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Hypoxia facilitates the proliferation of colorectal cancer cells by inducing cancer-associated fibroblast-derived IL6

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Colorectal cancer (CRC) is the most common malignancy worldwide, and its underlying molecular mechanisms remain largely unexplored. Accumulating evidences indicate cancer-associated fibroblasts (CAFs), abundant stromal cell population in the tumor microenvironment, play a key role in tumor development. Herein, we have successfully isolated CAFs and paired normal fibroblasts (NFs) from colorectal cancer tissues (n=10). By using a multiplex cytokine profiling assay, we have identified IL-6 as a major cytokine released by CAFs. Co-culturing of CAFs with CRC cell lines HCT116 or SW480 increases IL-6 release, and the secretion by CAFs can be further enhanced under hypoxia. By using the CCK-8 assay, we have found that HCT116 or SW480 cells treated with culture medium from CAFs, IL-6, or hypoxia showed a significant cell growth compared to control cells ($p<0.01$). Mechanistically, we have found that hypoxia could enhance the effect of the IL-6/STAT3 signaling on CRC cells, in part, through HIF-1a targeting PKM2. In conclusion, our data clearly proposes the interconnected mechanisms for constitutive activation of STAT3 signal by CAFs-derived IL-6 under hypoxia in colorectal cancer. The pharmacological inhibition of STAT3, PKM2, or HIF-1α can significantly reduce the oncogenic effect of IL-6, providing a potential therapeutic target for CRC patients.

Key words: colorectal cancer, cancer-associated fibroblast, IL-6, cell proliferation

Colorectal cancer (CRC) is one of the most common malignancies in the world [1]. The environment or genetic factors have been hypothesized to influence tumorigenesis of CRC. However, the driven mechanism(s) for promoting the development of CRC remains largely unexplored [2]. During the past decades, the tumor microenvironment (TME) has been demonstrated to play a key role in colorectal carcinogenesis [3]. TME is composed of different components, including immune cells, fibroblasts, macrophages, endothelial cells, and extracellular matrix (ECM) [4]. It is increasingly appreciated that the tumor stroma is an integral part of cancer initiation, growth, and progression.

Cancer-associated fibroblasts (CAFs), the most abundant stromal cell population, were observed to significantly contribute to the development of CRC [5]. It was reported that CAFs can be used as a promising marker to predict disease recurrence in CRC patients [6]. CRC patients with enriched CAFs tend to have more aggressive disease progression and experience metastasis or recurrence [7, 8]. Biologically, CAFs secrete growth factors or cytokines, to sufficiently promote multiple aggressive behavior of CRC cells, such as cell growth and stemness, migration and invasion, epithelial-mesenchymal transition, and even chemoresistance [9]. Despite these findings, the role of CAFs in CRC and their underlying mechanism(s) remains largely unexplored.

Hypoxia, or diminished oxygen availability, is a common feature of the tumor microenvironment, where the oxygen level is often below 1%. Hypoxia often triggers activation of several signaling pathways, affects angiogenesis and cell metabolisms, and remarkably facilitate tumor growth, progression, and metastasis [10]. It is of note that tumor cells can adapt to hypoxia through metabolic interplay with CAFs [11]. However, the alteration of biological functions of CAFs, such as cytokine secretion, under hypoxia remains unknown.

Herein, we aim to isolate CAFs in cancer tissues from CRC patients and identify key factors secreted by CAFs. We therefore, investigate the impact of hypoxia on the interaction between CRC and CAFs, and further decipher the mechanisms mediated by CAFs under hypoxia.

Materials and methods

Cell culture and materials. Human colorectal cancer cell lines HCT116, SW480, and CaCO-2 were obtained from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. CRC cells were cultured in a DMEM medium with 10% fetal bovine serum, 100 U/ml penicillin/streptomycin at 37 °C in 5% CO_2 humidified atmosphere. The recombinant interleukin (IL)-6 (Cat. No. GF338), STAT3 inhibitor (Cat. No. 573102), and HIF-1α inhibitor $CdCl_2$ (Cat. No. 239208) were purchased from Sigma-Aldrich LLC.

Isolation of NFs and CAFs cells. Primary normal fibroblasts (NFs) and cancer-associated fibroblasts (CAFs) were isolated from 10 colorectal cancer patients without treatment of radiation or chemotherapy. Briefly, tissues were washed with cleaning buffer (PBS with penicillin/streptomycin) and the epithelial and adipose tissues were removed. The remaining connective tissues were cut into 1×1×1 mm pieces. Tissues were then digested with 160 μg/ml collagenase I (Sigma-Aldrich, Cat. No. 1148089) and 25 μg/ml hyaluronidase (Sigma-Aldrich, Cat. No. 37326-33-3) at 37 °C for 2 h. To minimize inter-individual variations, the digested tissues were pooled together and cultured in DMEM supplemented with 5% FBS and 1% antibiotic-antimycotic solution and incubated at 37 °C in a humidified atmosphere containing 5% CO_2. The culture medium was replaced every 2 or 3 days. After the cellular fusion of the cells, the cell passage ratio was 1:2. The third-generation cells were used for verification.

Cell viability. Cell viability was detected by a CCK-8 assay (Cell Counting Kit-8, Beyotime, Cat. No. C0040). Briefly, human colorectal cancer cell lines HCT116 and SW480 (5×10^3 cells/well) were seeded into 96-well plates with 100 μl culture medium for 12 h. Then, HCT116 and SW480 cells were treated with corresponding treatment. Subsequently, 10 μl of CCK-8 solution was added to each well of the plate and the cells were incubated in a humidified incubator-containing 5% CO_2 at 37 °C for 1 h. Subsequently, the absorbance was detected by a microplate reader at 490 nm.

RNA extraction and quantitative real-time PCR. Total RNA was isolated by using TRIzol reagent (Invitrogen, Cat. No. 15596018) according to the manufacturer's instructions. The first-strand cDNAs were reverse transcribed from 1 μg total RNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Cat. No. K1621) and analyzed using TB Green® *Premix Ex Taq*™ (Takara, Cat. No. RR820B) by real-time PCR in 7500 Fast Real-Time PCR detection system. For the thermal cycles, the following reaction conditions were performed: 95 °C for 10 min and 45 cycles of 15 s at 95 °C and 1 min at 60 °C. The expression levels of target genes mRNA were normalized to the levels of β-actin gene transcript and calculated using the $2^{-\Delta\Delta ct}$ method. The primer sequences are shown in Supplementary Table S1.

Western blotting. In brief, the cells were harvest and lysed in the RIPA buffer (Beyotime Biotechnology, China). The protein concentrations were determined by the Bradford protein assay (Beyotime Biotechnology, Cat. No. P0006C). 10 μg of proteins were loaded, separated by 10% SDS-PAGE gel, and transferred to a PVDF (polyvinylidene fluoride) membrane (Millipore, Cat. No. IPVH00010). The membrane was blocked in 5% non-fat milk for 1 h, followed by incubation with the primary antibody overnight at 4 °C. The primary antibody, including anti-HIF-1α (Cat. No. 36169), anti-pSTAT3 (Cat. No. 9145), anti-STAT3 (Cat. No. 9139), anti-β-actin (Cat. No. 4970), and anti-PKM2 (Cat. No. 4053) were obtained from Cell Signaling Technology. After that, the membranes were incubated with secondary antibody at room temperature for 1 h and washed by TBS-T (TBS+0.5% Tween 20) 10 min 3 times. The target proteins were detected by Immobilon™ Western HRP reagent (Millipore, Cat. No. WBKLS). The β-actin protein served as an endogenous control.

Enzyme-linked immunosorbent assay (ELISA). A human IL-6 ELISA kit was purchased from R&D (Cat. No. D6050). In accordance with the vendor's instructions, supernatants of each group of cells with a serial dilution of standards were added to respective wells. The plate was sealed and incubated with gently shaking for 1 h at room temperature. After being washed, the plate was incubated with 100 μl tetramethylbenzidine substrate for 10 min in the dark at room temperature and 100 μl Stop solution for 1 min on a plate shaker. The intensity was measured at 450 nm employing spectrophotometry.

Establishment of a co-culture system. Transwell suspension chamber pre-covered with Polyester (PET) film was used for the co-culturing experiment. CAFs were inoculated in the upper chamber, while 1×10^5 of HCT116 or SW480 cells were inoculated in the lower chamber. Culture medium from the upper chamber cells was collected for further analysis.

Human cytokine screening. To screen cytokines, we collected 12.5 μl of culture medium and subsequently measured by using Bio-Plex Pro™ Human Cytokine Screening Panel (Bio-Rad, Cat. No. 12007283) according to the manufacturer's instruction.

Statistical analysis. Statistical analyses were performed using SPSS 20.0 software. All experiments were conducted independently at least three times. All results are illustrated as the means ± SEM. The significance of differences between groups was analyzed by Student's t-test. A p-value <0.05 was considered statistically significant.

Results

Cancer-associated fibroblasts (CAFs) secreted a high level of IL-6 in colorectal cancer. As mentioned above, CAFs act as major stromal cells to promote the development of colorectal cancer, we thus hypothesized that CAFs are likely to release several key cytokines that link to the development of CRC. Based on this hypothesis, we began to isolate CAFs and normal fibroblasts (NFs) from 10 colorectal tumor tissues and adjacent healthy tissues. To validate that we have success-

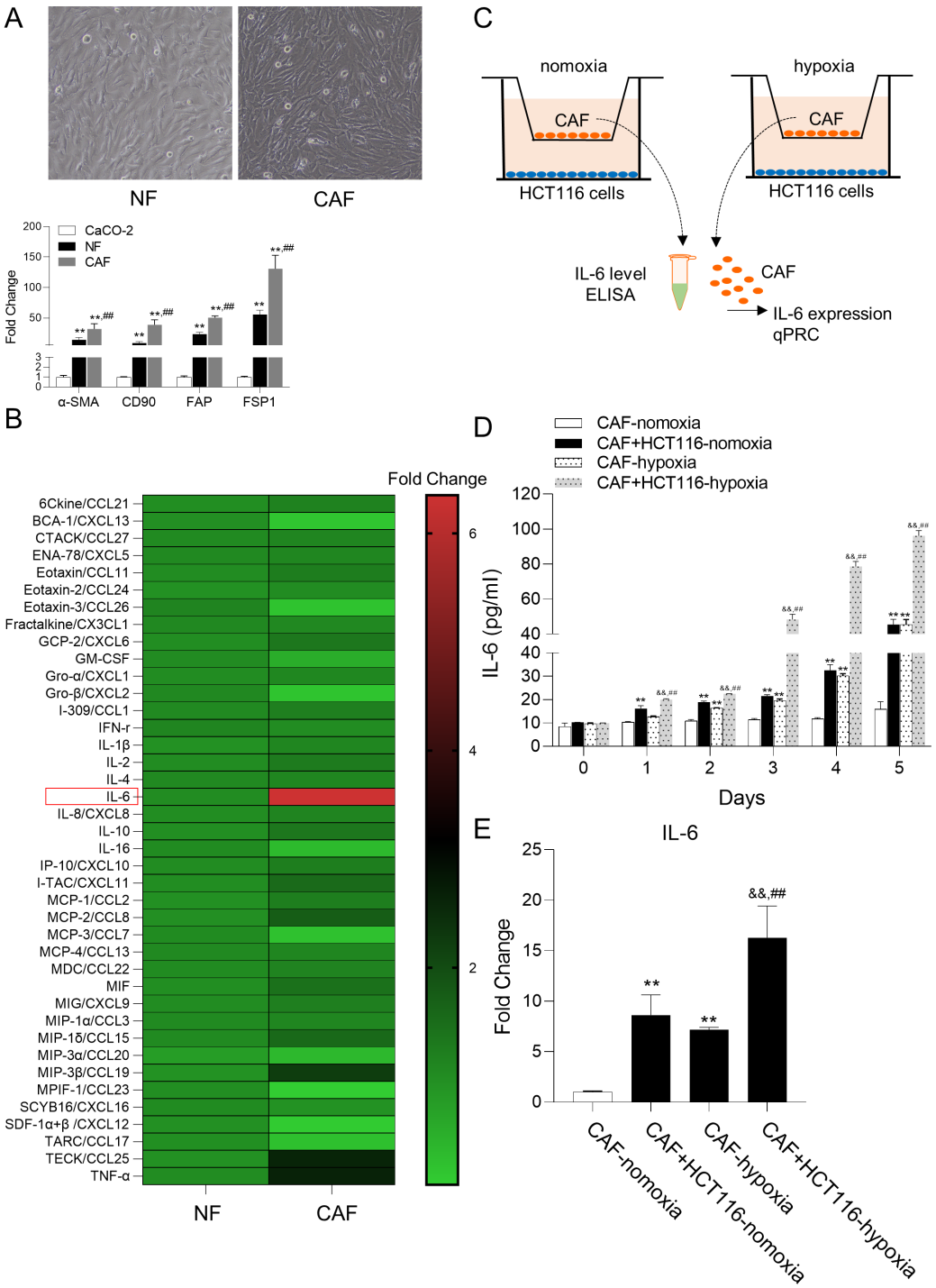

Figure 1. Hypoxia increased CAF-derived IL-6 level in CRC. A) The upper figure shows the images of isolated CAFs, NFs; the bottom figure shows the expression of CAF-specific genes in CAFs, NFs, and epithelial cell controls CaCO-2. qPCR was used to determine the expression level of α-SMA, CD90, FAP, and FSP1. **p<0.01, CAFs or NFs vs. CaCO-2. ##p<0.01, CAFs vs. NFs. B) Cytokine profiling by Bio-Plex assay. The culture medium was collected from CAFs and NFs and subjected to cytokine profiling by using Bio-Plex assay. C) The schematic illustration of the co-culture system. CAFs were seed in the upper chamber, while HCT116 cells in the bottom chamber. Culture medium from CAFs was collected for IL-6 measurement, and CAFs were collected for qPCR assay. D) IL-6 level in culture medium from CAFs cultured alone or with HCT116 cells, under normoxia or hypoxia. ELISA assay was performed for the IL-6 evaluation. **p<0.01, vs. CAFs cultured alone under normoxia; ##p<0.01, vs. co-culturing of CAFs with HCT116 cells under normoxia; &&p<0.01, vs. co-culturing of CAFs with HCT116 cells under hypoxia. E) IL-6 expression in CAFs with different treatments by using qPCR. **p<0.01, vs. CAFs cultured alone under normoxia; ##p<0.01, vs. co-culturing of CAFs with HCT116 cells under normoxia; &&p<0.01, vs. co-culturing of CAFs with HCT116 cells under hypoxia.

fully isolated CAFs and NFs, we subsequently measured the expression of several CAF-specific genes, including myofibroblast marker α-SMA, CD90, fibroblast activation protein (FAP) in CAFs, NFs, and epithelial cell controls CaCO-2. As shown in Figure 1A, CAFs and NFs expressed a higher level of α-SMA, CD90, FAP, and FSP1, compared to epithelial cell controls ($p<0.01$). Furthermore, the expression of these markers was more significantly upregulated in CAFs than those in NFs ($p<0.01$), suggesting that we have successfully isolated CAFs and NFs from colorectal tumors and adjacent normal tissues.

We, therefore, collected culture medium from CAFs and NFs for cytokine profiling by Bio-Plex assay. Interestingly, we observed several differential cytokines between CAFs and NFs (Figure 1B). Obviously, the level of IL-6 in CAF culture medium is up to 6-fold change (CAFs vs. NFs, $p<0.01$), while other differential cytokines are only up to 1 or 2-fold change, such as TNF-α, CCL25, CCL17, and IL-1β, implicating that IL-6 released by CAFs may play an important role in CRC.

CAFs released increased IL-6 as a response to hypoxia. Previous studies have shown that hypoxia has a significant impact on CRC carcinogenesis [12, 13], we, therefore, evaluated whether hypoxia is able to affect CAFs to secrete IL-6. To mimic tumor microenvironment (TME), CAFs were co-cultured with HCT116 cells under either normoxic (20% O_2) or hypoxic (1% O_2) conditions (Figure 1C). We collected culture medium from CAFs from day 1 to day 5 and measured IL-6 level by ELISA assay. In spite of the fact that IL-6 level from co-culture of CAFs and HCT116 cells is significantly higher than the level from CAFs cultured alone under normoxia, hypoxia, however, led to a pronounced increased IL-6 level from CAFs cultured alone or together with HCT116 cells (Figure 1D). Also, we collected CAFs and found the expression of IL-6 is dramatically induced by hypoxia (Figure 1E), suggesting that hypoxia substantially contributes to the release of IL-6 by CAFs.

CAF-derived IL-6 promotes cell proliferation of colorectal cancer cell lines HCT116 and SW480. We next investigated whether CAF-derived IL-6 can affect CRC cell growth *in vitro*. Therefore, we collected conditional medium from CAF (CM-CAF) or NF (CM-NF) and treated colorectal cancer cell lines HCT116 and SW480 with CM-CAF or NF for 72 h. The cell proliferation was determined by using the CCK-8 assay. As shown in Figure 2A, HCT116 and SW480 cells with CM-CAF showed an obvious rapid growth. To investigate whether IL-6 exhibits similar biological effects of CM-CAF on colorectal cancer cells, we treated HCT116 cells with recombinant IL-6 (10 ng/ml or 20 ng/ml). As shown in Figure 2A, IL-6 has the capacity to promote the proliferation of HCT116 and SW480 cells in a dose-dependent manner, suggesting that CAF-derived IL-6 contributes to the cell growth of CRC.

Since hypoxia promotes CAFs to release IL-6, and IL-6 has tumor-promoting activity as shown above, we next interrogated the correlation between hypoxia and proliferation of colorectal cancer cells. Therefore, HCT116 and SW480 cells were co-cultured with CAFs under hypoxia for 72 h and collected for the CCK-8 assay. As expected, cells under normoxia showed lower cell growth than those under hypoxia (Figure 2A), highlighting the role of hypoxia in colorectal carcinogenesis.

Hypoxia enhanced IL-6/STAT3 signaling in CRC cells. We next investigated the mechanisms for crosstalk of IL-6-mediated-carcinogenesis with hypoxia. Accumulating evidences have demonstrated that the IL-6/STAT3 pathway is aberrantly hyperactivated in a variety of cancer types, and its hyperactivation is often associated with a poor prognosis. Biologically, IL-6/STAT3 signaling acts to drive the proliferation, invasiveness, and metastasis of cancer cells [14, 15]. Consistently, HCT116 or SW480 cells treated with STAT3 inhibitor showed lower cell proliferation than control cells showed (Figure 2B).

HIF-1α is well recognized as a major transcription factor induced by the presence of hypoxia. We then measured expression of HIF-1α in HCT116 or SW480 cells, which were co-cultured alone or with CAFs. As shown in Figure 2C, the HIF-1α expression in HCT116 or SW480 cells is significantly induced in the presence of hypoxia, confirming that HIF-1α can be sufficiently induced by hypoxia. Moreover, we noticed that CAFs can significantly induce STAT3 phosphorylation in HCT116 or SW480 cells under normoxia, and strikingly, STAT3 phosphorylation can be further upregulated in the presence of hypoxia (Figure 2C), indicating that hypoxia is able to enhance the IL-6/STAT3 signaling in CRC cells.

To confirm that hypoxia can regulate IL-6/STAT3 signaling, HCT116 or SW480 cells were pre-treated with HIF-1α inhibitor cadmium chloride ($CdCl_2$) for 12 h, and subsequently cultured with CAFs under hypoxia. As Figure 2D showed, the expression of HIF-1α is significantly reduced by $CdCl_2$ in HCT116 and SW480 cells. Moreover, STAT3 phosphorylation is also suppressed by $CdCl_2$, highlighting the role of hypoxia in enhancing IL-6/STAT3 signaling in CRC.

HIF-1α target pyruvate kinase M2 is involved in the activation of IL-6/STAT3. Numerous studies have shown that HIF-1α target pyruvate kinase M2 (PKM2) promotes the activation of STAT3. Therefore, we hypothesized that the enhanced activity of IL-6/STAT3 by hypoxia maybe, in part, correlated with PKM2. HIF-1α and PKM2 were observed upregulated in HCT116 cells under hypoxia (Figure 3A). Moreover, inhibition of HIF-1α by $CdCl_2$ can suppress hypoxia-induced PKM2, confirming that PKM2 is a direct target of HIF-1α.

To examine whether PKM2 increases the STAT3 phosphorylation, HCT116 cells were treated with PKM2 siRNA under hypoxia. The results clearly showed that the knockdown of PKM2 inhibited STAT3 phosphorylation under hypoxia (Figure 3B). We then investigated whether the knockdown of PKM2 can suppress the biological effect of CAFs or IL-6 in CRC cells. As shown in Figure 3C, the suppression of PKM2

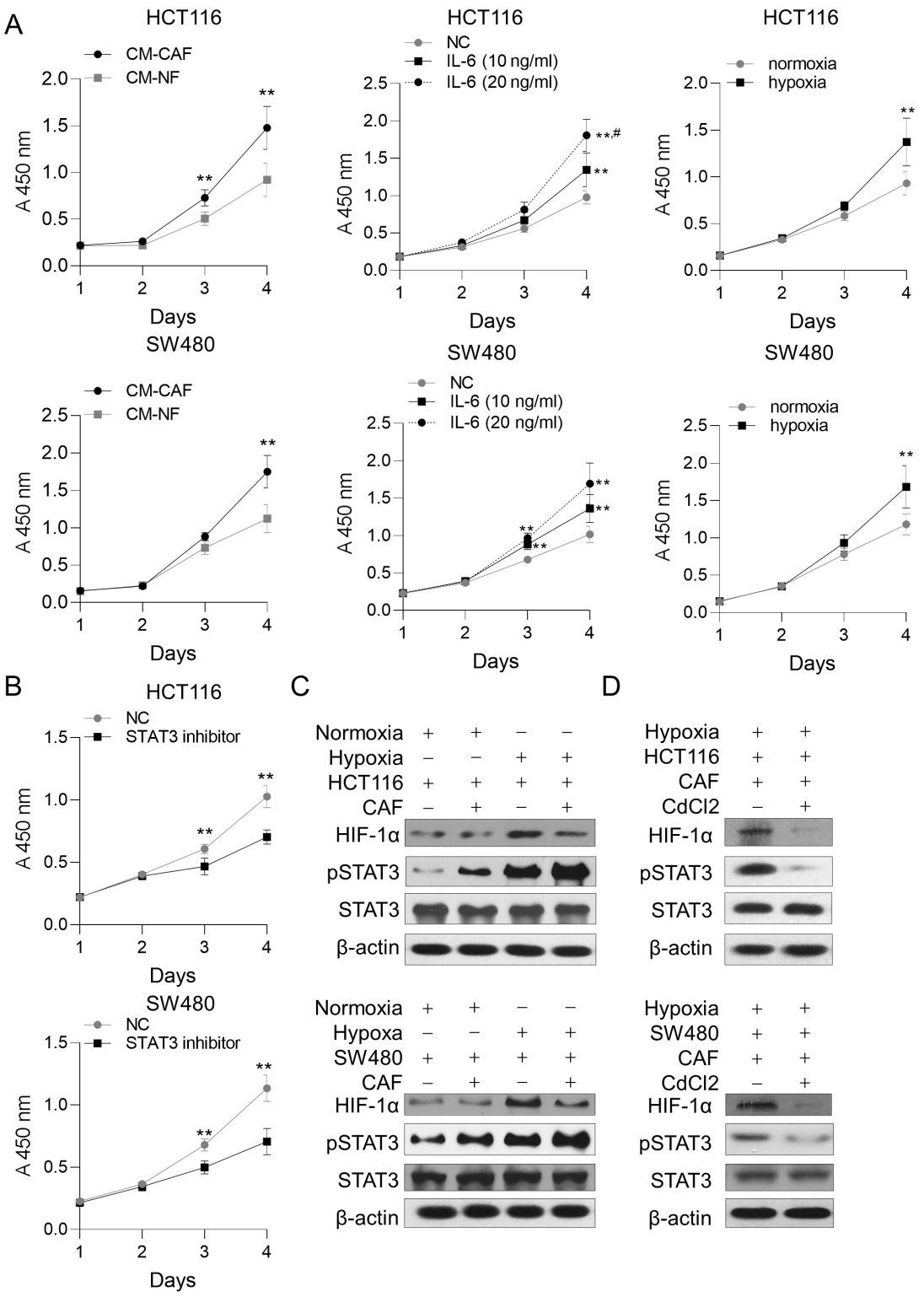

Figure 2. Hypoxia enhanced the IL-6/STAT3 signaling to promote the proliferation of colorectal cancer cells. A) The cell viability of HCT116 and SW480 cells with different treatments. CM-CAF: culture medium from CAFs, CM-NF: culture medium from NFs, NC: negative control. **p<0.01, CM-CAF vs. CM-NF; IL-6 vs. NC; hypoxia vs. normoxia. B) The cell viability of HCT116 and SW480 cells with STAT3 inhibitor. **p<0.01, STAT3 inhibitor vs. NC. C, D) The protein expression of HIF-1α, phosphorylation STAT3, and total STAT3 in HCT116 and SW480 cells with different treatments by using western blot.

obviously inhibited the STAT3 phosphorylation in HCT116 cells co-cultured with CAFs or treated with IL-6 under hypoxia.

Discussion

Accumulating evidences have demonstrated that tumor stroma or tumor microenvironment (TME) significantly contributes to cancer development. CAFs, representing the major cell component of TME, have been reported to exert tumor-promoting function through various mechanisms [17, 18]. In this study, we have successfully isolated and characterized CAFs from CRC patients. Furthermore, we have identified interleukin-6 (IL-6) as a key CAF-specific cytokine, and the secretion of IL-6 by CAFs can be enhanced by hypoxia. Interestingly, we also found the activation of IL-6/STAT3 signaling substantially promotes cell proliferation of CRC cells. Hypoxia can remarkably enhance the activity of IL-6/STAT3 signaling, in part, through HIF-1α PKM2. These findings indicate that the inhibition of IL-6/STAT3, PKM2, or HIF-1α may act as a potential therapeutic target to interplay between colorectal cancer cells and CAFs under hypoxia.

Several pro-inflammatory cytokines have been shown to regulate cancer cell growth and thereby contribute to tumor promotion and progression. IL-6 seems to take a center stage in human cancer development. Numerous studies have shown an increased expression of IL-6 in patients with CRC, where IL-6 levels are elevated in both serum and tumor tissues. Moreover, IL-6 expression can be associated with tumor stage, metastasis, and prognosis in sporadic and colitis-associated CRC [16–18]. In this study, we have successfully isolated CAFs from CRC tissues and observed that CAFs have the capacity to secret a high level of IL-6 compared to normal fibroblasts, suggesting that CAFs may be a major source of IL-6 in the tumor microenvironment in CRC.

Interestingly, we found that CAFs can increase IL-6 release under hypoxia. It was demonstrated that induction of IL-6 by hypoxia may be driven by the nuclear factor IL-6 (NF-IL-6) DNA binding site in the promoter [19–21]. Moreover, IL-6/STAT3/HIF-1α autocrine loop was observed in cancer cells [22], highlighting that induction of IL-6 expression may be an important adaptive mechanism triggered in CAFs by hypoxia. During past decades, amount of evidences IL-6 mediates activation of important

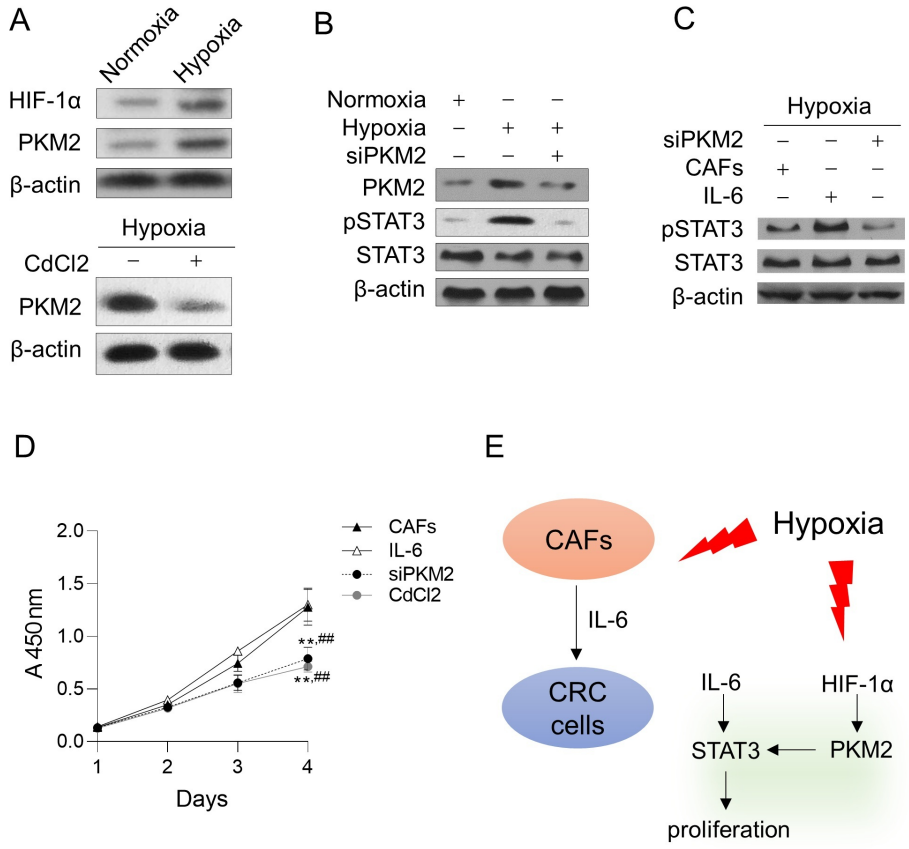

Figure 3. PKM2 is involved in the activation of IL-6/STAT3. A) Western blot was used for the PKM2 expression in HCT116 cells with different treatments. B) The impact of PKM2 knockdown in HCT116 cells under hypoxia. C) The cell viability of HCT116 with different treatments. **p<0.01, vs. IL-6. ##p<0.01, vs. cultured with CAFs. E) The schematic illustration of the interaction between CAFs, IL-6/STAT3, and CRC cells.

oncogenic pathways in cancer cells. Among these, IL-6/STAT3 as critical mediators of cancer cell proliferation. In this study, we have found that the STAT3 pathway was significantly activated by co-culturing of CRC cells with CAFs or IL-6. In AOM/DSS animal model, Grivennikov et al. [23] found reduced tumor development in IL-6 –/– mice exposed to AOM/DSS. Moreover, Bollrath et al. [24] showed an increased tumor growth following the AOM+DSS treatment in gp130Y757F mice, which have STAT3 hyperactivation, and attenuated tumor development in conditional knockout mice with a specific deletion of STAT3 in intestinal epithelial cells. The effect of STAT3 on tumor cells was mediated through the expression of various regulators of the G1/S and the G2/M cell cycle progression. Therefore, IL-6/STAT3 provides important growth signaling for CRC cells, and indeed, we have found that the inhibition of STAT3 strikingly suppressed cell growth *in vitro*.

Reprogramming of cell metabolism is essential for tumorigenesis. Hypoxia may trigger a shift in cellular metabolism away from oxidative phosphorylation towards aerobic glycolysis, a phenomenon known as the Warburg effect. PKM2, a direct target of HIF-1α, can promote aerobic glycolysis in cancer cells under hypoxia [25]. Recently, a number of studies reported that PKM2 promotes STAT3 hyperactivation [26–28]. We observed that PKM2 expression can be induced by hypoxia and knockdown of PKM2 effectively inhibited IL-6 mediated STAT3 activation, implicating that the STAT3 activation mediated by CAF-derived IL-6 is PKM2-dependent.

In summary, our work proposes the interconnected mechanisms that CAFs exert their functions on colorectal cancer under hypoxia. We reported a constitutive activation of STAT3 signal by CAFs-derived IL-6, which can be further enhanced by hypoxia in colorectal cancer. The pharmacological inhibition of STAT3, PKM2, or HIF-1α can significantly reduce the production of IL-6, which provides a potential drug target for colorectal cell proliferation.

Supplementary information is available in the online version of the paper.

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Hypoxia facilitates the proliferation of colorectal cancer cells by inducing cancer-associated fibroblast-derived IL6

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Supplementary Information

Supplementary Table S1. Primer sequences.

Target Gene	Forward Primer (5'→3')	Reverse Primer (5'→3')
α-SMA	GTGTTGCCCCTGAAGAGCAT	GCTGGGACATTGAAAGTCTCA
CD90	ATCGCTCTCCTGCTAACAGTC	CTCGTACTGGATGGGTGAACT
FAP	TGAACGAGTATGTTTGCAGTGG	GGTCTTTGGACAATCCCATGT
FSP1	GATGAGCAACTTGGACAGCAA	CTGGGCTGCTTATCTGGGAAG
IL-6	ACTCACCTCTTCAGAACGAATTG	CCATCTTTGGAAGGTTCAGGTTG
GAPDH	ACAACTTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC