Metabolic Reprogramming of Cancer-Associated Fibroblasts by IDH3α Downregulation

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Highlights
- IDH3α is uniformly decreased in CAFs
- Downregulation of IDH3α promotes metabolic switch to glycolysis in CAFs
- Decreased IDH3α reduces the ratio of α-KG to fumarate and succinate
- Reduced α-KG leads to the stabilization of HIF-1α under normoxic conditions

Graphical Abstract

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In Brief
Zhang et al. provide conclusive evidence that CAFs are prone to glycolysis and that this metabolic change is responsible for the tumor-promoting effect of CAFs. In addition, their study identifies a detailed molecular mechanism underlying glycolytic reprogramming in CAFs.

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Metabolic Reprogramming of Cancer-Associated Fibroblasts by IDH3α Downregulation

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SUMMARY

Cancer-associated fibroblasts (CAFs) provide critical metabolites for tumor growth and undergo metabolic reprogramming to support glycolysis. However, the molecular mechanisms responsible for this change remain unclear. Here, we report that TGF-β1- or PDGF-induced CAFs switch from oxidative phosphorylation to aerobic glycolysis. We identify down-regulation of isocitrate dehydrogenase 3α (IDH3α) as a marker for this switch. Furthermore, miR-424 downregulates IDH3α during CAF formation. Down-regulation of IDH3α decreases the effective level of α-ketoglutarate (α-KG) by reducing the ratio of α-KG to fumarate and succinate, resulting in PHD2 inhibition and HIF-1α protein stabilization. The accumulation of HIF-1α, in turn, promotes glycolysis by increasing the uptake of glucose, upregulating expression of glycolytic enzymes under normoxic conditions, and inhibiting oxidative phosphorylation by upregulating NDUFA4L2. CAFs from tumor samples exhibit low levels of IDH3α, and overexpression of IDH3α prevents transformation of fibroblasts into CAFs. Our studies reveal IDH3α to be a critical metabolic switch in CAFs.

INTRODUCTION

The development and progression of tumors are controlled not only by tumor cells but also by their surrounding stromal cells (Carmeliet and Jain, 2000; Rennov-Jessen et al., 1996; Tlsty, 2001). Cancer-associated fibroblasts (CAFs) are activated fibroblasts that form a large component of tumor stromal cells (Cousens and Werb, 2002; Kalluri, 2003; Rennov-Jessen et al., 1995). Through specific communications with cancer cells, CAFs directly promote tumor initiation (Bhowmick et al., 2004; Orimo et al., 2009), progression (Dimanche-Boitrel et al., 1994; Orimo et al., 2005), and metastasis (Grum-Schwensen et al., 2005; Olaso et al., 1997). CAFs produce ECM-degrading enzymes and secrete growth factors and cytokines, which collectively promote tumor development and progression (Boire et al., 2005; Ding et al., 2010).

Increasing evidence suggests that CAFs also can secrete metabolites to fuel the growth of tumor cells. Previous studies have shown that CAFs secrete large amounts of lactate and ketone bodies, utilized by tumor cells for anabolic metabolism or oxidative phosphorylation (Fiaschi et al., 2012). In addition, β-hydroxybutyrate, one of the ketone bodies, increased cancer cell proliferation approximately 3-fold compared to the control group, and lactate promoted angiogenesis in tumor model (Bonuccelli et al., 2010a; Fiaschi et al., 2012). However, it remains unclear why CAFs are prone to glycolysis and which mechanism(s) controls this metabolic reprogramming. We previously found that CAFs can be induced in vitro from fibroblasts (Li et al., 2013), which provides a unique system to investigate the factors that drive the metabolic transformation of fibroblasts to CAFs.

Here, we report that a reduction in the expression of the α subunit of the isocitrate dehydrogenase 3 complex (IDH3α) is associated with the metabolic switch from oxidative phosphorylation to glycolysis, and that overexpression of IDH3α prevents fibroblasts from transforming into CAFs. A decrease in IDH3α reduces the ratio of α-ketoglutarate (α-KG) to succinate and fumarate, resulting in a stabilization of HIF-1α, which, in turn, promotes glycolysis in CAFs. Our results identified IDH3α as the key molecular switch for glycolysis, and they provide an insight into the initiation of the Warburg effect in CAFs, a critical stromal component in tumorigenesis.

RESULTS

CAFs Are Prone to Glycolysis and Enhance Tumor Cell Growth

We and others showed previously that TGF-β and PDGF can promote the formation of CAFs from fibroblasts. For example,
PDGF can efficiently induce the conversion of fibroblasts to CAFs, evidenced by the expression of FSP1, a specific marker of CAFs (Figure S1A). TGF-β treatment in fibroblasts provides similar effect (data not shown). When melanoma A375 cells were co-cultured (1:1) with control fibroblasts or CAFs (TGF-β induced), CAFs induced a greater increase in A375 cell proliferation than did control fibroblasts (Figure S1B). However, pretreatment with the glycolysis inhibitor 2-deoxyglucose (2-DG) significantly compromised this growth-promoting effect. Interestingly, CAFs had an increased uptake of 2-DG compared to control fibroblasts, suggesting that CAFs have a higher capacity for glucose uptake than do fibroblasts (Figure S1C).

To extend this observation in vivo, we mixed A375 cells with control fibroblasts or with PDGF-induced CAFs, and injected them, respectively, into the abdominal or forelimb amput of nude mice; this injection protocol eliminates variation among mice to ensure comparable results in different groups. As expected, PDGF-induced CAFs did not form tumors in vivo, and co-injection with fibroblasts did not increase tumor growth (Figure S1D). However, CAFs greatly enhanced the growth of tumor cells in vivo. When A375 cells mixed with isolated primary CAFs (from colon cancer), or with TGF-β-induced CAFs, were injected using the same protocol above, we found that A375 cells mixed with primary CAFs or TGF-β-induced CAFs grew faster than A375 cells mixed with fibroblasts (Figure 1A). Tumors obtained from the isolated primary CAF group showed no significant differences compared to the tumors from the TGF-β-induced CAF group in tumor size. These data demonstrated that the induced CAFs function similarly to the isolated primary CAFs, and both can promote tumor growth in vitro and in vivo.

To investigate the tumor-promoting effect of CAF, we isolated primary fibroblasts from foreskin and treated them with TGF-β or PDGF-BB (Li et al., 2013). We observed that the color of the culture medium turned from pink to orange and pH dropped from 7.8 to 7.1 (Figure 1B). To exclude the possibility that the lower pH was a result of rapid cell growth, we quantified cell proliferation using BrdU and found that the cell proliferation ratio (treated versus non-treated) decreased rather than increased (Figure 1B, right) in TGF-β- or PDGF-treated fibroblasts. These results suggested that induced CAFs produce elevated levels of acidic catabolites compared to fibroblasts.

To determine whether these observations were due to a metabolic switch from oxidative phosphorylation to glycolysis in CAFs, we measured the glucose uptake, lactate production, and oxygen consumption in control fibroblasts and TGF-β- or PDGF-induced CAFs. Lactate measurements, obtained at three time intervals (1, 2, and 3 hr), showed lactate production was increased 2.5-fold in both TGF-β- and PDGF-induced CAFs compared to the control fibroblasts (Figure 1C). Glucose uptake also increased approximately 2-fold in both types of induced CAFs compared to control fibroblasts (Figure 1D). Interestingly, basal oxygen consumption decreased in both types of induced CAFs to approximately 50% of the level of control fibroblasts (p < 0.001) (Figure 1E). Moreover, the mitochondrial function of oxidative phosphorylation in the induced CAFs also was impaired, as reflected by the decrease in oxygen consumption (approximately 55 to 25 pmol/min) and by the decrease of respiratory capacity (approximately 80 to 40 pmol/min). These observations suggest that glucose metabolism has switched from oxidative phosphorylation to glycolysis in both forms of induced CAFs.

**CAFs Have Reduced IDH3α Expression**

To explore the mechanism underlying this metabolic reprogramming, we performed unbiased gene expression profiling of induced CAFs by deep sequencing (GSE61797). Genes that regulate cell metabolism (upregulated or downregulated for more than 2-fold) in CAFs were selected (Figure 2A; Table S1). The two induced CAFs showed similar gene expression changes, suggesting that metabolic reprogramming is a common feature of CAFs. Of the several downregulated enzymes involved in the TCA cycle, isocitrate dehydrogenase (IDH1 and IDH3α) were of particular interest as both were downregulated in the induced CAFs. To extend these observations, we performed western blot analysis and found that the protein level of IDH3α decreased dramatically 2 days after TGF-β treatment (Figure 2B). However, no significant IDH1 downregulation was observed (data not shown). The downregulation of IDH3α correlated with the gain of FSP1 expression, suggesting that the decreased IDH3α expression promotes the transition of fibroblasts to CAFs. A time-course experiment revealed that the IDH3α protein levels started to drop at 12 hr of TGF-β (8 ng/ml) treatment and reached a maximum in 4 days (Figure 2C, left). The downregulation of IDH3α was the result of decreased IDH3α mRNA as examined by real-time PCR (Figure 2C, right). In line with this idea, no change of IDH3α was found in cells after treatment with MG132 or chloroquine (proteasome or lysosome inhibitors, respectively) in TGF-β-treated or untreated fibroblasts (Figure 2D). These data suggest that IDH3α downregulation occurred mainly through transcriptional regulation.

To confirm that IDH3α also is downregulated in the CAFs of human colon cancer and melanoma, we performed immunofluorescence and immunohistochemistry staining and found that IDH3α expression was indeed decreased in CAFs in both colon cancer and melanoma, compared with fibroblasts in normal tissue (Figures 2E and S2A). In addition, CAFs isolated from fresh human colon cancer were characterized with positive expression of F-actin and FSP-1, and exhibited a tumor-promoting effect in vivo (Figure S2B). The CAFs isolated from tumors samples displayed an increased expression of glycolysis-related enzymes, such as hexokinase 2 (HK2) and 6-phosphofructokinase-liver type (PFKL) (Figure 2F). In addition, caveolin (CAV1), a potential onco-protein (Sotgia et al., 2009, 2012), was downregulated in these CAFs. These data demonstrated that the CAFs isolated from clinical samples function similarly to the induced CAFs, display reduced IDH3α expression, and promote tumor growth in vivo.

To determine whether IDH3α downregulation switches oxidative phosphorylation to glycolysis in CAFs, we generated primary fibroblasts with IDH3α either knocked down or overexpressed. We found that IDH3α knockdown increased glucose uptake and lactate production (Figure 2G). In addition, IDH3α knockdown reduced oxygen consumption in fibroblasts (Figure 2I). Other small hairpin RNA (shRNA) targeting IDH3α showed similar results (data not shown). In contrast, IDH3α overexpression not only reduced the basal level of lactate production but also...
Figure 1. Cell Metabolism Switches to Glycolysis during CAF Formation

(A) Tumor growth-promoting effects of isolated CAFs and induced CAFs as shown in the growth curve plot (*p < 0.01; #p < 0.01); the image to the right displays representative tumors.

(B) The pH value changes during CAF formation. (Left) Color of culture medium with or without TGF-β1/PDGF treatment. The first two plots display pH values with or without the indicated treatments (*p < 0.05). The last two plots show the relative proliferation ratio (proliferation value in induced CAFs divided by the proliferation value in control fibroblast) with indicated treatments.

(C) Lactate production in induced CAF cells, with the concentration measured by colorimetric method at the indicated time points (1, 2, and 3 hr) (*p < 0.001). The last two plots display pH values with or without the indicated treatments (*p < 0.05). The last two plots show the relative proliferation ratio (proliferation value in induced CAFs divided by the proliferation value in control fibroblast) with indicated treatments.

(D) Glucose uptake in fibroblasts or TGF-β1-induced CAFs with or without treatment of [3H] labeled 2-DG.

(E) Basal oxygen consumption, oxidative phosphorylation (with oligomycin treatment), and maximum respiration capacity (with FCCP treatment) of fibroblasts. Bar plots show the basal oxygen consumption (mean ± SD) of fibroblasts with or without stimulation (*p < 0.001).
Figure 2. Downregulation of IDH3α Promotes Glycolysis in Fibroblasts

(A) Heat map of gene expression in normal fibroblasts and TGF-β-induced CAFs. A list of the predominant genes involved in glucose metabolism that are up- or downregulated is presented.

(B) Western blot analysis of IDH3α expression in fibroblasts treated with indicated concentrations of TGF-β1.

(legend continued on next page)
Inhibited TGF-β-induced lactate production in fibroblasts (Figure 2H, left). IDH3α overexpression also reduced the basal level of and TGF-β-stimulated glucose uptake (Figure 2H, right). While TGF-β treatment suppressed oxygen consumption in fibroblasts, IDH3α overexpression increased basal oxygen consumption and partially restored TGF-β-mediated downregulation of oxygen consumption (Figure 2J). Taken together, these data demonstrate that IDH3α downregulation is critical for switching energy metabolism from oxidative phosphorylation to glycolysis in CAFs.

**miR-424 Regulates IDH3α Expression**

To determine the mechanism of IDH3α downregulation in TGF-β-induced CAFs, we utilized Starbase, a web-based software, to predict microRNAs (miRNAs) targeting the IDH3α 3'UTR. We also analyzed miRNA expression in CAFs by deep sequencing. Both approaches lead to the identification of miR-181a and miR-424 for targeting IDH3α (Figure 3A). However, only miR-424 reduced firefly luciferase in the IDH3α 3'UTR luciferase reporter assay. When the four nucleotides (Figure 3B, indicated in red) in the IDH3α 3'UTR that were predicted to associate with miR-424 were mutated, miR-424 lost the ability to inhibit firefly luciferase expression. These data suggest that miR-424 is involved in IDH3α downregulation. Moreover, the miR-424 level was increased in fibroblasts after 12 hr of TGF-β treatment (Figure 3A, bottom), indicating that miR-424 mediates the TGF-β-induced IDH3α downregulation.

To confirm that miR-424 regulates IDH3α expression, specific oligomers targeting miR-424 were transfected into fibroblasts. Knockdown of miR-424 increased the level of IDH3α (Figure 3C), whereas miR-424 overexpression reduced the IDH3α in fibroblasts (Figure 3C, middle). Moreover, miR-424 knockdown prevented the TGF-β-induced IDH3α downregulation (Figure 3C, right). These data indicate that miR-424 inhibits IDH3α expression and is responsible for TGF-β-induced IDH3α downregulation in CAFs.

To determine whether miR-424 regulates glycolysis in fibroblasts, we analyzed glycolysis-related enzymes by quantitative PCR in miR-424-overexpressing fibroblasts. miR-424 overexpression increased the expression of glycolysis-related enzymes, including GLUT1, HK2, and PFKM. In contrast, expression of IDH3α decreased the expression of these enzymes (Figure 3D, top left). Rescue expression of IDH3α also blocked the miR-424-induced upregulation of these molecules. We also investigated lactate production, glucose uptake, and oxygen consumption in fibroblasts overexpressing miR-424. Lactate production was upregulated in miR-424-overexpressing fibroblasts by approximately 2-fold compared to control fibroblasts (Figure 3D, top right). Glucose uptake, as measured by 3H-labeled deoxyglucose, was increased more than 2-fold in miR-424-overexpressing fibroblasts compared to control fibroblasts (Figure 3D, bottom left). Overexpressing miR-424 also decreased oxygen consumption in these fibroblasts (Figure 3D, bottom right). Furthermore, oxidative phosphorylation, as reflected by the maximum respiration capacity, decreased in fibroblasts overexpressing miR-424. Together, these data indicate that miR-424 promotes glycolysis in CAFs by downregulating IDH3α.

**Downregulation of IDH3α Inhibits Proline Hydroxylase Activity**

Proline hydroxylase (PHD), a member of the dioxygenase family, hydroxylated HIF-1α at prolines 402 and 564. This hydroxylation led to the binding of E3 ligase VHL and consequent ubiquitination and degradation of HIF-1α (Figure 4A). The activity of PHD requires oxygen and α-KG, but succinate and fumarate, structural metabolic analogs of α-KG, can inhibit this activity. Because IDH3α converts isocitrate into α-KG, we speculated that downregulation of IDH3α in CAFs reduces PHD activity through changing the ratio of α-KG to succinate/fumarate (the effective α-KG) (Figure 4A). To test this idea, we measured the cellular content of these metabolites by gas chromatography-mass spectrometry (GC-MS). The level of both succinate and fumarate increased about four times in CAFs and fibroblasts with IDH3α knockdown. Unexpectedly, the level of α-KG also was increased, although the magnitude of this increase was significantly lower than that of succinate and fumarate (Figure 4B).

As a result, the ratio of α-KG to succinate and fumarate was greatly reduced in CAFs and fibroblasts with IDH3α knockdown. Because α-KG also is derived from glutamate, we examined the expression of enzymes converting glutamate to α-KG, including glutamic pyruvate transaminase (ALT) and aspartate aminotransferase (AST), by quantitative PCR and western blot analysis in CAFs. We found that the expression of ALT2 was significantly increased in induced CAFs (Figure 4C), suggesting that the slight elevation of α-KG is likely through the glutamate pathway by feedback upregulation of ALT2 in CAFs and fibroblasts with IDH3α knockdown.

To examine whether IDH3α affects PHD activity, we employed ODD (oxygen-dependent degradation domain) luciferase assay to measure the PHD activity in fibroblasts with IDH3α knockdown; cells treated with cobalt chloride to mimic hypoxia were

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(C) IDH3α expression at the indicated time points after TGF-β1 stimulation. The middle plot shows densitometry analysis of the IDH3α protein level and the right plot presents the quantitative mRNA level of IDH3α (p < 0.01).
(D) Analysis of IDH3α protein stability after MG132 and chloroquine treatments.
(E) IDH3α is downregulated in colon CAFs. Paraffin-embedded colon cancer samples were co-stained with IDH3α and FSP1 antibodies.
(F) Expression of IDH3α (western blot) and glycolysis-related genes (mRNA analysis) in CAF cells isolated from colon cancer samples (p < 0.01).
(G) Analysis of lactate production and glucose uptake in fibroblasts depleted of IDH3α. Lactate production was measured at indicated time points (p < 0.01).
(H) Analysis of lactate production and glucose uptake in fibroblasts overexpressing IDH3α. Lactate production was measured at indicated time points (p < 0.01).
(I) Basal oxygen consumption, oxidative phosphorylation, and maximum respiration capacity of fibroblasts after IDH3α knockdown. The histogram displays the basal level of oxygen consumption (p < 0.01).
(J) Basal oxygen consumption, oxidative phosphorylation, and maximum respiration capacity of fibroblasts with IDH3α overexpression. The histogram displays the basal level of oxygen consumption (p < 0.01; #p < 0.05).
used as a positive control. We found that IDH3α downregulation as well as CoCl2 treatment increases ODD luciferase activity (Figure 4D), suggesting that IDH3α knockdown suppresses PHD activity. In addition, when we examined fibroblasts treated with α-KG along with increasing concentrations of succinate, α-KG decreased ODD luciferase activity, as expected. However, succinate restored ODD activity in a dose-dependent manner (Figure 4E), suggesting that succinate can compromise the activity of PHD, and that the ratio of α-KG/succinate is critical in determining this activity in vivo. To extend this contention, we measured the tri-methylation of histone H3K4 (H3K4me3), a substrate of the Jumonji family of histone demethylases (JMHD, another member of the dioxygenase family) in CAFs and fibroblasts with IDH3α knockdown. We found methylation of H3K4 PHD and measured hydroxylation of HIF-1α (proline 564) in vivo. We found that IDH3α knockdown as well as CoCl2 treatment reduces the HIF-1α hydroxylation (Figure 4F). In contrast, the overexpression of IDH3α increases HIF-1α hydroxylation. To determine which isofrom of PHD (PHD1, PHD2, or PHD3) was responsible for HIF-1α hydroxylation, we knocked down three individual PHD isoforms in fibroblasts and found that PHD2, but not PHD1 or PHD3, significantly reduced HIF-1α hydroxylation (Figure 4G). In addition, knockdown of IDH3α also significantly inhibited HIF-1α hydroxylation. These results indicate that PHD2 is the predominant enzyme responsible for HIF-1α hydroxylation, and that downregulation of IDH3α is a major factor contributing to the suppression of HIF-1α hydroxylation in CAFs.
Increasing HIF-1α and HIF-1β expression increased, whereas miR-424 knockdown decreased HIF-1α mRNA levels unaffected by both succinate, fumarate, and IDH3α knockdown increased HIF-1α, whereas IDH3α overexpression reduced HIF-1α, with HIF-1α mRNA levels unaffected by both treatments (Figure 5C).

To determine whether HIF-1α is responsible for IDH3α-mediated glycolysis, we treated fibroblasts with the HIF-1α inhibitor YC-1. IDH3α knockdown increased the expression of HK2 and Glut1 and promoted lactate generation; however, YC-1 treatment attenuated these increases (Figure 5C, bottom). In addition, miR-424 overexpression increased, whereas miR-424 knockdown decreased HIF-1α protein, but not mRNA (Figure 5D). Overexpression of miR-424 also increased the expression of Glut1 and HK2 and promoted lactate generation; however, YC-1 inhibited Glut1 and HK2 expression and abrogated the miR-424 overexpression-induced lactate production (Figure 5D, bottom). These data support the contention that HIF-1α is responsible for the

**IDH3α Downregulation Promotes Glycolysis by Increasing HIF-1α Stability**

As HIF-1α and c-Myc are known transcription factors in regulating glycolysis, we hypothesized that these factors may mediate the IDH3α-induced glycolytic response in CAFs. To examine this idea, we investigated the expression of c-Myc and HIF-1α. We found decreased expression of c-Myc, rather than an increased one, in CAFs (Figure 5A). However, expression

![Figure 4. Downregulation of IDH3α Inhibits PHD Activity](image)

(A) Potential mechanism by which IDH3α regulates HIF-1α stability.

(B) Total content of α-KG, succinate, and fumarate were analyzed by GC-MS. The histograms display the relative content of α-KG, succinate, and fumarate in CAFs or fibroblasts depleted of IDH3α (*p < 0.01).

(C) Enzymes involved in glutamate conversion into α-KG were screened by quantitative PCR (bar plot). The increase of ALT2 expression was further validated by dose-response experiment in fibroblasts treated with TGF-β or PDGF.

(D) ODD luciferase activity was measured in fibroblasts depleted of IDH3α or treated with 100 μM CoCl2.

(E) ODD luciferase activity was measured in fibroblasts treated with 100 μM α-KG or succinate, or mixtures of α-KG (100 μM) with various concentrations of succinate (100, 200, 300, or 400 μM, respectively); the values were normalized to control.

(F) Effect of IDH3α on hydroxylation of HIF-1α. The hydroxylation level of HIF-1α was detected in fibroblasts depleted of or overexpressing IDH3α, using antibody against hydroxylated HIF-1α at proline 564. Fibroblasts were treated with 5 mg MG132 for 6 hr before analysis.

(G) PHD2, but not PHD1 or PHD3, regulates HIF-1α stability.
Figure 5. Downregulation of IDH3α Promotes Glycolysis by Increasing HIF-1α Stability

(A) Expression of HIF-1α, but not HIF-2α, HIF-3α, or c-Myc, increases in CAF cells.

(B) Stabilization of HIF-1α protein in induced CAF cells. Western blot shows increased protein level of HIF-1α, whereas mRNA expression is not changed.

(C) IDH3α regulates HIF-1α stabilization. (Top) Western blot shows the protein level of HIF-1α after overexpression or knockdown of IDH3α; mRNA level after IDH3α knockdown. (Bottom) Western blot shows expression of glycolysis-related proteins in fibroblasts treated with the HIF-1α inhibitor YC-1 or depleted of IDH3α; lactate production in fibroblasts with IDH3α knockdown and YC-1 treatment.

(D) miR-424 affects HIF-1α protein stabilization. (Top) Western blot (left) and quantitative PCR (right) of HIF-1α; lactate production in fibroblasts with miR-424 overexpression and YC-1 treatment. (Bottom) Western blot shows expression of proteins involved in glycolysis in fibroblasts treated with the HIF-1α inhibitor YC-1 or depleted of IDH3α; lactate production in fibroblasts with miR-424 overexpression and YC-1 treatment.

(E) HIF-1α activator CoCl2 restores IDH3α-inhibited glycolysis. Western blot shows the effect of CoCl2 on the expression of genes involved in glycolysis; histogram shows the effects of CoCl2 on lactate production in fibroblasts with IDH3α knockdown or IDH3α overexpression.

Changes in glycolysis mediated by IDH3α downregulation or miR-424 upregulation. In line with this observation, CoCl2 increased the expression of HK2 and Glut1 (as detected by western blot) and promoted lactate production in fibroblasts. However, neither IDH3α overexpression nor miR-424 knockdown blocked the HIF-1α-mediated increase of HK2/Glut1 expression and lactate production (Figure 5E, right).

IDH3α Downregulation Inhibits Oxidative Phosphorylation by Upregulating NDUFA4L2

We have shown that IDH3α knockdown or miR-424 overexpression increases glycolysis and reduces oxygen consumption. Gene expression profiling also indicated that NDUFA4L2 (NADH dehydrogenase [ubiquinone] 1 alpha subcomplex, 4-like 2), a negative regulator of mitochondrial complex 1, was upregulated in CAFs (Figure 2A). A previous study showed that HIF-1α upregulates NDUFA4L2 expression during hypoxia in mouse embryonic fibroblasts (MEFs) and tumor cells (Tello et al., 2011). To determine whether IDH3α regulates oxidative phosphorylation through NDUFA4L2, genes that are involved in oxidative phosphorylation and are upregulated or downregulated in expression profiling analysis (>2-fold), were further verified individually by quantitative PCR. NDUFA4L2 was dramatically upregulated (>4-fold) in fibroblasts upon IDH3α knockdown; the increase of NDUFA4L2 in fibroblasts exhibited a dose- and time-dependent response to TGF-β treatment (Figure 6A, bottom). However, the
expressions of PGC1α and TFB1M, two proteins that are master regulators of mitochondrial biogenesis and gene expression, were not altered (Figure 6A).

We next determined whether IDH3α regulates NDUFA4L2, using quantitative PCR and western blots, in fibroblasts with IDH3α knockdown or YC-1 treatment. Both quantitative PCR and
western blot analyses showed that, under normoxic conditions, IDH3α knockdown increased, whereas YC-1 inhibited NDUFA4L2 expression (Figure 6B). In addition, YC-1 treatment blocked NDUFA4L2 upregulation mediated by IDH3α knockdown. These data suggest that HIF-1α regulated NDUFA4L2 expression under normoxic conditions, whereas IDH3α knockdown decreased the basal oxygen consumption, whereas IDH3α knockdown decreased the basal oxygen consumption; NDUFA4L2 knockdown partially rescued (70%) the repressed oxygen consumption mediated by IDH3α knockdown (Figure 6D). Moreover, IDH3α knockdown inhibited oxidative phosphorylation and reduced oxygen consumption under normoxia. NDUFA4L2 knockdown rescued the decrease of oxidative phosphorylation mediated by IDH3α knockdown in comparison with control fibroblasts (Figure 6D). Taken together, these data demonstrate that IDH3α regulates the expression of NDUFA4L2, which in turn modulates oxidative phosphorylation and oxygen consumption.

**IDH3α Downregulation Is Responsible for the Tumor-Promoting Effects of CAFs**

To investigate whether the IDH3α downregulation is responsible for the tumor-promoting effects of CAFs in vivo, we established fibroblasts with IDH3α knockdown or overexpression. A375 melanoma cells or HCT116 colon cancer cells were mixed with control or IDH3α-silenced fibroblasts, and injected into the abdominal or forelimb amputis of nude mice (six mice/group) (Figures 7A and S4). While both control fibroblasts and fibroblasts with IDH3α knockdown did not grow into tumors, A375 cells that contained fibroblasts with IDH3α knockdown grew much faster and produced bigger tumors than those with control fibroblasts. Similarly, A375 cells that contained fibroblasts with IDH3α knockdown grew much faster and produced bigger tumors than those with control fibroblasts (Figure 7B); however, overexpression of IDH3α in CAFs significantly abolished the growth-promoting effect of CAFs. Furthermore, A375 cells that contained fibroblasts with miR-424 overexpression also grew faster than those with control fibroblasts (Figure 7C). Taken together, our data demonstrate that the reduction of IDH3α contributes to the tumor-promoting effects of CAF.

**DISCUSSION**

Previous studies have demonstrated that glycolytic enzymes, such as LDH and PKM2, and products such as lactate are upregulated in breast cancer CAFs under normoxic conditions. However, it is not clear whether aerobic glycolysis is a general metabolic feature of CAFs, and, if so, what regulates this aerobic glycolysis. In this study, we examined and compared lactate production, glucose uptake, and oxygen consumption in fibroblasts and CAFs. In two induced-CAF models (TGF-β induced and PDGF induced) and isolated CAFs from human colon cancer and melanoma, lactate production and glucose uptake were increased, whereas oxygen consumption was decreased, clearly demonstrating that aerobic glycolysis was increased in both the induced CAFs and the isolated CAFs. Our data suggest that aerobic glycolysis is a common feature of CAFs. Moreover, CAV1, a potential marker of CAFs in human breast cancers and associated with tumor recurrence, metastasis, and poor clinical outcome (Bonuccelli et al., 2010a; Sotgia et al., 2009), was downregulated in CAFs, whether induced or isolated, suggesting that CAV1 downregulation is a consequence rather than a cause of CAF formation.

The mechanism of an oxygen-independent metabolic switch from oxidative phosphorylation to aerobic glycolysis in CAFs is not well defined. Here we report that IDH3α downregulation triggers the metabolic switch from oxidative phosphorylation to glycolysis. CAFs isolated from colon cancers and melanomas are glycolytic. In vitro TGF-β- or PDGF-induced CAFs promote a switch from oxidative phosphorylation to aerobic glycolysis. IDH3α is a critical rate-limiting enzyme in the TCA cycle and commonly decreased in CAFs. Although IDH1 also was downregulated in CAF, our functional studies revealed that knockdown of IDH3α, but not IDH1 or IDH2, increased glycolysis and inhibited oxidative phosphorylation in fibroblasts. Moreover, overexpression of IDH3α prevented fibroblasts from transforming into CAFs.

Hypoxia has been implicated in the metabolic reprogramming of cancer cells, and HIF-1α plays an important role in the regulation of glycolysis (Denko, 2008; Fiaschi et al., 2012; Mimura et al., 2012; Sonveaux et al., 2008). Our data demonstrate that HIF-1α, but not HIF-2α or HIF-3α, is increased in CAFs both in vitro and in vivo, and that downregulation of IDH3α results in HIF-1α protein stabilization. HIF-1α stability is regulated by ubiquitin-dependent proteasome degradation. Ubiquitination of HIF-1α is triggered by hydroxylation at the P402 and/or P564 of HIF-1α. PHD2 was found to directly regulate HIF-1α hydroxylation (Lee et al., 2008; Minervini et al., 2013; Tennant et al., 2009). PHD2 activity is activated by oxygen and α-KG but suppressed by fumarate and succinate, two inhibitory metabolic structural analogs of α-KG. Our results showed that IDH3α downregulation decreased the level of effective α-KG by reducing the ratio of α-KG to fumarate and succinate, resulting in PHD2 inhibition and HIF-1α stabilization in CAFs.

Downregulation of IDH3α increases the protein level of HIF-1α. The accumulation of HIF-1α, in turn, promotes glycolysis, by upregulating the uptake of glucose and expression of glycolytic enzymes under normoxic conditions, and inhibits oxidative phosphorylation by upregulating NDUFA4L2, the negative regulator of complex I. Although the IDH3α-HIF-1α-signaling axis regulates NDUFA4L2 expression under normoxic conditions, we have not excluded the possibility that NDUFA4L2 is regulated by other transcription factors. This is because IDH3α downregulation reduces the effective level of α-KG, which suppresses the activity of other dioxygenases, such as the Jumonji family of enzymes under normoxic conditions, whereas IDH3α regulated NDUFA4L2 expression. Consistent with this idea, IDH3α overexpression decreased the expression of NDUFA4L2 (Figure 6B, right). The effect of miR-424 on NDUFA4L2 expression was also determined. Both quantitative PCR and western blot analyses revealed that, under normoxic conditions, miR-424 overexpression increased NDUFA4L2 expression while YC-1 inhibited this increase (Figure 6C). These data indicate that IDH3α regulates NDUFA4L2 expression through HIF-1α and that NDUFA4L2 is responsible for reduced oxidative phosphorylation mediated by IDH3α downregulation.
histone demethylases. IDH3α can regulate NDUFA4L2 expression through these histone demethylases in CAFs, an interesting possibility that should be explored in the future.

Although tumor cells also are known to be addicted to glycolysis, our study suggests that the roles of glycolysis in tumor cells and CAFs are different. The major function of glycolysis in tumor cells is to produce building blocks for biosynthesis and to maintain ATP production under hypoxia. Our results indicate that CAFs also are prone to glycolysis and that the increased glycolytic traits in CAFs are responsible for the tumor-promoting effect of CAFs. Because the proliferation of CAFs decreases, rather than increases, compared to primary fibroblasts, it seems likely that the increased glycolysis in CAFs is not for the proliferation and growth of CAFs, but for the rapid proliferation of tumor cells by providing the required building blocks and supporting niche. Our results further identified IDH3α as a molecular switch promoting aerobic glycolysis (Figure 7D), and provided insights into the initiation of the Warburg-like effect in CAFs. Our study indicates that the increased glycolytic traits in CAFs are responsible for the tumor-promoting effect of CAFs. Targeting the glycolytic events in both tumor cells and their nearby CAFs may represent a novel and effective approach for inhibiting tumor development and progression.

**EXPERIMENTAL PROCEDURES**

**Cells and Reagents**

A375 melanoma cells (ATCC) were cultured in DMEM with 10% fetal bovine serum (FBS). Glycine-deprived DMEM was purchased from Gibco. Human recombinant TGF-β1 was purchased from Peprotech. SYBR Green PCR master mix and the TaqMan microRNA reverse transcription kit were purchased from ABI. The lactate and cell proliferation assay kits were purchased from BioVision Technologies and Roche, respectively. Monoclonal antibodies against IDH3α, β-actin, and GAPDH were purchased from OriGene.

**Clinical Samples**

Tissue collection was approved by the Medical Ethical Committee of the Shanghai Jiao Tong University School of Medicine. Foreskin tissues were collected after informed consent from children aged 7 to 12 years at the affiliated Xinhua Children Hospital of the Shanghai Jiao Tong University School of Medicine. Fresh and paraffin-embedded colon cancer blocks or peripheral...
normal colon tissue were collected after informed consent from patients undergoing surgery at the affiliated Ruijin Hospital of the Shanghai Jiao Tong University School of Medicine. Paraffin-embedded primary melanoma blocks or normal skin tissue were collected after informed consent from patients undergoing surgery at the affiliated Renji Hospital of the Shanghai Jiao Tong University School of Medicine.

Isolation of Human Normal Primary Fibroblasts and CAFs

Human normal primary fibroblasts and CAFs were isolated from foreskin or from colon cancer tissues, respectively. After postectomy, the foreskins were immediately transported to the laboratory on ice. The foreskins were minced and then digested with 0.1% type I collagenase and trypsin. After digestion, the tissue was filtered with a 400-mesh sieve, and the filtrate was centrifuged at 1,000 × g for 10 min. Cells obtained from the pellet were cultured with DMEM containing 10% FBS for 2 hr; the attached cells, verified by F-actin staining (Figure 1), were fibroblasts. After three passages, the cells were frozen in liquid nitrogen for further experiments.

Luciferase Assay

For the 3′-UTR luciferase assay, the full-length 3′ UTR of the human IDH3α gene (1129–2655 nt, GenBank accession number NM_005530.2) was amplified using a human cDNA library as a template. To generate a mutant IDH3α 3′ UTR, site-directed mutagenesis was performed. The primers for mutagenesis were sense, 5′-CTTTGTTATTACAGAGTCATCCTCTGTTT-3′, and antisense, 5′-AACAGAGTGATGACCCTGCTAAACAAAG-3′. In the mutant 3′ UTR, the nucleotide sequence that is complementary to the seed region of miR-424 was replaced with the sequence shown in Figure 4B (CTGCTCATT to CAGGTCCTC). The 293T cells were co-transfected with 50 ng pGL3- IDH3α 3′ UTR (wild-type or mutant) and 500 ng miR-424 overexpression plasmid. The empty vector was used as a control, and the renilla plasmid pRL-TK was used as an internal control. Cells were harvested 48 hr after transfection and analyzed with the Dual-Luciferase Reporter Assay Kit. Firefly luciferase values were normalized to renilla luciferase values from the same cell samples. The assays were repeated at least three times in independent experiments.

For the ODD luciferase assay, the ODD domain of HIF-1α (GenBank accession number U9499) was fused to the 5′ end of the firefly luciferase reporter gene (Dewhirst et al., 2007). As a control, a luciferase expression vector in which the luciferase gene is driven by the CMV promoter was used.

Quantitative Real-Time PCR

Total cellular RNA was extracted using the guanidinium isothiocyanate and phenol-chloroform method. For the quantification of mRNA, 1 μg total RNA was reverse transcribed into cDNA with MMLV reverse transcriptase according to the manufacturer’s instructions. Quantitative real-time PCR reactions were performed in triplicate in a 96-well plate using 1 μl cDNA. The β-actin gene was used as an internal control for normalization. For the quantification of miRNA, the TaqMan microRNA reverse transcription kit was used with the stem-loop primer according to the manufacturer’s instructions, and the results were normalized to RNU48.

Oligo- or Lentivirus-Mediated shRNA Knockdown and Lentivirus-Mediated Gene Overexpression

A quantity of 50 nM oligomer (inhibitor) targeting miR-424 was transfected using RNAi Max (Invitrogen) according to the manufacturer’s directions. Primary human fibroblasts were seeded in 60-mm dishes at 30% confluence. After 48 hr, the cells were analyzed or further treated with 8 ng/ml TGF-β1. Specific shRNA sequences targeting IDH3α were inserted into the pSpZP lentiviral vector (Open Biosystems); the IDH3α cloning sequence was cloned into the pHR-SIN lentiviral vector. These lentiviral plasmids were co-transfected into 293T cells with psiPAX2 and pMD2G to generate the lentiviruses using Lipofectamine 2000 (Invitrogen). Viral stocks were collected from the transduced 293T cells 72 hr post-transfection, and were used to infect human fibroblasts with 6 μg/ml polybrene. Lentivirus-mediated miR-424 knockdown was performed with the pPACKH1 lentivector packaging system (System Biosciences), according to the manufacturer’s instructions.

Immune Staining

Immunofluorescence staining was performed on cultured cells. After fixation and permeabilization, the cells were incubated with primary antibodies at 4°C overnight, followed by the appropriate Alexa Fluor 488-conjugated secondary antibodies. The cells were visualized by fluorescence microscopy.

Immunohistochemical staining was used for paraffin-embedded clinical samples. After deparaffinization and rehydration, the tissue sections were incubated with antibodies overnight at 4°C, followed by incubation with peroxidase-labeled anti-chicken antibodies. Finally, antibody binding was visualized with a DAB substrate (Immunopure).

Cell Proliferation Assay

Human fibroblasts were cultured in media containing D-glucose or galactose (4.500 mg/dl) for 24 hr, after which the supernatants were collected. A375 melanoma cells were co-cultured with this supernatant. After incubation for the indicated times, BrdU cooperation assays (Roche) were performed. The absorbance at 370 nm was measured using a microplate reader.

Aerobic Glycolysis Analysis

Extracellular lactate was measured with a lactate assay kit (BioVision Technologies). Briefly, 5 × 10^5 fibroblasts were seeded in 60-mm dishes overnight. The cells were incubated in DMEM without FBS prior to assay, the supernatants were collected at the indicated time points (1, 2, or 3 hr), and lactate levels were quantified by the colorimetric assay, according to the manufacturer’s instructions.

The glucose uptake rate was determined as previously described (Yamamoto et al., 2011). Cells were incubated in the media containing 12 mM glucose for 24 hr. After three washes with KR buffer, the cells were incubated for 30 min in KR buffer and then incubated in KRP containing 0.5 μCi/ml [3H]-deoxyglucose (PerkinElmer) for 10 min. After washing, the cells were lysed with 1 ml of 0.1 N NaOH and incubated at 37°C for 2 hr. An aliquot of 0.5 ml was used for scintillation counting.

The oxygen consumption rate (OCR) was measured using a Seahorse XF24 technology (Seahorse Bioscience). Briefly, 2.5 × 10^5 cells were seeded on the special plate for 24 hr. The OCR was measured under basal conditions and after the addition of oligomycin (1 μM), FCCP (1 μM), and rotenone (1 μM)/antimycin (1 μM).

Cellular Succinate, Fumarate, and α-KG Detection

The cellular content of succinate, fumarate, and α-KG were analyzed by GC-MS as previously described (Chan et al., 2009). The cells (1 × 10^5) were harvested and suspended in chloroform-methanol-water (2:1:1, v/v). The derivatized sample was injected into a Shimadzu QP 2010 GC tandem quadrupole mass spectrometer. The GC separation was performed on an Agilent DB-5 mass spectrometer fused silica capillary column (30 m × 0.25 mm × 0.25 μm). The column temperature was 70°C for the first 3 min and then increased at 5°C/min to 310°C for 5 min. The injection temperature was set at 300°C, and the injection volume was 1 μl with a 10:1 split ratio. Helium (99.9995%) was applied as a carrier gas. The column flow was 1.2 ml/min, and the column was equipped with a linear velocity control. The mass spectra scanning scope was set to 33–600 m/z in the full-scan mode with a scan speed of five scans s–1 and a solvent cut time of 5.6 min based on the retention time of the pyridine solvent. The temperatures of the interface and the ion source were adjusted to 280°C and 240°C, respectively. The detector voltage was maintained at 1.2 kV, and the electron impact (EI) model was selected to achieve ionization of the metabolites at 70 eV.

Mice Tumor Xenografts

Four-week-old BALB/c nude mice were obtained from a Shanghai animal laboratory. The animals were handled according to the protocol approved by the Institutional Animal Care and Use Committee of the Shanghai Jiao Tong University School of Medicine. Tumor xenografts were generated by subcutaneously co-injecting human fibroblasts with A375 cells (the ratio of fibroblasts to A375 cells was 3:1, and the total cell number in each injection was 4 × 10^5) into the armpits bilaterally. The tumor volume was determined weekly using digital caliper measurements and the following formula: tumor volume = length × width^2/2.
volume (mm³) = ½ × longest diameter² × shortest diameter. After 5 weeks, the mice were sacrificed and the tumors were excised.

**Statistical Analysis**

The animal data are presented as the medians ± SD, whereas other data are presented as the means ± SD. All data are representative of at least three independent experiments. The differences between groups were assessed by Student’s t test; all reported differences are p < 0.05 unless otherwise stated.

**ACCESSION NUMBERS**

The accession number for the RNA sequencing data of TGF-β1 and PDGF-induced CAFs and their corresponding fibroblasts reported in this paper is GSE61797.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes four figures and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.02.006.

**AUTHOR CONTRIBUTIONS**

D.Z., Z.S., and P.S. performed most of the experiments. Y.W. and X.H. performed some of the experiments. J.Z. analyzed the gene expression profiling data. B.P.Z. and S.Z. provided reagents and revised the paper. J.M. designed the project and wrote the article. All authors reviewed the manuscript.

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