extracted from ALDH-sorted cells using the QIAGEN RNeasy Mini Kit. A portion of 10 μ g RNA was used for the synthesis of cDNA using the Superscript Kit (BRL), which was then purified using the GeneChip sample cleanup module from Affymetrix. Biotinylated cRNA was then synthesized using the GeneChip Expression 3'-Amplification Reagent kit (Affymetrix), purified using the RNeasy columns, and finally hybridized to Human Genome U133 + 2 GeneChip oligonucleotide arrays (Affymetrix). The GeneChip was subsequently stained with streptavidin R-phycoerythrin (Molecular Probes) using the GeneChip[®] Fluidics Station 450 and scanned with an Affymetrix GeneChip Scanner 3000. Microarrays were analyzed using MicroArray Suite 5.0 software. Data analysis was done as previously described.¹⁵

2.4 | Quantitation of mitochondrial DNA

DNA was extracted from ALDH FACS-sorted cells using the QIAamp DNA Blood Mini Kit (QIAGEN, 51106). Quantitation of mitochondrial DNA and nuclear DNA was done as described in.¹⁶ An Applied Biosystem Real-time PCR machine was used for the real-time PCR analysis.

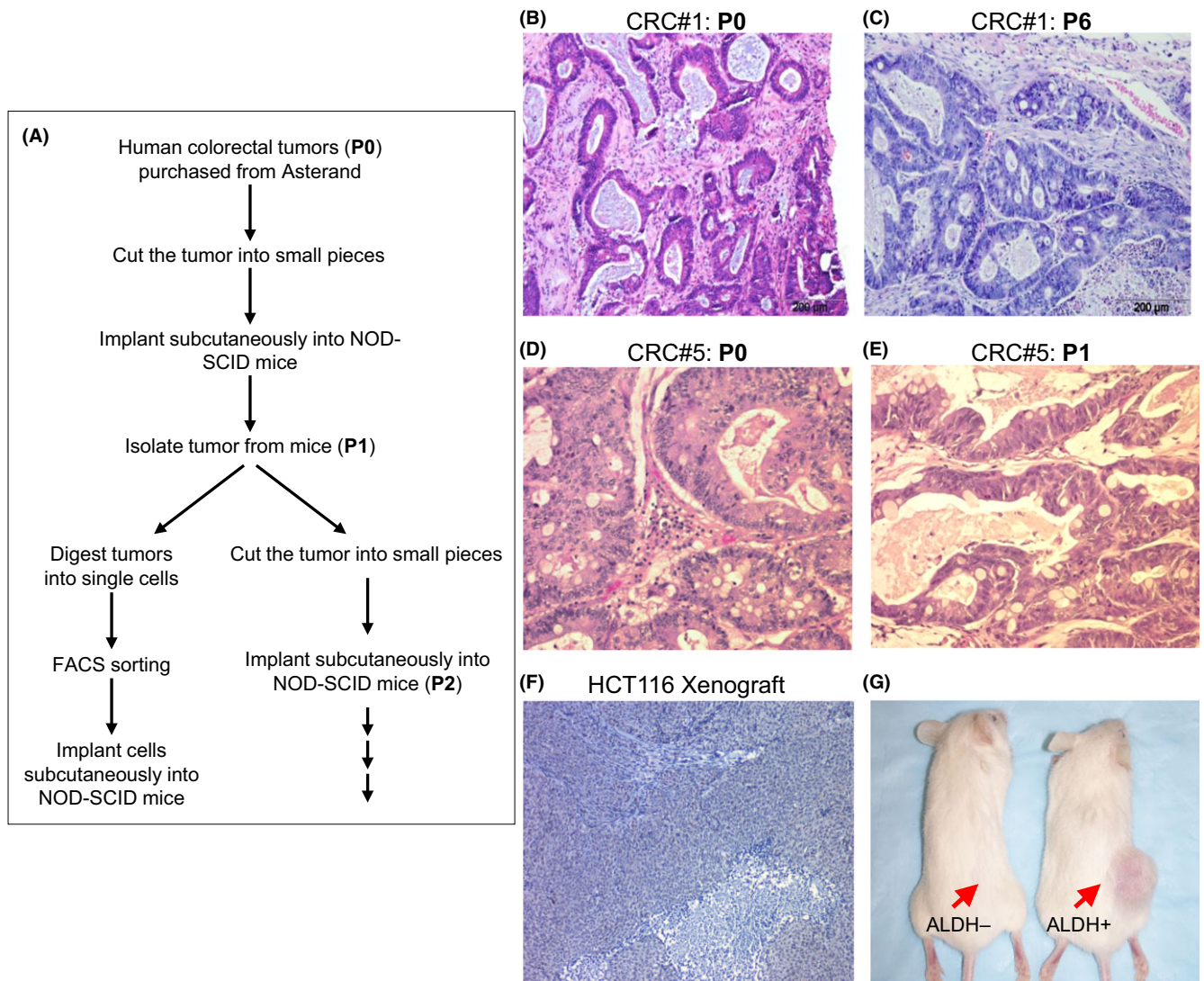


FIGURE 1 Human colorectal tumors implanted into NOD-SCID mice preserve most of the tumor cell hierarchy. A, A flow chart of processing colorectal tumors. (B and C) H&E staining of CRC#1 at Passage 0 (P0) and Passage 6 (P6). (D and E) H&E staining of CRC#5 at Passage 0 (P0) and Passage 1 (P1). F, H&E staining of xenograft tumors generated from a colorectal cancer cell line, HCT116. G, ALDH FACS-sorted CRC#2 cells were subcutaneously injected into the flank of NOD-SCID mice

TABLE 1 Summary of patient information

	CRC #1	CRC #2	CRC #3	CRC #4	CRC #5	CRC #6
Age/Sex/Race	49/Female/Caucasian	84/Male/?	76/Male/Caucasian	83/Female/?	60/Female/Caucasian	82/Female/Caucasian
Tumor type	Adenocarcinoma of the colon	Adenocarcinoma of the colon	Adenocarcinoma of the colon	Mucinous adenocarcinoma of the colon	Adenocarcinoma of the large intestine	Adenocarcinoma of the large intestine
AJCC/UICC stage	T3N2M0	T3N1MX	T3N1MX	T4N0MX	T4N0MX	T3N2MX
AJCC/UICC stage group	IIIC	IIIB	IIIB	IIIB	IIIB	IIIC

AJCC, American Joint Committee on Cancer (cancerstaging.org/Pages/default.aspx); UICC, Union for International Cancer Control (www.uicc.org); T-primary tumor, N-regional lymph node, M-distal metastasis; ?, patient's ethnicity was not identified at the time of surgery.

2.5 | Mitochondria Complex activity assay

10 000 to 40 000 ALDH-sorted cells were used for Complex I (MitoScience, MS130) and Complex IV (MitoScience, MS430) assays. Assays were done as recommended by the manufacturer.

2.6 | Quantitation of cellular ATP

Cellular ATP was measured using the ATP Determination Kit (ThermoFisher, A22066). 1000 to 5000 ALDH-sorted cells were used for ATP measurement as recommended by the manufacturer. A Perkin Elmer Victor plate reader was used to measure the luminescence.

3 | RESULTS

3.1 | Isolation and validation of cancer stem cells in human colorectal tumors

In order to isolate CRC-CSCs, we first purchased a panel of human colorectal tumor samples from Asterand Bioscience. We implanted small pieces of tumors into nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice within 24-48 hours after the surgery (Figure 1A). Within a year, we successively established 6 PDX CRC lines, which are designated as CRC#1 to CRC#6 (Table 1). The surgeries for all 6 CRC patients, 2 males and 4 females, were performed in US hospitals. The median age of the 6 patients was 79 years old. For the ethnicity of the patients, 4 were Caucasian and 2 were not identified at the time of their surgeries. Based on the cancer classification of American Joint Committee on Cancer (AJCC) and Union for International Cancer Control (UICC), there are 2 Stage II colorectal cancers (CRC#4 and #5) and 4 Stage III colorectal cancers (CRC#1, #2, #3, and #6). The tumor, node, and metastasis (TNM) staging for the 6 patients is also listed in Table 1. Similar to other reports, we found that the tumor cell hierarchy is well preserved in PDX (Figure 1B-E). In stark contrast, the xenograft tumor generated using established colorectal cancer cell line, HCT116, completely lacks tumor cell hierarchy (Figure 1F), suggesting that the PDX tumors more resemble the pathology of the original patient tumors than that from the established cancer cell line.

Most studies isolated CRC-CSC using FACS based on either the expression of cell surface proteins, for example CD133 and CD44, or ALDH activity.^{17,18} We have sorted PDX CRC cells using FACS based on higher expression of CD133, CD44, and higher ALDH activity and then subcutaneously implanted them into NOD-SCID mice to test their tumorigenicity. We found that FACS-sorted CRC cells based on their ALDH activity more consistently produce tumors (Figures 1G and S1, Table 2). Therefore, we chose ALDH FACS as our method of isolating CRC-CSCs. A representative ALDH FACS profile of CRC cells is shown in Figure 2A. We first validated the ALDH+ CRC cells as the CRC-CSC by injecting serial diluted ALDH+ cells subcutaneously into NOD-SCID mice. As shown in Table 2, as few as 50 ALDH+ cells from CRC#2 are sufficient to regenerate CRC tumors in 7 out of the 10 mice injected. In general, 2000

TABLE 2 Tumorigenic activity of ALDH-sorted colorectal cancer cells

# Cells/mouse	CRC #1	CRC #2	CRC #4	CRC #5	CRC #6
ALDH+					
10 000			3/4	4/5	5/5
2000	10/10	10/10	1/5	2/5	5/5
200		7/10			
50		7/10			
ALDH-					
10 000		0/5	0/5	0/5	2/5 (very small)
2000	3/10 (very small)	0/10	0/5	0/5	0/5

ALDH+ cells are sufficient to regenerate tumors. Interestingly, the ability of 2000 ALDH+ cells to regenerate tumors seems to correlate pretty well with their Stage ranking: the higher the tumor Stage, the higher the tumor-take rate in mice. For example, for the 3 Stage III CRC lines (CRC#1, #2, and #6), the tumor-take rate of 2000 ALDH+ cells is 100%. The tumor-take rate for the 2 Stage II lines, CRC#4 and CRC#5, is only 20% and 40%, respectively. In stark contrast, 2000 to 10 000 ALDH- cells from the corresponding tumor either failed to regenerate any tumors (Figure S1 and Table 2), or produced tumors that are much smaller than that from the ALDH+ cells (CRC#1 and #6 in Table 2). For unknown reasons, tumors derived from ALDH+ cells of CRC#3 grew much slower than others. Therefore, we did not perform the serial dilution experiment using CRC#3. In summary, our data demonstrated that a few of ALDH+ cells FACS sorted from most CRCs are sufficient to regenerate tumors in mice, suggesting that, in most CRCs, cancer cells with higher ALDH activity are potentially the cancer stem cells.

Another essential feature of CSCs is that, once injected into mice, they should produce a spectrum of cells similar to the pool of tumor cells that they were originally isolated from (ie, the Differentiation Test). To test the differentiation ability of the ALDH+ cells, we sorted ALDH+ cells from CRC#2 and then subcutaneously reimplanted them back into NOD-SCID mice. When the tumors grew up, we reanalyzed their ALDH profile. As seen in Figure 2B, the new tumors (labeled as F1-1 and F1-2) showed ALDH profile almost identical to the parental tumor (labeled as Parental), indicating the ALDH+ cells from the parental tumor not only maintain the ALDH+ population, but also are capable of differentiating to ALDH- cells in the next passage of tumors. Our data demonstrated that the ALDH+ cells isolated from PDX tumors have the ability to differentiate into ALDH- cells, thus satisfying the Differentiation Test.

To further confirm that the ALDH+ CRC cells are CRC-CSCs, we examined the commonly used stem cell/cancer stem cell markers in them. As shown in Figure 2C (RNA) and 2D (protein), ALDH+ CRC cells indeed express higher levels of stem cell/cancer stem cell markers than the ALDH- CRC cells. A member of the ATP-binding cassette (ABC) superfamily of transporters, ABCG2, was previously proposed to be a potential CSC maker.¹⁹ However, in the 3 CRC lines examined, the ALDH- cells actually expressed

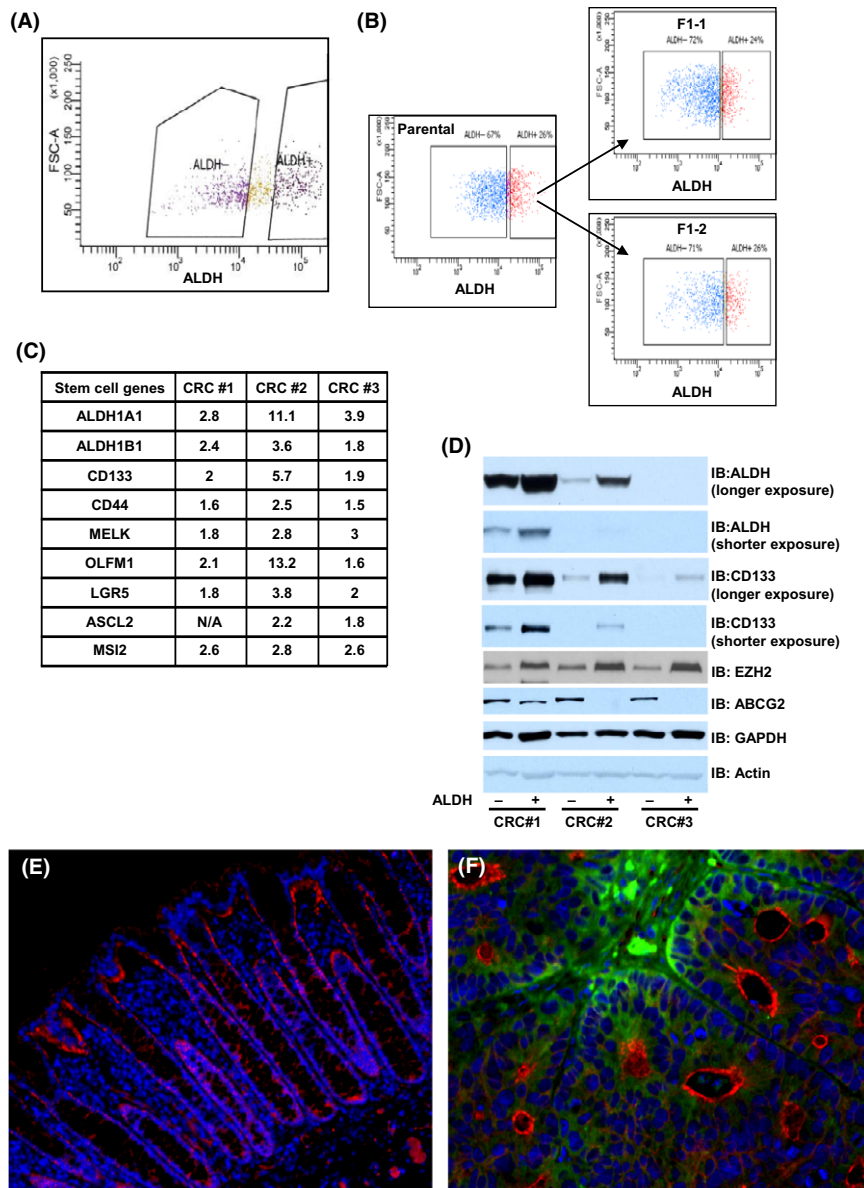


FIGURE 2 ALDH⁺ CRC cells manifest many features of cancer stem cells. A, A representative ALDH FACS profile. B, ALDH⁺ cells are capable of regenerating both ALDH⁻ cells and ALDH⁺ cells in mice. ALDH⁺ cells sorted from a CRC#2 tumor (labeled as Parental) were injected subcutaneously into the flank of NOD-SCID mice. After they grew up, tumors were isolated from 2 mice (F1-1 and F1-2) and then reanalyzed by flow cytometry. C, Increased expression of selected stem cell/cancer stem cell genes in ALDH⁺ cells. The numbers listed indicate the ratio between the relative RNA levels from ALDH⁺ cells vs that from ALDH⁻ cells. D, ALDH⁺ cells express higher level of stem cell/cancer stem cell protein biomarkers than ALDH⁻ cells. Proteins from equal numbers of ALDH FACS-sorted cells were analyzed by Western Blot. The antibodies used for immunoblotting are indicated on the right. (E and F) normal colon (E) or CRC#2 tumor (F) was stained with antibodies recognizing ABCG2 (red), and/or CD133 (green). Nuclei were stained with DAPI (blue)

higher level of ABCG2 than the ALDH⁺ cells by immunoblotting (Figure 2D). We also performed immunofluorescent staining using normal colon tissues and found that ABCG2 expression is higher in cells residing in the upper part of the colon crypts, most of which are differentiated cells (Figure 2E). Furthermore, when CRC tumor samples were costained with antibodies recognizing ABCG2 and CD133 (Figure 2F), most ABCG2-positive cells are not positive of CD133, suggesting that the ABCG2-expressing cells most likely belong to the non-CRC-CSCs, or differentiated cells.

Therefore, ABCG2 could be used as a marker for differentiated cells within the colon and/or rectum.

3.2 | Colorectal cancer stem cells possess unique metabolic properties

To investigate the metabolic properties of the CRC-CSC, we first compared the gene expression profile of ALDH⁺ cells with that of ALDH⁻ cells with a focus on metabolism-related genes. When we

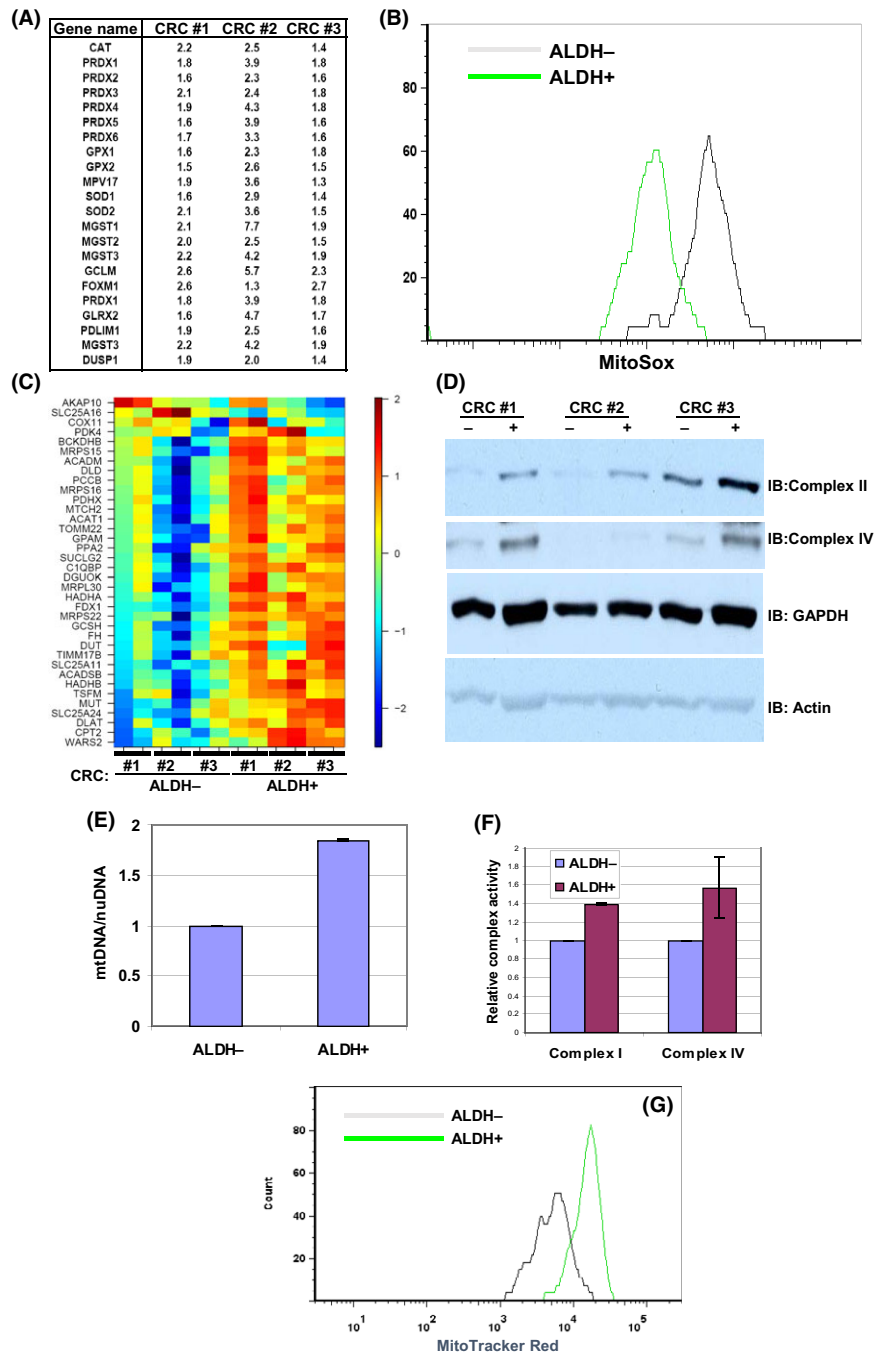


FIGURE 3 ALDH+ CRC cells possess more mitochondria but contain low level of ROS. A, Increased expression of antioxidant genes in ALDH+ cells. The numbers listed in the table are the ratio between the relative RNA levels from ALDH+ cells and that from ALDH- cells for each tumor. B, ALDH FACS-sorted cells were first labeled with MitoSox and then analyzed by flow cytometry. C, Heat-map of mitochondrial genes. D, Increased expression of mitochondrial proteins in ALDH+ cells. Proteins from equal numbers of ALDH FACS-sorted cells were analyzed by Western Blot. The antibodies used for immunoblotting are indicated on the right. E, ALDH+ cells from CRC#2 contained more mitochondrial DNA than ALDH- cells. F, ALDH+ cells from CRC#2 showed higher ETC. complex activity than ALDH- cells. G, ALDH+ cells from CRC#2 showed higher mitochondrial membrane potential than ALDH- cells. ALDH FACS-sorted cells were first labeled with MitoTracker Red and then analyzed by flow cytometry. All error bars are standard deviation obtained from 3 different experiments

performed pathway analysis of metabolic genes that showed differential expression between ALDH+ cells and ALDH- cells, we noted that many antioxidant genes are upregulated in ALDH+ cells (Figure 3A), suggesting that ALDH+ cells may have reduced level of reactive oxygen species (ROS). To directly measure the amount of

ROS in ALDH FACS-sorted cells, we first labeled them with MitoSox and then analyzed the incorporation of MitoSox using a flow cytometer.²⁰ The amount of MitoSox incorporated into a cell is proportional to its ROS level. As shown in Figure 3B, ALDH+ cells indeed contain less ROS than ALDH- cells.

In the pathway analysis, we also noticed that ALDH⁺ cells express considerably higher level of genes involved in mitochondrial biogenesis (Figure 3C). When we examined the mitochondrial protein (Figure 3D) as well as the mitochondrial DNA (Figure 3E) in ALDH FACS-sorted cells, we found that ALDH⁺ cells indeed contain more mitochondria than the ALDH⁻ cells. Consistently, ALDH⁺ cells also showed higher Electron Transportation Chain (ETC.) complex activity (Figure 3F) and higher membrane potential (Figure 3G), as measured by the increased incorporation of MitoTracker. Since mitochondrion is the major energy producing organelle, not surprisingly, ALDH⁺ cells also contain more ATP (Figure 4A). Intriguingly, this seems to be true only for the ALDH⁺ cells sorted from PDX colorectal tumors because the ATP level is the same between the ALDH⁺

cells and the ALDH⁻ cells sorted from a human colorectal cancer cell line, Colo205 (Figure 4B).

In summary, we demonstrated that CRC-CSCs contain more mitochondria and produce more ATP yet show reduced level of ROS, likely because they express higher level of antioxidant genes than the non-CRC-CSCs.

3.3 | Increased AMPK activity in CRC-CSCs promotes their survival

AMPK is often considered a low-energy sensor because it is robustly activated by the binding of AMP to its regulatory subunit when more cellular ATP is converted to AMP.¹³ Since we observed that

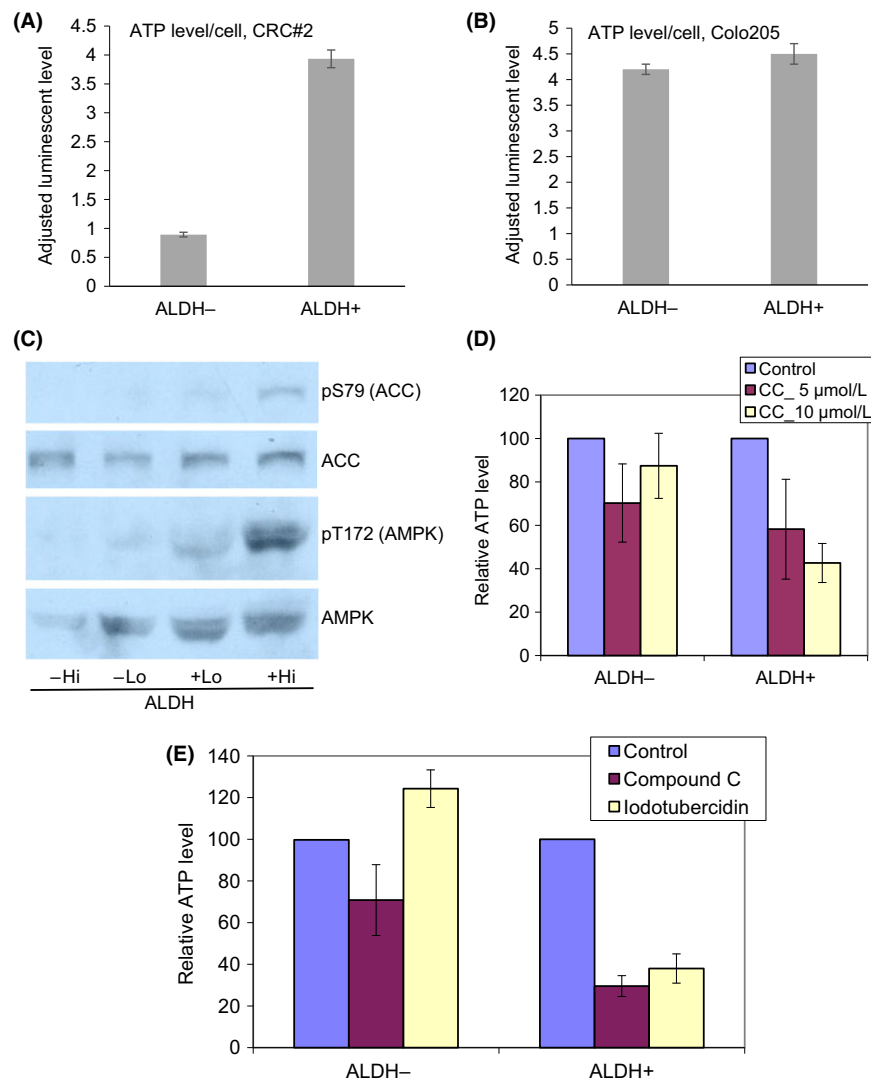


FIGURE 4 Increased AMPK activity in ALDH⁺ cells contributes to their survival. (A and B) ATP level in ALDH FACS-sorted cells from either CRC#2 (A), or from a colorectal cancer cell line, Colo205 (B). C, Increased AMPK activity in ALDH⁺ cells. CRC#2 tumor cells were FACS sorted into 4 fractions based on their ALDH activity: -Hi < -Lo < +Lo < +Hi. Proteins from equal numbers of ALDH FACS-sorted cells were analyzed by Western Blot. The antibodies used for immunoblotting are indicated on the right. (D and E) AMPK inhibitors preferentially target ALDH⁺ cells. Equal numbers of ALDH FACS-sorted cells from CRC#2 were treated with 5 μM or 10 μM Compound C, or 10 μM Iodotubercidin, for 24 h. Cellular ATP was then quantified and normalized against DMSO (Control)-treated samples as a reflection of cell viability. All error bars are standard deviation obtained from 3 different experiments

ALDH⁺ cells contain 4 times more ATP than ALDH⁻ cells (Figure 4A), we reasoned that the AMPK activity should be much lower in the ALDH⁺ cells. To our surprise, when we examined the AMPK activity by monitoring the phosphorylation of the Tyrosine-172 of its catalytic subunit, which is required for its activity,²¹ as well as the phosphorylation of one of its best-known targets, acetyl-CoA carboxylase (ACC) at Serine-79,²² we found that the ALDH⁺ cells actually showed the highest AMPK activity (Figure 4C). Next, we tested whether the higher AMPK activity is important for the cell viability of CRC-CSCs. We treated FACS-sorted ALDH⁺ cells and ALDH⁻ cells with 2 different AMPK inhibitors, Compound C²³ and Iodotubercidin,²⁴ and monitored cell viability based on their ATP level. Amazingly, inhibition of AMPK preferentially affected the viability of ALDH⁺ cells (Figure 4D and 4E), suggesting that activated AMPK promotes the survival of CRC-CSCs.

4 | DISCUSSION

To investigate the metabolic properties of cancer stem cells, we first isolated cancer stem cells in human colorectal cancers based on their ALDH activity and validated their identities by reimplanting them subcutaneously into the NOD-SCID. For all 6 PDX CRC lines, 2000 ALDH⁺ cells are sufficient to regenerate tumors. Amazingly, for one of the lines, CRC#2, even as few as 50 ALDH⁺ cells were sufficient to regenerate tumors. The tumor-take rate of ALDH⁺ cells seems to correlate nicely with patient's tumor malignancy, ie, the higher grade of the patient's CRC, the more potent of its cancer stem cells in regenerating tumors. This could potentially be used to classify how malignant the patient's CRC is. Considering the ALDH⁺ cells as CRC-CSCs, we then investigated their metabolic properties. We found that CRC-CSCs contain more mitochondria and produce more cellular ATP than the non-CRC-CSCs, yet they were able to keep the amount of ROS at a lower level. The CRC-CSCs likely achieve this by upregulating many antioxidant genes. Most intriguingly, we found that CRC-CSCs manifested higher AMPK activities that are very important for their viability. Consistent with our findings, Nakada and colleagues recently showed that activation of AMPK protects leukemia initiating cells and promotes leukemogenesis.²⁵ Together, these data suggest that AMPK inhibitors may selectively target the cancer stem cells.

Here, we uncovered 3 unique metabolic features of CRC-CSCs: higher AMPK activity, higher mitochondria mass/activity, and lower ROS. Based on existing evidence, it is very likely that the higher AMPK activity contributes to the increased mitochondrial mass/activity and lower ROS in the CRC-CSCs. For example, using primary mouse muscle cells and PGC1 α genetic knockout mice, Spiegelman and colleagues demonstrate that deletion of PGC1 α almost completely abolished the positive effects of AMPK on mitochondrial biogenesis and function.²⁶ Intriguingly, in our gene expression analysis, we found that PGC1 α , but not PGC1 β , is robustly upregulated in ALDH⁺ cells in all 3 CRC lines (Figure S2). Therefore, it is possible that under the metabolically stressed microenvironment, CRC-CSCs activate AMPK, which then induces the expression of PGC1 α and

then promotes mitochondrial biogenesis. Increased mitochondrial mass/activity would provide CRC-CSCs with ample nutrients and energy for their proliferation.^{11,14} In addition, AMPK also plays an important role in attenuating the cellular ROS in cancer cells. Recently, Hay and colleagues showed that AMPK increases NADPH level by downregulating one of the major NADPH consuming pathways — fatty acid synthesis pathway.²⁷ NADPH is required to synthesize a variety of antioxidants, including glutathione. In addition, Bae and colleagues showed that inactivation of AMPK in pancreatic cancer cells increased intracellular ROS, which then led to DNA damage and cell death.²⁸ Finally, Jones and colleagues demonstrated that in mouse embryonic fibroblasts, depletion of the catalytic subunit of AMPK increased the level of ROS while treatment of MEFs with AMPK activator attenuated cellular ROS.²⁹ Therefore, activated AMPK protects cancer stem cells by reducing their exposure to high level of intrinsic or extrinsic ROS, accumulation of which could be very harmful to their DNA and proteins.

It has been noted that AMPK may play a dual role during tumorigenesis depending on the context. Under certain conditions and/or at certain stage of tumorigenesis, AMPK may play a role in preventing tumor formation.^{30,31} However, more and more evidence indicate that AMPK is probably more important for the survival of cancer cells and cancer stem cells by reprogramming their metabolism in order to adapt to their uncontrolled proliferating nature and better cope with the intrinsic and extrinsic metabolic stress.³⁰⁻³⁴ Therefore based on the latter role of AMPK, we propose that inhibition of AMPK may help to eradicate cancer stem cells and prevent tumors from metastasizing and relapsing.

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CONFLICT OF INTEREST

None.

AUTHOR CONTRIBUTIONS

All listed authors meet the requirements for authorship. BG and DZ conceived and designed the experiments; BG, XH, DT, SH, and DZ performed the experiments. BG and DZ performed the experiments and wrote the main manuscript text. BG, XH, and DZ performed the tumor transplant, processed the tumors. BG and DZ performed the FACS analysis. All authors have read and approved the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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