Hypoxia Facilitates the Proliferation of Colorectal Cancer Cells by Inducing Cancer-associated Fibroblast-derived IL6.

Ying Xu
  Tongren Hospital Shanghai Jiaotong University School of Medicine

Rong Kuai
  Tongren Hospital Shanghai Jiaotong University School of Medicine

Yimin Chu
  Tongren Hospital Shanghai Jiaotong University School of Medicine

Lu Zhou
  Tongren University

Hai-qin Zhang
  Tongren Hospital Shanghai Jiaotong University School of Medicine

Ji Li (✉ lj1723@shtrhospital.com)
  Tongren Hospital

Research

Abstract

Background: Colorectal cancer (CRC) is 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.

Methods: We have successfully isolated CAFs and paired normal fibroblasts (NFs) from colorectal cancer tissues (n=10). By using multiplex cytokine profiling assay, we have identified IL-6 as a major cytokine released by CAFs. Coculturing of CAFs with CRC cell lines HCT116 or SW480 increase IL-6 release, and the secretion by CAFs can be further enhanced under hypoxia. By using CCK-8 assay, we have found 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).

Results: Mechanistically, we have found hypoxia can enhanced effect of IL-6/STAT3 signaling on CRC cells, in part, throughHIF-1a targets PKM2.

Conclusions: In conclusion, our data clearly proposes the interconnected mechanisms for a constitutive activation of STAT3 signalby CAFs-derived IL-6 under hypoxia in colorectal cancer. The pharmacological inhibition of STAT3, PKM2 or HIF-1α can significantly reduce oncogenic effect of IL-6, providing a potential therapeutic target for CRC patients.

Trail registration: Not applicable

Introduction

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 development of CRC remains largely unexplored(2). During the past decades, tumor microenvironment (TME) has been demonstrated to play a key role in colorectal carcinogenesis(3). TME is a composed of different components, including immune cells, fibroblasts, macrophage, 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), most abundant stromal cell population, were observed significantly contribute to development of CRC(5). It was reported that CAFs can be used as 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 secret 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 of 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 pathway, 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 interaction between CRC and CAFs, and further deciphered the mechanismsmediated 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 DMEM medium(Thermo Fisher Scientific, Inc) with 10% fetal bovine serum(Thermo Fisher Scientific, Inc.), 100 U/ml penicillin/streptomycin at 37°C in 5% CO_2humidified atmosphere. The recombinant interleukin (IL)-6, STAT3 inhibitor and HIF-1α inhibitor CdCl2 purchased from Sigma-Aldrich LLC.

Isolation of NFs and CAFs cells

Primary normal fibroblasts (NFs) and cancer-associated fibroblasts (CAFS) cells 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 piece. Tissues were then digested with 160μg/ml collagenase I(Sigma, USA) and 25μg/ml hyaluronidase at 37°C for 2h(Sigma, USA). 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% CO2. The culture medium was refreshed 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, Dojindo Laboratories, Japan). Briefly, human colorectal cancer cell lines HCT116 and SW480 (5×10^3cells/well) were seeded into 96-well plates with 100μl culture medium for 12h. Then, HCT116 and SW480 were treated with corresponding treatment. Subsequently, 10μl of CCK-8 solution was added to each well of the plate and the cells were incubated in a humidified incubator-containing 5%CO2 at 37°C for 1h. Subsequently, the absorbance was detected by a Microplate Reader (Bio-Rad, USA) at 490nm.

RNA extraction and quantitative real-time PCR

Total RNA was isolated by using TRIzol reagent (Invitrogen, USA) according to manufacturer's instruction. The first-strand cDNAs were reverse transcribed from 1μ total RNA using RevertAid First Strand cDNA Synthesis Kit(Thermo Fisher Scientific, USA) and analyzed using TB Green® *Premix Ex Taq*™(Takara, Japan) by real-time PCR in 7500 Fast Real-Time PCR detection system(ABI, USA). For the thermal cycles, the following reaction conditions were performed: 95°C for 10 minutes and 45 cycles of 15 seconds at 95°C and 1 minute at 60°C.The expression levels of target genes mRNA were normalized to the levels of β-actin gene transcript and calculated using $2^{-\Delta\Delta ct}$ method. The primer sequences were shown in Supplementary table 1 (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, China). 10μg of proteins were loaded, separated by 10% SDS-PAGE gel, and transferred to a PVDF (polyvinylidene fluoride) membrane (Millipore, Germany). The membrane was blocked in 5% non-fat milk for 1h, followed by incubation with the primary antibody overnight at 4°C. The primary antibody, including anti-HIF-1α, anti-pSTAT3, anti-STAT3, anti-β-actin and anti-PKM2 were obtained from Cell Signaling Technology, USA. After that, the membranes were incubated with secondary antibody at room temperature for 1h, and washed by TBS-T (TBS+%0.5Tween 20, Sangon, China) in 10 minutes for 3 times. The target proteins were detected by Immobilon™ Western HRP reagent (Millipore, USA). The β-actin protein was served as endogenous control.

Enzyme-linked immunosorbent assay(ELISA)

A human IL-6 ELISA kit was purchased from R&D(USA). 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 1h at room temperature. After being washed, the plate was incubated with 100μL tetramethyl benzidine substrate for 10min in the dark at room temperature and 100μL Stop solution for 1 min on a plate shaker. Intensity was measured at 450nm employing spectrophotometry.

Establishment of co-culture system

Transwell suspension chamber pre-covered with Polyester (PET) film was used for co-culturing experiment. CAFs were inoculated in the upper chamber, while $1×10^5$ of HCT116 or SW480 cells inoculated in the lower chamber. Culture medium from the upper chamber cells were 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, USA) manufacturer's instruction

Statistical analysis

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

Results

Cancer-associated fibroblasts (CAFs) secreted high level of IL-6 in colorectal cancer.

As mentioned above, CAFs act as major stromal cells to promote development of colorectal cancer, we thus hypothesized that CAFs is likely to release several key cytokines that links 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 successfully isolated CAFs and NFs, we subsequently measured 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 Fig. 1A, CAFs and NFs expressed higher level of α-SMA, CD90,FAPand FSP1, compared to epithelial cell controls(P< 0.01). Furthermore, the expression of these markers is more significantly up-regulated in CAFs than those in NFs (P< 0.01), suggesting that we have successfully isolated CAFs and NFs from colorectal tumor 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 B). 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 is only up to 1–2 fold change, such as TNF-α, CCL25, CCL17, and IL-1β, implicating that IL-6 that released by CAFs may play an important role in CRC.

Cancer-associated fibroblasts (CAFs) released increased IL-6 toresponse to hypoxia.

Previous studies have shown that hypoxia has significant impact on CRC carcinogenesis(12, 13), we therefore evaluate 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 normoxia (20% O2) or hypoxic (1% O2)condition (Fig. 1C). We collected culture medium from CAFs from Day1 to Day5, and measured IL-6 level by ELISA assay. In spite of the fact that IL-6 level from co-culture of CAFs and HCT116 is significantly higher than the level from CAFs cultured aloneunder normoxia, hypoxia, however, led to a pronounced increased IL-6 level from CAFs cultured alone or together with HCT116 (Fig. 1D). In consistent, we also collected CAFs cells and found the expression of IL-6 is dramatically induced by hypoxia (Fig. 1E), suggesting that hypoxia substantially contributes to the release of IL-6 by CAFs.

CAF-derived IL-6promotes 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 line HCT116 and SW480 with CM-CAF or NF for 72hrs. The cell proliferation was determined by using CCK-8 assay. As shown in Fig. 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 (10ng/ml or 20ng/ml). As shown in Fig. 2A, IL-6 has the capacity to promote proliferation of HCT116 and SW480 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 72hrs, and collected for CCK-8 assay. As expected, cells under normoxia showed lower cell growth than those under hypoxia (Fig. 2A), highlighting 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 IL-6/STAT3 pathway is aberrantly hyper activated in a variety of cancer type, and its hyper activation is often associated with poor prognosis. Biologically, IL-6/STAT3 signaling acts to drive the proliferation, invasiveness and metastasis of cancer cells(14, 15). Consistently, HCT116 or SW480 treated with STAT3 inhibitor showed lower cell proliferation than control cells showed (Fig. 2B).

HIF-1α is well recognized as a major transcription factor, that induced by the presence of hypoxia. We then measured expression of HIF-1α in HCT116 or SW480, which co-cultured alone or with CAFs. As shown in Fig. 2C, the HIF-1α expression in HCT116 or SW480 is significantly induced in the presence of hypoxia, confirming thatHIF-1α can be sufficiently induced by hypoxia. Moreover, we noticed that CAFs can significantly induceSTAT3 phosphorylation in HCT116 or SW480 under normoxia, and strikingly, STAT3 phosphorylation can be further up-regulated in the presence of hypoxia (Fig. 2C), indicating that hypoxia is able to enhance IL-6/STAT3 signaling in CRC cells.

To confirm hypoxia can regulate IL-6/STAT3 signaling, HCT116 or SW480 cells were pre-treated with HIF-1α inhibitor chloride (CdCl2) for 12hrs, and subsequently cultured with CAFs under hypoxia. As Fig. 2D showed, the expression of HIF-1α is significantly reduced by CdCl2 in HCT116 and SW480 cells. Moreover, STAT3 phosphorylation is also suppressed byCdCl2, highlighting the role of hypoxia in enhancingIL-6/STAT3 signaling in CRC.

HIF-1α target pyruvate kinase M2 is involved in 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 was observed up-regulated in HCT116 cell sunder hypoxia

(Fig. 3A). Moreover, inhibition of HIF-1α by CdCl2 can suppress hypoxia-induced PKM2, confirming that PKM2 is direct target of HIF-1α.

To examine whether PKM2 increase STAT3 phosphorylation, HCT116 were treated with PKM2 siRNA under hypoxia. The results clearly showed that knockdown of PKM2 inhibited STAT3 phosphorylation under hypoxia (Fig. 3B). We then investigated whether knockdown of PKM2 can suppress the biological effect of CAFs or IL-6 on CRC cells. As shown in Fig. 3C, suppression of PKM2 obviously inhibited 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 contribute to the cancer development. CAFs, representing 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 CAF from CRC patients. Furthermore, we have identified 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 enhanced activity of IL-6/STAT3 signaling, in part, through HIF-1α PKM2. These findings indicate that 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. Interleukin-6 (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 CAFs have capacity to secret high level of IL-6 compared to normal fibroblasts, suggesting that CAF maybe a major source of IL-6 in tumor microenvironment in CRC.

Interestingly, we found 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 oncogenic pathways in cancer cells. Among these, IL-6/STAT3 as critical mediators of cancer cell proliferation. In this study, we have found that 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 increased tumor growth following 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 G1/S and G2/M cell cycle progression. Therefore, IL-6/STAT3 provide important growth signaling for CRC cells, and indeed, we have found 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 promotes aerobic glycolysis in cancer cells under hypoxia(25). Recently, a number of studies reported that PKM2 promotes STAT3hyperactivation(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 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 colorectalcancer under hypoxia. We reported a constitutive activation of STAT3 signal by CAFs-derived IL-6, which can be further enhanced by hypoxia in colorectalcancer. The pharmacological inhibition of STAT3, PKM2 or HIF-1α can significantly reduce production of IL-6, which provides a potential drug targets for colorectal cell proliferation.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

All data generated or analysed during this study are included in this published article.

Competing interests

The authors declare they have not any competing interests.

Funding

Author's contribution

Study concept and design: Ying Xu, Rong Kuai, Ji Li; Experiments: Ying Xu, Rong Kuai, Yi-min Chu, Lu Zhou, Hai-qin Zhang; Statistical analysis: Ying Xu; Drafting of the manuscript: Ying Xu, Rong Kuai, Ji Li.

References

1. Timar J, Kashofer K. Molecular epidemiology and diagnostics of KRAS mutations in human cancer. Cancer Metastasis Rev. 2020.

2. Martini G, Dienstmann R, Ros J, Baraibar I, Cuadra-Urteaga JL, Salva F, et al. Molecular subtypes and the evolution of treatment management in metastatic colorectal cancer. Ther Adv Med Oncol. 2020;12:1758835920936089.

3. Guo L, Wang C, Qiu X, Pu X, Chang P. Colorectal Cancer Immune Infiltrates: Significance in Patient Prognosis and Immunotherapeutic Efficacy. Front Immunol. 2020;11:1052.

4. Pietras K, Ostman A. Hallmarks of cancer: interactions with the tumor stroma. Exp Cell Res. 2010;316(8):1324-31.

5. Mukaida N, Sasaki S. Fibroblasts, an inconspicuous but essential player in colon cancer development and progression. World J Gastroenterol. 2016;22(23):5301-16.

6. Tsujino T, Seshimo I, Yamamoto H, Ngan CY, Ezumi K, Takemasa I, et al. Stromal myofibroblasts predict disease recurrence for colorectal cancer. Clin Cancer Res. 2007;13(7):2082-90.

7. Huijbers A, Tollenaar RA, v Pelt GW, Zeestraten EC, Dutton S, McConkey CC, et al. The proportion of tumor-stroma as a strong prognosticator for stage II and III colon cancer patients: validation in the VICTOR trial. Ann Oncol. 2013;24(1):179-85.

8. Henry LR, Lee HO, Lee JS, Klein-Szanto A, Watts P, Ross EA, et al. Clinical implications of fibroblast activation protein in patients with colon cancer. Clin Cancer Res. 2007;13(6):1736-41.

9. Tommelein J, Verset L, Boterberg T, Demetter P, Bracke M, De Wever O. Cancer-associated fibroblasts connect metastasis-promoting communication in colorectal cancer. Front Oncol. 2015;5:63.

10. Muz B, de la Puente P, Azab F, Azab AK. The role of hypoxia in cancer progression, angiogenesis, metastasis, and resistance to therapy. Hypoxia (Auckl). 2015;3:83-92.

11. Wanandi SI, Ningsih SS, Asikin H, Hosea R, Neolaka GMG. Metabolic Interplay between Tumour Cells and Cancer-Associated Fibroblasts (CAFs) under Hypoxia versus Normoxia. Malays J Med Sci. 2018;25(3):7-16.

12. Vadde R, Vemula S, Jinka R, Merchant N, Bramhachari PV, Nagaraju GP. Role of hypoxia-inducible factors (HIF) in the maintenance of stemness and malignancy of colorectal cancer. Crit Rev Oncol Hematol. 2017;113:22-7.

13. Nagaraju GP, Bramhachari PV, Raghu G, El-Rayes BF. Hypoxia inducible factor-1alpha: Its role in colorectal carcinogenesis and metastasis. Cancer Lett. 2015;366(1):11-8.

14. Johnson DE, O'Keefe RA, Grandis JR. Targeting the IL-6/JAK/STAT3 signalling axis in cancer. Nat Rev Clin Oncol. 2018;15(4):234-48.

15. Kang S, Tanaka T, Narazaki M, Kishimoto T. Targeting Interleukin-6 Signaling in Clinic. Immunity. 2019;50(4):1007-23.

16. Chung YC, Chang YF. Serum interleukin-6 levels reflect the disease status of colorectal cancer. J Surg Oncol. 2003;83(4):222-6.

17. Galizia G, Orditura M, Romano C, Lieto E, Castellano P, Pelosio L, et al. Prognostic significance of circulating IL-10 and IL-6 serum levels in colon cancer patients undergoing surgery. Clin Immunol. 2002;102(2):169-78.

18. Knupfer H, Preiss R. Serum interleukin-6 levels in colorectal cancer patients--a summary of published results. Int J Colorectal Dis. 2010;25(2):135-40.

19. Yan SF, Ogawa S, Stern DM, Pinsky DJ. Hypoxia-induced modulation of endothelial cell properties: regulation of barrier function and expression of interleukin-6. Kidney Int. 1997;51(2):419-25.

20. Yan SF, Tritto I, Pinsky D, Liao H, Huang J, Fuller G, et al. Induction of interleukin 6 (IL-6) by hypoxia in vascular cells. Central role of the binding site for nuclear factor-IL-6. J Biol Chem. 1995;270(19):11463-71.

21. Grassl C, Luckow B, Schlondorff D, Dendorfer U. Transcriptional regulation of the interleukin-6 gene in mesangial cells. J Am Soc Nephrol. 1999;10(7):1466-77.

22. Kim HL, Cassone M, Otvos L, Jr., Vogiatzi P. HIF-1alpha and STAT3 client proteins interacting with the cancer chaperone Hsp90: therapeutic considerations. Cancer Biol Ther. 2008;7(1):10-4.

23. Grivennikov S, Karin E, Terzic J, Mucida D, Yu GY, Vallabhapurapu S, et al. IL-6 and Stat3 are required for survival of intestinal epithelial cells and development of colitis-associated cancer. Cancer Cell. 2009;15(2):103-13.

24. Bollrath J, Phesse TJ, von Burstin VA, Putoczki T, Bennecke M, Bateman T, et al. gp130-mediated Stat3 activation in enterocytes regulates cell survival and cell-cycle progression during colitis-associated tumorigenesis. Cancer Cell. 2009;15(2):91-102.

25. Wong N, Ojo D, Yan J, Tang D. PKM2 contributes to cancer metabolism. Cancer Lett. 2015;356(2 Pt A):184-91.

26. Li Y, Wang Y, Liu Z, Guo X, Miao Z, Ma S. Atractylenolide I Induces Apoptosis and Suppresses Glycolysis by Blocking the JAK2/STAT3 Signaling Pathway in Colorectal Cancer Cells. Front Pharmacol. 2020;11:273.

27. Damasceno LEA, Prado DS, Veras FP, Fonseca MM, Toller-Kawahisa JE, Rosa MH, et al. PKM2 promotes Th17 cell differentiation and autoimmune inflammation by fine-tuning STAT3 activation. J Exp Med. 2020;217(10).

28. Ma R, Liu Q, Zheng S, Liu T, Tan D, Lu X. PKM2-regulated STAT3 promotes esophageal squamous cell carcinoma progression via TGF-beta1-induced EMT. J Cell Biochem. 2019.
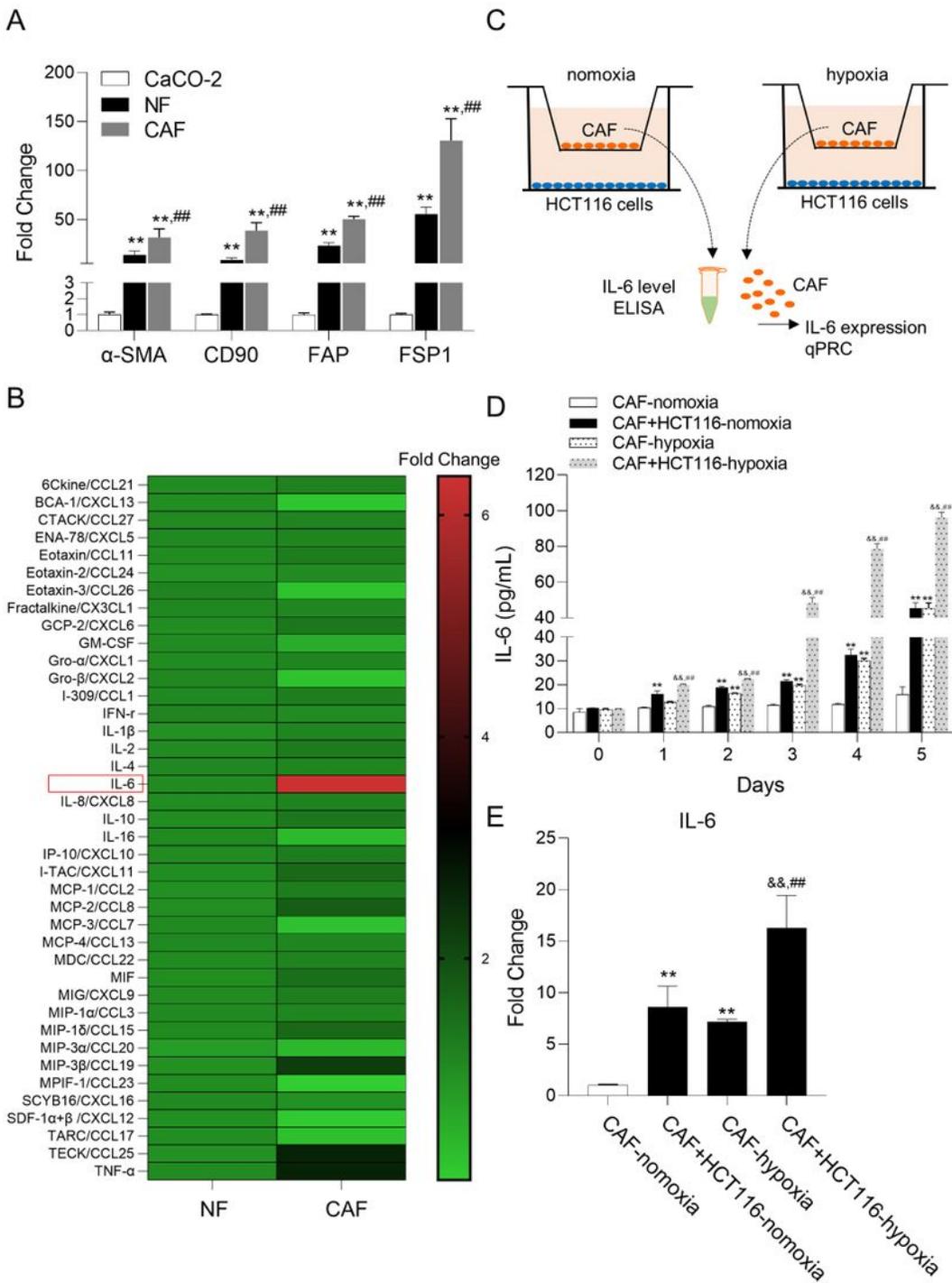
Figures

Figure 1

Figure 1

Hypoxia increase CAF-derived IL-6 level in CRC. A: The expression of CAF-specific genes in CAFs, NFs andepithelial cell controls CaCO-2. qPCR was used to determine 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 subject to cytokine profiling by using Bio-Plex assay. C: The schematic illustration of co-culture system. CAFs were seed in the upper chamber,

while HCT116 cells in the bottom chamber. Culture medium from CAFs were collected for IL-6 measurement, and CAFs cell were collected for qPCR assay. D: IL-6 level in culture medium from CAFs cultured alone or with HCT116, under normoxia or hypoxia. ELISA assay was performed for IL-6 evaluation. **P<0.01, vs CAFs cultured alone under normoxia; ##P<0.01, vsCo-culturing of CAFs with HCT116 under normoxia; &&P<0.01, vsCo-culturing of CAFs with HCT116 under hypoxia. E: IL-6 expression in CAFs with different treatment by using qPCR. **P<0.01, vs CAFs cultured alone under normoxia; ##P<0.01, vsCo-culturing of CAFs with HCT116 under normoxia; &&P<0.01, vsCo-culturing of CAFs with HCT116 under hypoxia.
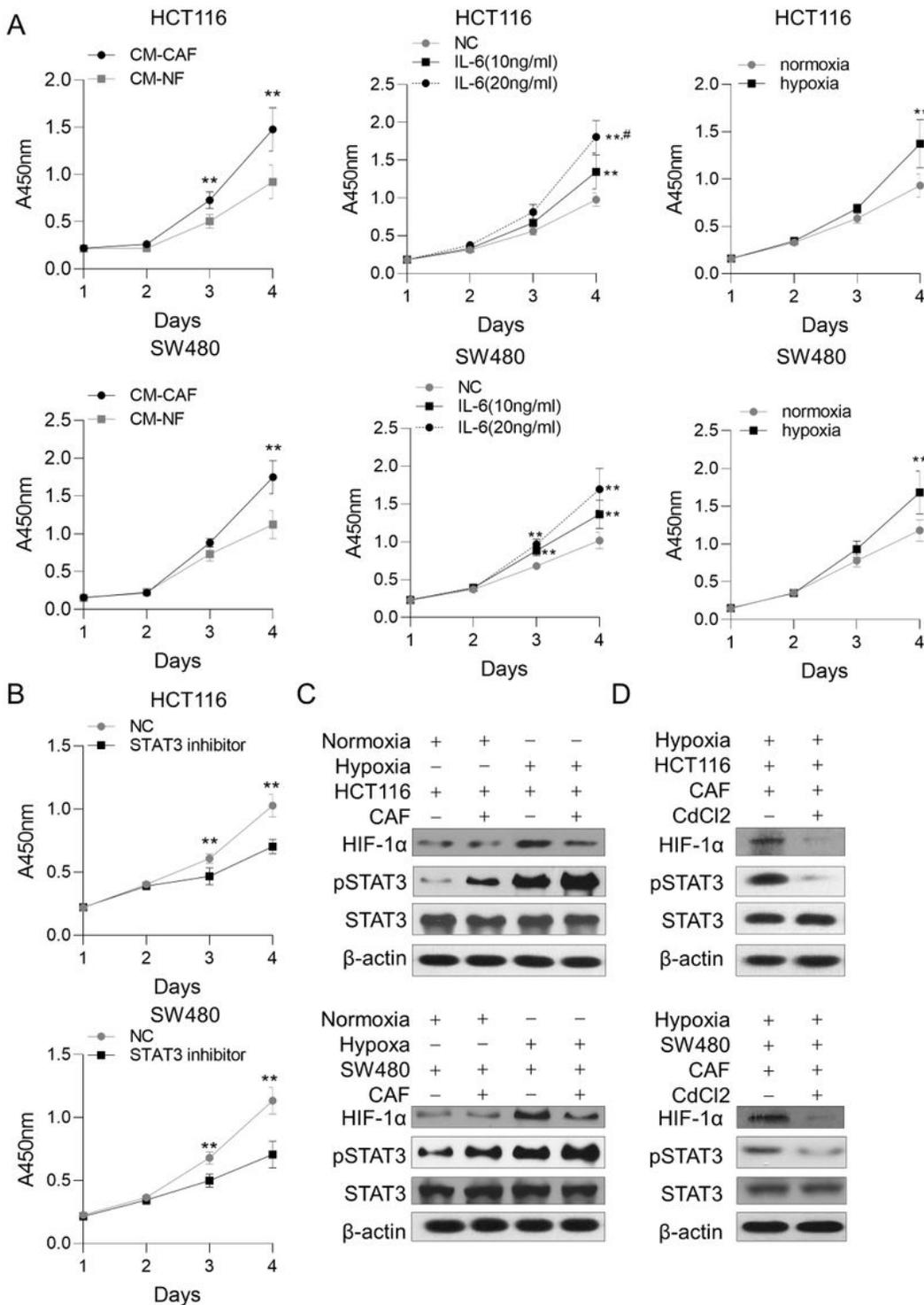
Figure 2

Figure 2

Hypoxia enhanced IL-6/STAT3 signaling to promote proliferation of colorectal cancer cells. A: The cell viability of HCT116 and SW480 with different treatment. CM-CAF: culture medium from CAFs, CM-NF: culture medium from NFs, NC: negative control. ** $P<0.01$,CM-CAF vsCM-NF; IL-6 vs NC; hypoxia vs normoxia. B: The cell viability of HCT116 and SW480 with STAT3 inhibitor. ** $P<0.01$,STAT3 inhibitor

vsNC. C and D: The protein expression of HIF-1α, phosphorylation STAT3 and total STAT3 in HCT116 and SW480 cells with different treatment by using western blotting assay.
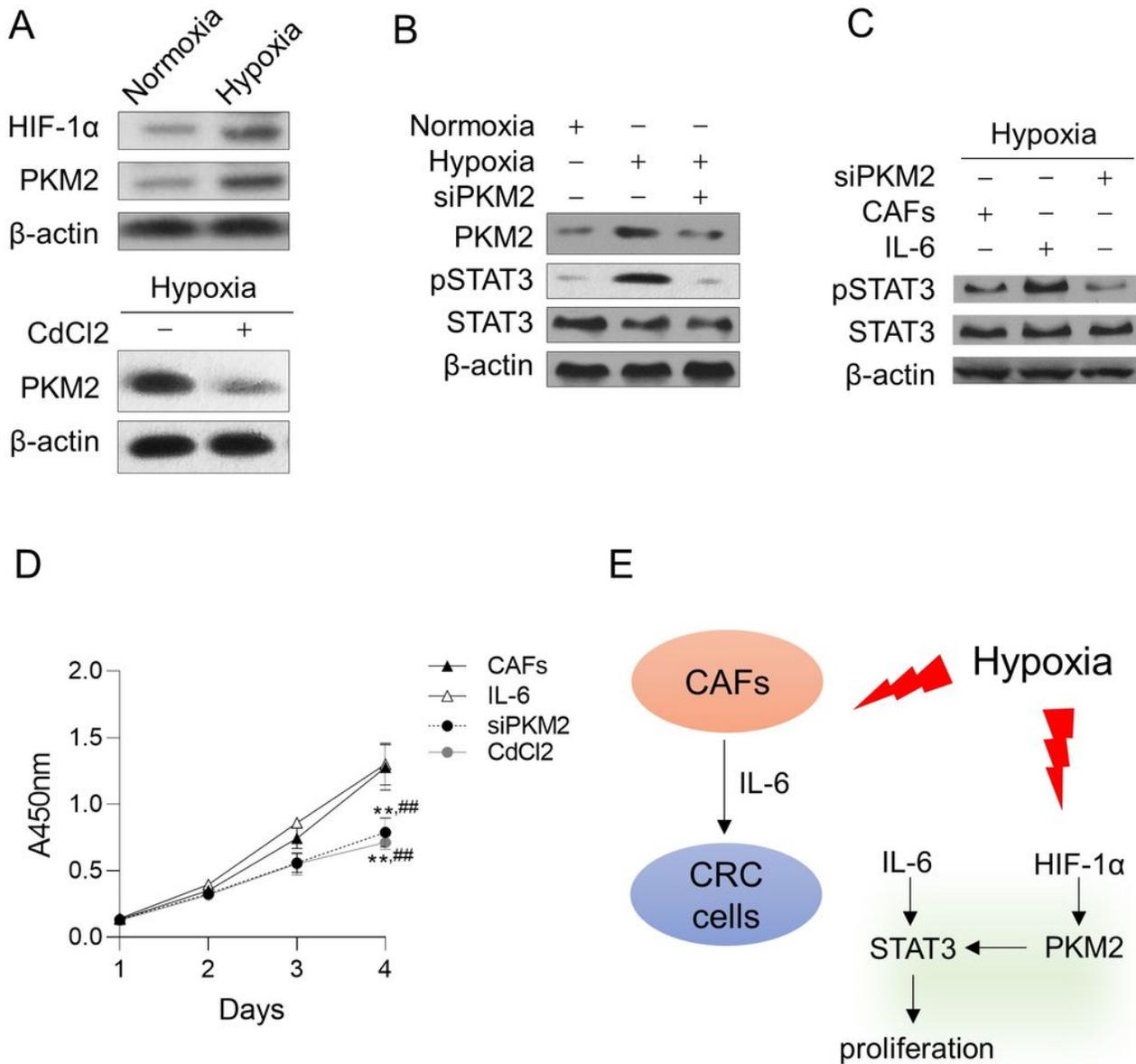
Figure 3

Figure 3

PKM2 is involved in activation of IL-6/STAT3 A: Western blotting assay was used for PKM2 expression in HCT116 with different treatment. B: The impact of PKM2 knockdown on HCT116 under hypoxia. C: The cell viability of HCT116 with different treatment. ** P<0.01,vsIL-6. ##P<0.01,vscultured with CAFs. E: The schematic illustration of interaction between CAFs, IL-6/STAT3 and CRC cells.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Xuetal.SupplTableS1.docx