RESEARCH ARTICLE

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Role of Oxidative Stress-Dependent *C/EBPβ* Expression on CAF Transformation Inducing HCT116 Colorectal Cancer Cell Progression; Migration and Invasion

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Abstract

Objective: To investigate oxidative stress-related CAF transformation through C/EBPβ, which affects CRC progression and may have a potential implication for CRC treatment. **Methods:** The conditioned media (CM) from HCT116, CRC cells, was used to activate CCD-18Co, colon fibroblasts, then the ability of activated FBs to induce HCT116 growth and progression was assessed using MTT assay, transwell migration, and matrix invasion assay. Alteration of the cytokine profile and oxidative stress of the activated FBs were studied with cytokine arrays and DCFH-DA assay, respectively. The protein expressions of the CAF markers (α-SMA and FAP) and *C/EBPβ* were investigated with immunofluorescence and western blotting. **Result:** It was found that CM from HCT116 cells induced oxidative stress, change of cytokine profile, CAF markers, and the *C/EBPβ* expression of activated FBs. Furthermore, when the oxidative stress of the activated FBs was suppressed, FAP and *C/EBPβ* expression were downregulated, correlating with the disabling of their capability to support the cancer progression. The *C/EBPβ* and prognosis for CRC patients were accessed using the GEPIA dataset, in which high *C/EBPβ* expression was associated with a poor prognosis. **Conclusion:** These findings suggest that *C/EBPβ* expression has a role in CAF transformation in an oxidative stress-related manner and might be used as a target to improve aggressive CRC treatment outcomes.

Keywords: *C/EBPβ*- colorectal cancer- cancer-associated fibroblasts- oxidative stress

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Introduction

Cancer is one of the leading causes of death worldwide, after cardiovascular disease, while colorectal cancer (CRC) is the second most common cause of cancer-related death (Keum and Giovannucci, 2019). The challenge of CRC treatment is distant metastasis, which involves a poor prognosis for cancer (Tauriello et al., 2017). Nowadays, the tumor microenvironment (TME), consisting of cells and extracellular matrix components, has been proven to influence the aggressiveness of cancer (Wu and Dai, 2017). Chronic inflammation, one of the hallmarks of the TME, causes oxidative stress to cancer cells which contribute to cancer development and progression (Lim and Moon, 2016; Neganova et al., 2021). The inflammatory environment of cancer is caused by various factors such as inflammatory molecules secreted by the cancer cells themselves or by the cells in the TME and infiltrating leukocytes (Wang et al., 2021).

Among all the cell types present in the TME, cancer-associated fibroblasts (CAFs) are the major component and are considered to be an important factor assisting in the critical steps of cancer progression, including cancer development, proliferation, invasion, and metastasis (Fiori et al., 2019). Furthermore, the CAFs can maintain the inflammatory environment by recruiting immune cells and secreting pro-inflammatory cytokines (Lim and Moon, 2016). This corresponds with the finding that an accumulation of CAFs around CRC is found to be associated with a poor prognosis (Henry et al., 2007; Wikberg et al., 2013).

Previous studies have shown that CAFs are activated fibroblasts (FBs) induced by cancer (Alkasalias et al., 2018; Zhang et al., 2020). When FBs are activated to become CAFs, they have more mesenchymal phenotypes and contractility. Additionally, increased oxidative stress in FBs had been found to induce FBs to gain more CAF characteristics by inducing CAFs marker expression, including alpha-smooth muscle actin (α-SMA) and fibroblast activation protein (FAP) (Toullec et al., 2010).

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However, there has been little research on how oxidative stress can induce FBs to become CAFs. Therefore, identifying the oxidative stress-related regulators involved in how FBs are regulated to become CAFs would be beneficial for aggressive cancer treatment. Since, the TME has a niche that resembles the site of an unhealing wound, consisting of chronic inflammation and fibrosis, myofibroblasts are commonly present in the TME, which is considered to be activated FBs or CAFs (Rybinski et al., 2014). Thus, in the CAF transformation process, FB activation may resemble the activation of myofibroblasts in chronic inflammation and fibrosis sites.

CCAAT/enhancer binding protein β (C/EBPβ) is a transcriptional factor that regulates diverse cell processes and cell differentiation. Interestingly, the dysregulation of *C/EBPβ* is associated with oncogenic roles in many cancers, including gastrointestinal malignancy (Regalo et al., 2016). Moreover, some studies have mentioned that *C/EBPβ* is found to be upregulated in activated FBs in the fibrosis area of the lung and cardiac myofibroblasts of autoimmune myocarditis. These studies also suggested that *C/EBPβ* plays a role in the myofibroblast transformation (Hu et al., 2007; Li et al., 2018). However, the role of *C/EBPβ* in CAF transformation is still unknown and needs to be elucidated.

The aim of this study was to investigate the oxidative stress-related CAF transformation mechanism via *C/EBPβ* expression, which affects CRC progression. Therefore, HCT116, a high progressive capacity CRC cell line, was used to activate the FBs. Then, the CAF characteristics of the activated FBs in the aspect of morphology, CAF markers, and cytokine profile were examined. Furthermore, the ability to induce the HCT116 CRC progression of activated FBs was studied along with cellular oxidative stress and *C/EBPβ* expression. Additionally, the prognosis for CRC patients with differential *C/EBPβ* expression was accessed using the Gene Expressing Profiling Interactive Analysis (GEPIA) database.

Materials and Methods

Cell lines and cell culture

HCT116 (ATCC® CCL-247TM), human colon cancer cells which have a high growth rate and are commonly used for migration and invasion experiments (Meng et al., 2019; Tao et al., 2019; Chen and Liu, 2021), were cultured with McCoy's 5A. CCD-18Co (ATCC® CRL-1459™), human colorectal fibroblasts, was cultured with EMEM. Both mediums were supplemented with 10% FBS, 1% L glutamine, 1% penicillin-streptomycin, and non-essential amino acid. The cells were maintained at 37°C in a humidified atmosphere that was supplied with 5% CO_2 in an incubator.

Conditioned medium (CM)

The cells were cultured until they reached 70-80% confluency. The culture medium was replaced with a serum free medium, and the cells were cultured for 24 h. Then the culture medium was collected and centrifuged at 2,000 rpm for 10 min. After that, the supernatant was separated and filtered through a 0.22 µm filter, aliquoted, and kept at -20 °C for a few weeks. Finally, the CM was diluted with completed medium at a ratio of 1:1 when used for the experiments.

Fibroblast with morphological change counting

CCD-18Co cells were seeded in 6 well plates (10,000 cells/well) and cultured for 24 h. The cells were treated with CM from HCT116 for 24 h and were observed under an inverted light microscope at 40X magnification and randomly count for 100 cells from each independent experiment. The activated FBs with morphological change are considered as the criteria in Table 1.

Cytokine arrays

The cytokine array membranes (ab133996, Abcam, UK) were incubated with 1X blocking buffer at RT for 30 min. Then, the CM was incubated with membranes at 4 °C overnight and washed five times at RT for 5 min each time. The membranes were incubated with 1X Biotin-conjugated Anti-cytokines at 4 °C overnight and washed five times at RT for 5 min each time. After that, the membranes were incubated with 1X HRP-Conjugated Streptavidin at RT for 2 h and washed five times. Finally, the membranes were mixed with detection buffers, incubated at RT for 2 min, washed, detected with a Gel documentation analyzer, and had their densitometry analyzed using ImageJ software.

The densitometry was normalized with the control. According to the instruction of the cytokine array membrane, the normalized values were calculated as Eq.1.

$$X_{Ny} = X_y * P_1 / P_y \quad \text{Eq.1}$$

P_1 = mean signal density of positive control spots on reference array

P_y = mean signal density of positive control spots on array 'y'

X_y = mean signal density of spot 'X' on array of sample 'y'

XN_y = normalized signal intensity of spot 'X' on array 'y'

3-(4,5-dimethythiazol-2-yl)-2,5 diphenyltetrasodium bromide (MTT) assay

HCT116 cells were seeded in 96-well plates (20,000 cells/well) and cultured for 24 h. The cells were treated with CM from activated FBs for 24 h. Then, the medium was replaced by 100 µl per well of serum free medium, which contained 10 µl of 5 mg/ml MTT stock solution and incubated for 2 h. After that, the medium was replaced with 100 µl of dimethyl sulfoxide (DMSO) and the formazan soluble was detected by a microplate reader at an absorbance of 570 nm in wavelength. The results were shown as percentages of the viable cells (% cell viability) of the control.

Transwell migration and matrix invasion assay

CCD-18Co cells were cultured with CM from HCT116 in the lower part of the 24-well plates for 24 h. For the transwell migration assay, HCT116 cells (100,000 cells/ insert) were seeded into cell culture inserts (Corning,

USA) with 8.0 μm pores and co-culture with activated FBs for 24 h, while for matrix invasion assay, the HCT116 cells were seeded into cell culture inserts coated with extracellular matrix (ECM) gel (Sigma-Aldrich, USA) and co-cultured with activated FBs for 72 h (Justus et al., 2014). The cell cultured inserts were collected, and the upper part was washed with PBS. Then, the lower part of the inserts was fixed with 4% paraformaldehyde and stained with crystal violet before being observed under a light microscope at 200X magnification and count with the ImageJ.

Wound healing assay

The HCT116 cells were seeded into 6-well plates and cultured until the cells reached 100% confluence with the monolayer. Then the wound gap was created using a pipette tip and the cells were further incubated with or without H_2O_2 50 μM at 37°C in a humidified atmosphere supplied with 5% CO_2. After 12 h and 24 h the closure of the wound gap was recorded randomly in three fields. The closure of the wound was measured using ImageJ and calculated as a % of the control, as Eq.2.

$$\text{Cell migration \% of control} = (D_i - D_t)/D_i * 100 \quad \text{Eq.2}$$

D_i = distance of wound gap at initiation
D_t = distance of wound gap at time t

DCFH-DA assay

Briefly, CCD-18Co cells were seeded in 96-well plates (4,000 cells/well) and incubated for 24 h. Then cells were treated with CM from HCT116, CM with H_2O_2, or CM with vitamin C for 24 h. The cultured medium was removed and replaced with 10 μM of DCFH-DA media and incubated for 1 h. In this process, DCF fluorescence intensity was assessed for cellular oxidants with a fluorescent microscope and a fluorescence microplate reader at excitation/emission wavelengths of 485/535 nm.

Immunofluorescent assay

CCD-18Co cells were cultured on a coverslip in 6-well plates for 24 h and treated with CM from HCT116 for 24 h. The cells were fixed in 100% methanol at 4 °C for 30 min, and then washed with PBS. After that, the cells were incubated with blocking buffer (1% BSA in PBS) for 1 h at RT, washed, and stained with primary antibodies: α-SMA (1:500, ab5694; Abcam), FAP (1:100, ab28244; Abcam), *C/EBPβ* (1:100, SAB2702381; Sigma-Aldrich) for 1 h at RT. The cells were washed and stained with secondary antibody conjugated with Alexa Fluor 488 (1:500, ab150077, ab150113; Abcam) for 1 h at RT. The samples were washed with PBS. Then, the cells were counter stained with Hoechst fluorescent stain. Finally, coverslips were mounted, and the slides were observed under a fluorescence microscope (Model BX53, Olympus, Tokyo, Japan).

The quantification of nuclear localization was calculated by measuring the mean fluorescence intensity and %Nuclear as Eq.3 and Eq.4, respectively (Kelley and Paschal, 2019).

$$\text{Mean fluorescence intensity} = \frac{\text{Total fluorescence intensity measured}}{\text{Number of pixels measured}} \quad \text{Eq.3}$$

$$\text{\%Nuclear} = \frac{\text{Total nuclear intensity}}{\text{Total nucclear intensity + Total cytoplasmic intensity}} \quad \text{Eq.4}$$

Western blotting

The wash-trypsinized cells were combined with RIPA lysis buffer (Merck, Germany) and protease inhibitor (Sigma-Aldrich, USA), and then homogenized with a sonicator (Vibro cell, Sonics, USA). After that, the lysate cells were centrifuged at 14,000 rpm at 4℃ for 15 min to obtain the supernatant. Total protein concentration was determined with the Bradford assay. Equal amounts of proteins were electrophoresed in 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Germany). The membranes were blocked in 5% skim-milk in RT for 1 h. Then, the membranes were incubated with primary antibodies overnight at 4°C. The following antibodies were used: α-SMA (1:5,000, ab5694; Abcam), FAP (1:1,000, ab28244; Abcam), *C/EBPβ* (1:500, SAB2702381; Sigma-Aldrich). Finally, the membranes were incubated with a secondary antibody (1:10,000, ab205718; Abcam, 1:2,000, P0447; Dako) at RT 1 h. The immunoblots were detected using Luminata Forte western HRP substrate (Merck, Germany) for 5 min, and the bands were analyzed relative to β-actin using ImageJ.

Gene expression profiling interactive analysis (GEPIA)

Differentiating the mRNA expression of *C/EBPβ* in colon adenocarcinoma (COAD) and in rectum adenocarcinoma (READ) was performed by using online software, Gene Expression Profiling Interactive Analysis (GEPIA), based on comparing tumors and normal samples from the TCGA (The Cancer Genome Atlas). The overall survival analysis of COAD and READ patients with high and low *C/EBPβ* expression was performed based on the Kaplan Meier curve.

Statistical analysis

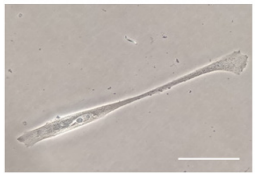
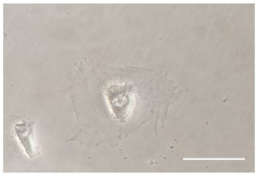
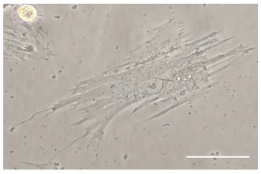
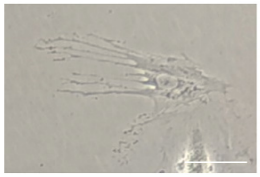
R (version 4.1.0) and Rstudio (version 1.4.1717) were used for all statistical analyses. Comparisons between two groups were based on analysis using the student T-test. Comparisons among more than 3 groups were based on analysis using one-way ANOVA and the Tukey's post-hoc test to identify the significant differences between each group. Data are expressed as means±SD (n=3). Significant statistical difference was considered when $P<0.05$.

Results

Conditioned media from HCT116 induced morphological change and CAF marker expression of fibroblasts

The morphology of activated FBs was observed, randomly counted as the criteria (Table 1) under inverted microscopy and presented as a percentage. When the FBs were treated with CM from HCT116 cells, the morphology of the FBs was changed. In the control, most FBs had an elongated-spindle shape with a smooth

Table 1. Criteria for Counting Activated FBs under Inverted Light Microscopy at 100X Magnification. The activated FBs with morphological change are considered when score≥2. Scale bar: 150 µm.

Criteria (Score)			
Elongated spindle (0)	Expanded cytoplasm (1)	Irregular shape (1)	Dendritic-like (1)
			

border and regular size, while in the CM-treated group, the activated FBs had expanded cytoplasm and irregular shapes with more dendritic borders than in the control group (Figure 1A). Moreover, when we observed the arrangement of α-SMA, the cytoskeleton of cells, we found that the activated FBs with morphological change had distinctly dense stress fibers with a crossing pattern when compared with the normal FBs (Figure 1B). Further investigation of the CAF markers, α-SMA, and FAP, by IFA showed that α-SMA was clearly upregulated in the

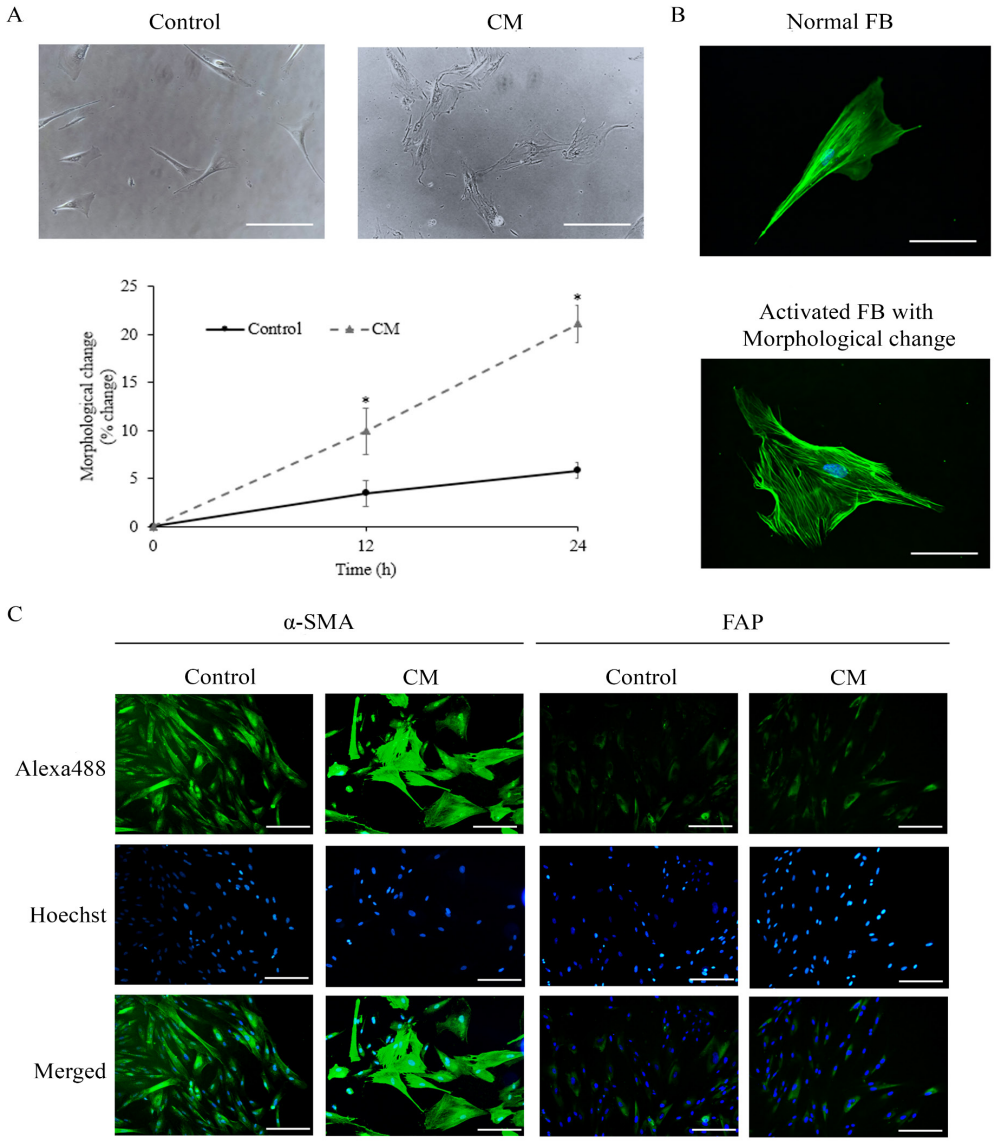


Figure 1. Morphology and CAF Markers of Activated FBs. (A) Morphology observed under inverted microscopy at 40X magnification after 24 h of CM treatment with the graph of percentage of morphological change at 12 h and 24 h. Scale bar: 400 µm (B) Cytoskeletal arrangement of normal FBs and activated FBs with morphological change. Scale bar: 100 µm (C) Immunofluorescence of α-SMA and FAP. Scale bar: 200 µm. Results were statistically significant at *P<0.001.

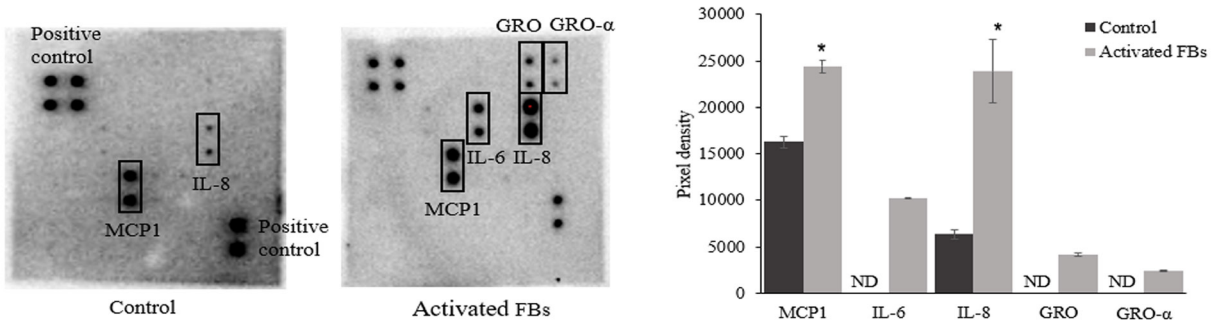


Figure 2. Cytokine Array of the CM from Activated FBs Comparing with the Control. Results were statistically significant at *P <0.05 and. ND = Not detected.

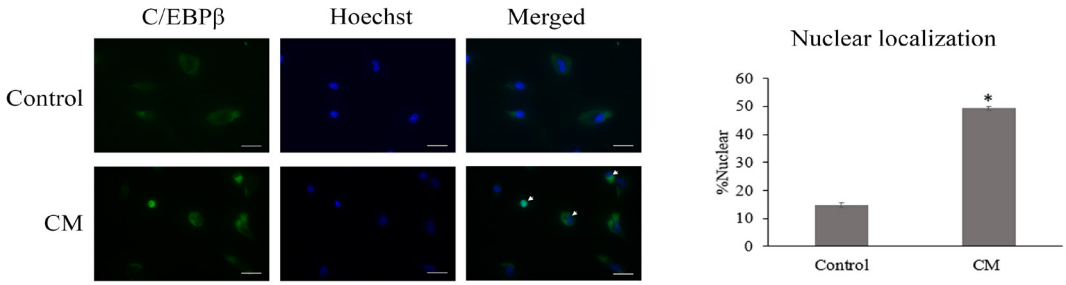


Figure 3. Immunofluorescence of C/EBPβ after being Incubated with CM from HCT116 for 24 h. The arrows sign indicated cells that C/EBPβ was localized in the nucleus. Scale bar: 200 μm. Results were statistically significant at *P <0.001.

activated FBs with morphological changes while the FAP was slightly upregulated when the FBs were treated with CM (Figure 1C).

The cytokine profile of activated FBs was changed

The CM from the activated FBs was investigated with cytokine arrays in order to study the cytokine profile and compared it with the control (Figure 2). The activated FBs released cytokines including MCP1, IL-6, IL-8, GRO (α, β, γ), and GRO-α, while in the control, only MCP1 and IL-8 were found and there were significantly more MCP1, IL-6, IL-8, GRO (α, β, γ), and GRO-α from the activated FBs than in the control.

C/EBPβ was localized around nucleus of activated FBs

To examine the *C/EBPβ* localization of the activated FBs, the FBs were incubated with the CM from HCT116 for 24 h and stained with *C/EBPβ* antibody (Figure 3). The results show that when the FBs were activated with CM, *C/EBPβ* was localized in the nucleus of the FBs more than was found in the control group.

Activated fibroblast induced cancer cell proliferation and progression

To confirm that activated FBs can be representative of the CAFs which have the ability to induce cancer proliferation and progression, CM from the activated FBs was used to treat cancer cells and observe the

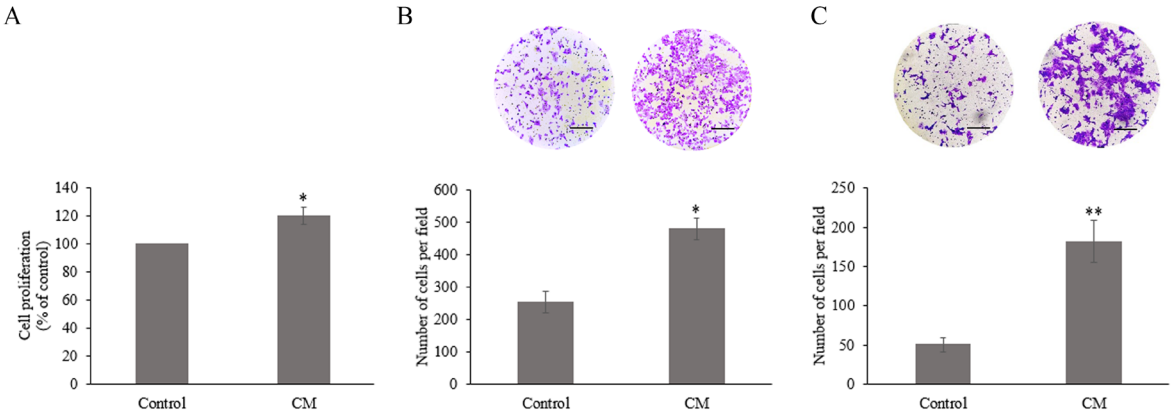


Figure 4. Cancer Proliferation and Progression. (A) MTT assay of HCT116 cells after incubated with CM from activated FBs for 24 h. (B) Transwell migration assay of HCT116 cells co-cultured with activated FBs for 24 h. (C) Matrix invasion assay of HCT116 cells co-cultured with activated FBs for 72 h. Results were statistically significant at *P<0.05 and **P<0.01.

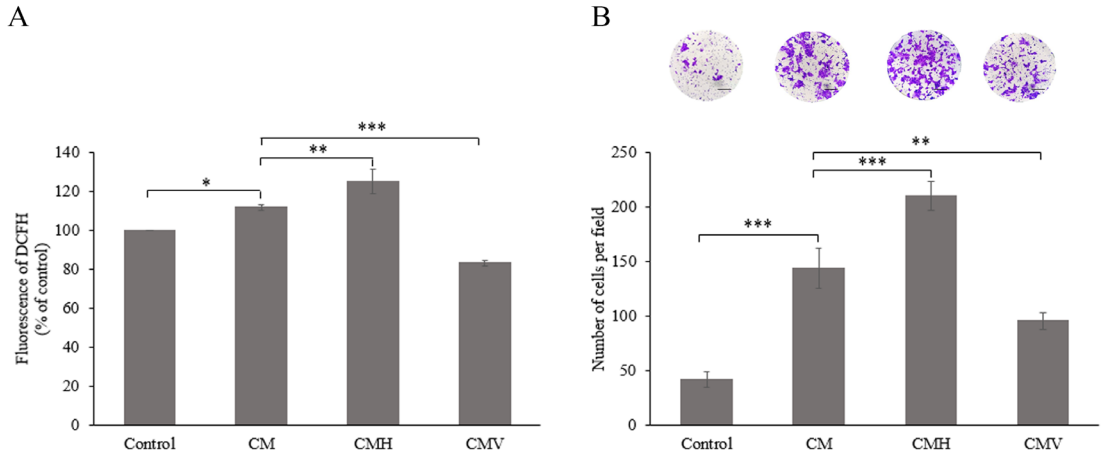


Figure 5. Oxidative Stress of Activated FBs and Effects of Activated FBs with Different Oxidative Stress on Cancer Progression. (A) DCFH-DA assay of activated FBs incubated with CM from HCT116 (CM), CM with H_2O_2 50μM (CMH), and CM with vitamin C 5μM (CMV). (B) Matrix invasion assay of HCT116 cells co-cultured with activated FBs in different oxidative stress conditions. Results were statistically significant at *P <0.05, **P<0.01, and ***P<0.001.

proliferation using MTT assay (Figure 4A). Furthermore, cancer progression was assessed by transwell migration (Figure 4B) and transwell invasion assay (Figure 4C). The results show that treated cancer cells could proliferate significantly more than when compared with the control group. Additionally, when the activated FBs were co-cultured with HCT116 cells, they were able to induce HCT116 cell migration and invasion significantly more than when compared with the control group.

Oxidative stress was increased in the activated fibroblasts and the decreased oxidative stress of activated FBs could suppress cancer progression

From studying the cellular oxidative stress of the activated FBs with DCFH-DA assay, it was found that oxidative stress increased significantly compared with the control. Additionally, to investigate the association between oxidative stress and CAF transformation, the activated FBs (CM) were manipulated under different oxidative stress conditions: oxidative stress was induced

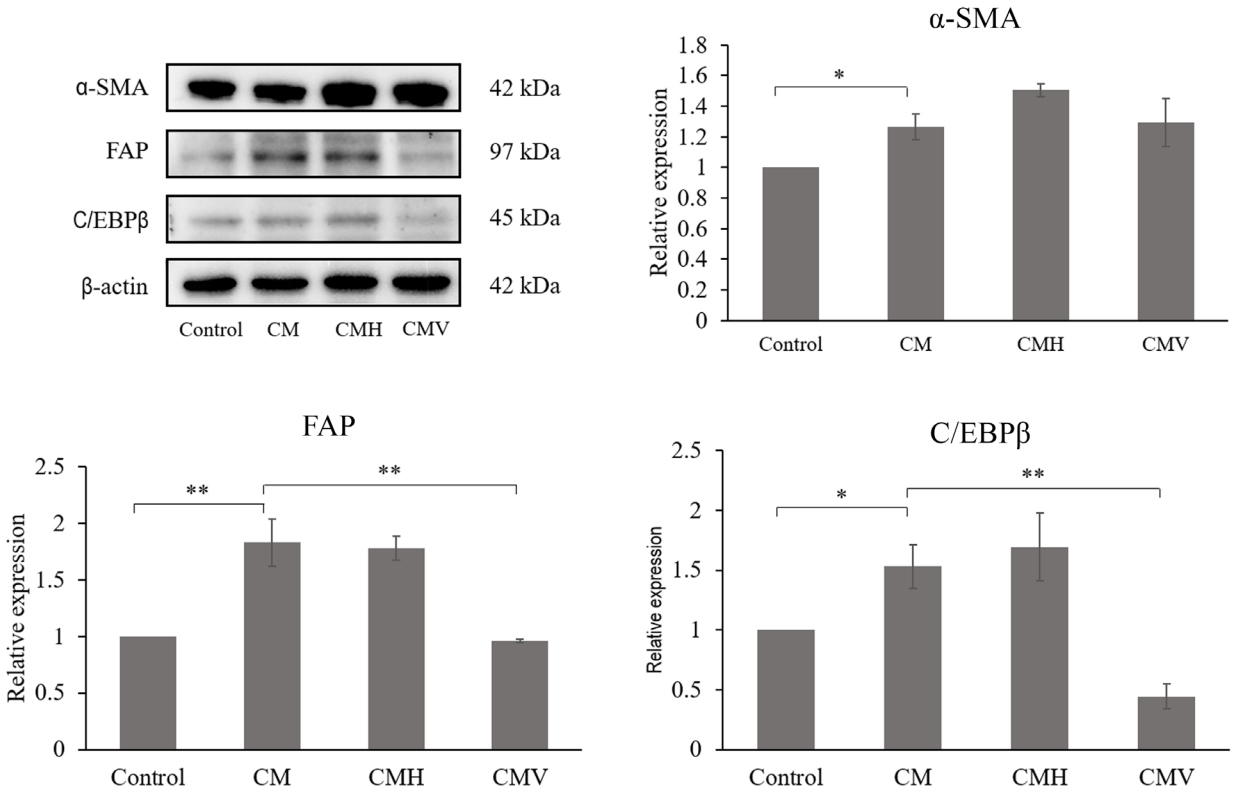


Figure 6. *C/EBPβ* and CAF Marker Expressions in Activated FBs Related with Oxidative Stress. CM from HCT116 promotes CAF markers (α-SMA and FAP) and *C/EBPβ* expression in different oxidative stress conditions. The CAF markers and *C/EBPβ* expression levels were measured with western blotting and used β-actin as the loading control. Results were statistically significant at *P<0.05 and **P<0.001.

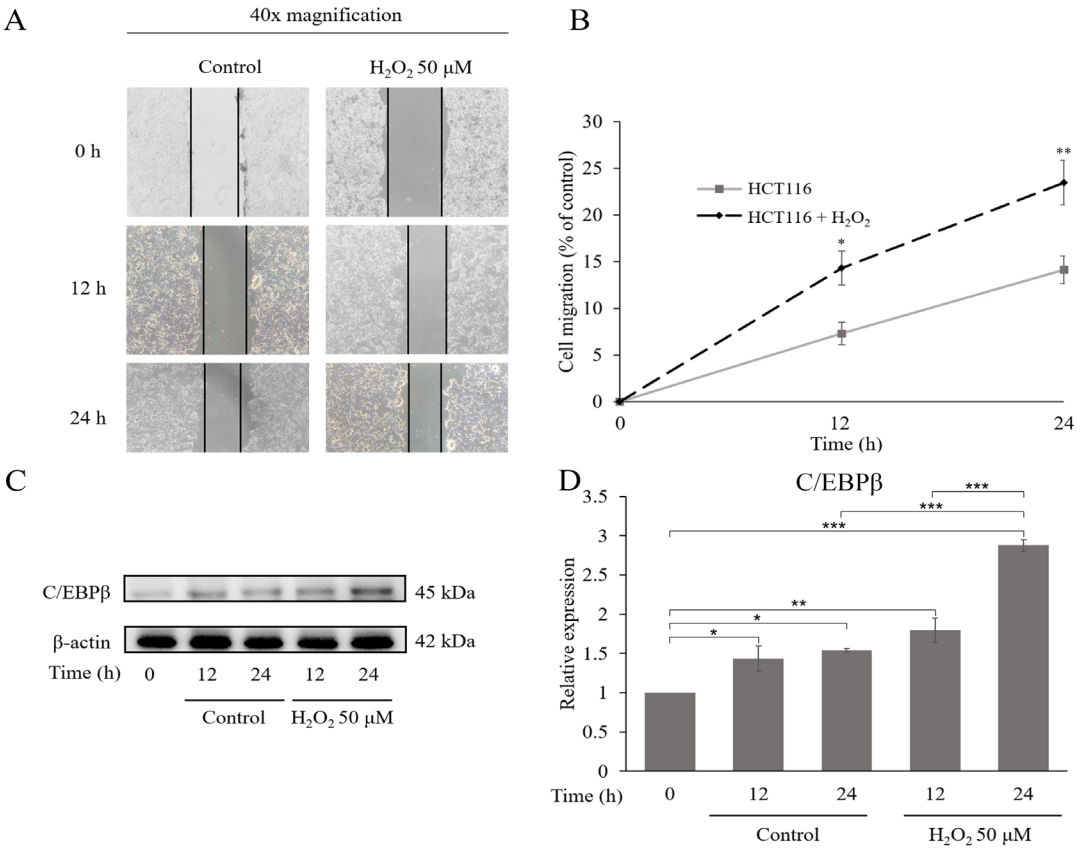


Figure 7. HCT116 Migration and *C/EBPβ* Expression of HCT116 with Oxidative Stress Induction. (A) Wound healing migration of HCT116 when induced with H2O2 50 μM at 12 h and 24 h. (B) Cell migration (% of control) of HCT116 when induced with H2O2 50 μM at 12 h and 24 h. (C) C/EBPβ expression of HCT116 at different time and oxidative stress conditions. (D) Relative C/EBPβ expression of HCT116 at different time and oxidative stress conditions. The C/EBPβ expression level was measured with western blotting and used β-actin as the loading control. Results were statistically significant at *P<0.05, **P<0.01, and ***P<0.001.

by 50μM H_2O_2 (CMH) and reduced oxidative stress with antioxidant, 5μM vitamin C (CMV) (Figure 5A). Furthermore, to investigate whether oxidative stress is related to the FB activation process, which affects cancer progression, activated FBs with different stress conditions were co-cultured with HCT116, and then the cancer invasion was observed. Interestingly, when the activated FBs were induced with oxidative stress, the HCT116 cells could invade the chamber significantly more than the HCT116 cells co-cultured with activated FBs could

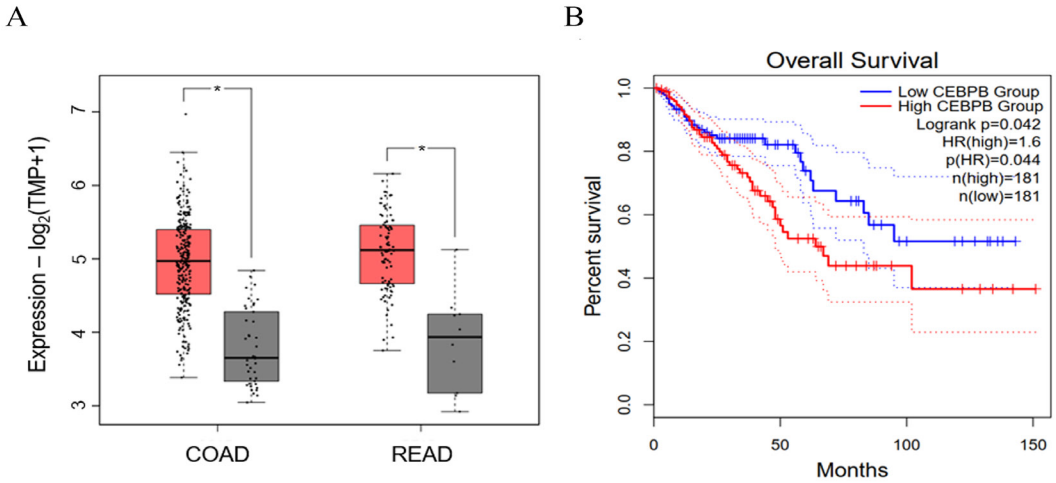


Figure 8. The mRNA Expression and Overall Survival Rate Analysis of Colon Adenocarcinoma (COAD) and Rectum Adenocarcinoma (READ) Patients Using the GEPIA Database. (A). The box plot shows the relative *C/EBPβ* expression in COAD and READ (red) compared with normal tissue near the tumor (gray). COAD; n (tumor)=275, n (normal)=41. READ; n (tumor)=92, n (normal)=10. (B) The *C/EBPβ* expression and overall survival analysis of COAD and READ patients. n=181/group. Results were statistically significant at *P<0.01.

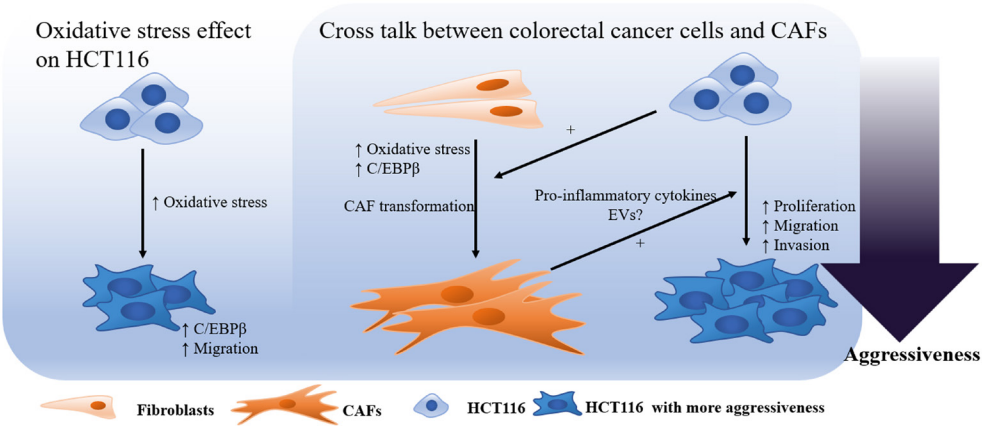


Figure 9. Roles of Oxidative Stress-Related *C/EBPβ* Expression in Aggressive CRC. The migration of HCT116, colorectal cancer cells, is influenced by oxidative stress-related *C/EBPβ* expression. Additionally, HCT116 induces CAF transformation through oxidative stress-related *C/EBPβ* expression and the CAFs eventually support cancer growth and progression (migration and invasion) by secreting pro-inflammatory cytokines.

do. Also, when the oxidative stress of the activated FBs was reduced, HCT116 cell invasion was significantly suppressed compared with the HCT116 cells co-cultured with activated FBs (Figure 5B).

C/EBPβ and CAF marker expressions were upregulated in activated FBs and associated with oxidative stress condition

After the activated FBs were manipulated under different stress conditions, the α-SMA, FAP, and *C/EBPβ* expression levels were investigated with western blotting. The expressions of the CAF markers and *C/EBPβ* were significantly upregulated in the activated FBs, which had higher oxidative stress than the control. When the oxidative stress of the activated FBs was increased, none of the markers showed significant change, but the *C/EBPβ* and α-SMA expression tended to be upregulated. Additionally, when the oxidative stress of the activated FBs was decreased, the expression of FAP and *C/EBPβ* was significantly downregulated, while there was no significant change to α-SMA (Figure 6).

C/EBPβ was upregulated in migrated HCT116 colorectal cancer cells and associated with oxidative stress

Because oxidative stress has been known to be involved with cancer progression, HCT116 cell migration induced with H_2O_2 50 μM was studied using a wound healing assay. Then, to study the correlation between oxidative stress and *C/EBPβ* expression on the aggressiveness of CRC, the *C/EBPβ* expression of HCT116 in different oxidative stress conditions harvested from wound healing assay, which migrated for different times, was investigated with western blotting. When HCT116 cells were induced with oxidative stress, they migrated faster than the control (Figure 7A, 7B). Furthermore, the *C/EBPβ* expression of HCT116 upregulate when HCT116 cells were induced with oxidative stress with the most significant upregulation observed at 24 h compared with the control at 0 h (Figure 7C, 7D).

C/EBPβ has higher expression in colorectal cancer and associated with low survival rate of colorectal cancer patients

The mRNA expression of the *C/EBPβ* in colon adenocarcinoma (COAD) and rectum adenocarcinoma (READ) was compared with normal tissue near the tumor using a GEPIA database (Figure 8A). The data showed that COAD and READ had significantly higher *C/EBPβ* expression. Furthermore, the overall survival rate of COAD and READ patients with high *C/EBPβ* expression was lower than for their counterparts with low *C/EBPβ* expression. In addition, the hazard ratio (HR) of COAD and READ patients with high *C/EBPβ* expression was more than 1, which suggests that high *C/EBPβ* expression increased the risk of death in COAD and READ patients (Figure 8B).

Discussion

Previous studies have shown that besides the aggressiveness of cancer itself, CAFs are a crucial player supporting cancer growth and progression related to the prognosis of cancer patients (Bussard et al., 2016; Gieniec et al., 2019; Sahai et al., 2020). Therefore, more knowledge about the transformation of FBs to CAFs is necessary in order to further support the achievement of the objective of inhibiting CAF formation, which may improve the treatment outcome of patients with aggressive cancer. Because the TME niche is considered to be an unhealing wound with chronic inflammation (Greten and Grivennikov, 2019), the process of FB activation into CAFs may resemble FB activation in cases of chronic inflammation. Thus, we hypothesized that any *C/EBPβ* found upregulated in the FBs of chronic inflammatory disease (Hu et al., 2007; Li et al., 2018) would be related to the FB activation process in CAFs. This study supports the hypothesis because when the FBs were activated with CM from CRC, the activated FBs showed morphological change similar to the finding of previous research, which mentioned that some activated FBs underwent a morphological change that left them appearing flat with

a leaf-like shape (Shimura et al., 2018). The cells which underwent a morphological change upregulated α-SMA and had cytoskeletal rearrangement, which represents an increase in the contractility of the cells (Ribatti and Tamma, 2019). When the cytokine profile of activated FBs was investigated, it was found that many pro-inflammatory cytokines, including GRO (α, β, γ), GRO-α, IL-6, IL-8, and MCP1, were upregulated compared with the normal FBs. These cytokines were observed in CAFs and found to be related to cancer proliferation, progression, and poor prognosis in cancer patients (Li et al., 2020; Chen et al., 2022; Cui et al., 2022; Gundlach et al., 2022). Moreover, the activated FBs showed CAF phenotypes, including upregulated CAF markers and enhanced cancer proliferation and progression, which correspond with the pro-inflammatory cytokine released by activated FBs.

Oxidative stress that is increased in chronic inflammation leads to cancer progression and is involved in the CAF transformation process (Lim and Moon, 2016; Tejada et al., 2019). Therefore, it has been suggested that oxidative stress might also be related to the regulation of signaling molecules in activated FBs. Previous research has shown that FBs with generated antioxidant defectives increased cellular oxidative stress and expressed more CAF phenotypes through HIF-1/CXCR4/CXCL12 pathway (Toullec et al., 2010). This is consistent with previous research which shows that prostate cancer induces the TGFβ-NOX4 pathway of FBs which then causes intracellular ROS production, resulting in CAF transformation (Sampson et al., 2018). Also, *C/EBPβ* was reported to be regulated by oxidative stress (Lei et al., 2020; Liu et al., 2021). Therefore, we assumed that in the process of CAF transformation, *C/EBPβ* might control the differentiation of the FBs in an oxidative stress-related manner.

Interestingly, the results partially support this assumption because activated FBs increased oxidative stress more than was observed with the control, while *C/EBPβ* expression was upregulated and re-located into the nucleus, which suggests an association between *C/EBPβ* and CAF transformation. When the oxidative stress of the activated FBs was increased by H_2O_2, the expressions of the *C/EBPβ* and CAF markers were not significantly upregulated. This might be due to the duration of the oxidative stress induction not being long enough to replicate the chronic inflammatory environment. However, when the oxidative stress was reduced with an antioxidant, vitamin C, the expressions of FAP and *C/EBPβ* were significantly downregulated, correlating with the capability of the activated FBs to induce cancer progression being disabled. These results suggest that oxidative stress is required for *C/EBPβ* expression and the CAF transformation process.

According to the results, even though activated FBs induced with H_2O_2 did not undergo significant increases in their CAF markers and *C/EBPβ* expression when compared with activated FBs, they could induce significantly more CRC cell invasion than the activated FBs could do. This may be explained by previous studies, which have reported that besides growth factors and cytokines, oxidative stress can induce cells to release extracellular vesicle (EV) secretions (Berumen Sánchez et al., 2021). The efficiency of the CAF-derived EV usually comes from the miRNAs contained in the EVs (Vokurka et al., 2022), which have various roles in relation to cancer cells, including influencing the cancer cells to increase their migration and invasion capability (He et al., 2021; Li et al., 2021). Therefore, the reduction of oxidative stress by an antioxidant might suppress CRC invasion through two mechanisms: suppressing both CAF transformation and EV release from the FBs. Another unexpected result is the α-SMA expression of activated FBs which was not suppressed when vitamin C was added. Because the CAFs still have no specific marker, using different combined markers is necessary to identify the CAFs (Shiga et al., 2015). The α-SMA is originally the marker of myofibroblasts, which was found in the wound healing process. One previous study found that vitamin C influences myofibroblast phenotypes including α-SMA expression and collagen secretion (Piersma et al., 2017). This effect of vitamin C other than as an antioxidant may explain why α-SMA expression was not regulated as had been expected.

The dysregulation of *C/EBPβ* has been found in various types of cancer, including gastrointestinal cancer (Regalo et al., 2016; Cao et al., 2021; Sterken et al., 2022). Moreover, increased cellular oxidative stress has been found in the cancer parenchymal and stromal parts (Prasad et al., 2017). For that reason, the effect of oxidative stress-related *C/EBPβ* expression on CRC progression was directly investigated in CRC cells. Because metastases are the most problematic aspect of CRC, causing a low 5-year survival rate among CRC patients (Chibaudel et al., 2015; Tauriello et al., 2017), HCT116 cells were selected to represent highly aggressive CRC for studying the role of oxidative stress-related *C/EBPβ* in CRC progression. The results show that HCT116 induced with oxidative stress migrated faster than the control, which corresponds to the findings for *C/EBPβ* expression. Previous research has reported that *C/EBPβ* could activate the ubiquitin4/Wnt/β-catenin signaling pathway, which promotes CRC progression (Tang et al., 2021). In addition, *C/EBPβ* can mediated IL-6 transcription (Poli, 1998), the well-known pro-inflammatory cytokine that has a role in the EMT of cancer cells (Bharti et al., 2016; Li et al., 2020). These are the possible pathways for oxidative stress-related *C/EBPβ* expression inducing HCT116 progression.

From the GEPIA dataset, the mRNA of *C/EBPβ* was found to be upregulated in CRC and associated with a poor survival rate among CRC patients. These data correspond with the results from this study which showed that *C/EBPβ* plays roles in CRC progression and CAF transformation, contributing to the aggressiveness of the cancer.

Because *C/EBPβ* is a transcription factor induced by pro-inflammatory cytokines and responds by binding with the promoter of inflammatory cytokines (Vanoni et al., 2017), the interplay between inflammation, the pro-inflammatory cytokines, oxidative stress, and *C/EBPβ* expression is the key to understanding the role of *C/EBPβ* in CAF transformation and CRC

progression. Therefore, besides the consistency of *C/EBPβ* expression, CAF transformation, and the aggressiveness of CRC, more knowledge about oxidative stress-related *C/EBPβ* pathways in CAF transformation will benefit TME-targeted therapy in patients with aggressive colorectal cancer.

In conclusion, this study proposed a novel aspect of *C/EBPβ* in CAF transformation which affects HCT116 CRC cell growth and progression (Figure 9) and showed that the expression of *C/EBPβ* depends on cellular oxidative stress in both CAFs and CRC cells. Furthermore, high *C/EBPβ* expression is associated with a poor prognosis for CRC patients. This knowledge can be applied to improve the treatment outcomes of aggressive CRC.

Author Contribution Statement

Conceptualization, P.S. and A.H.; methodology, A.H., R.T., and T.M.; validation, A.H., R.T. and T.M.; formal analysis, A.H.; investigation, A.H.; resources, P.S.; data curation, A.H.; writing-original draft preparation, A.H.; writing-review and editing, A.H., R.T., T.M., and P.S.; visualization, A.H.; supervision, P.S. and S.W.; project administration, P.S.; funding acquisition, P.S. All authors concur with the publication of this version of the manuscript.

Abbreviations

CAFs: Cancer-associated fibroblasts
C/EBP β: CCAAT/enhancer binding protein β
CM: Conditioned medium
CRC: Colorectal cancer
EV: Extracellular vesicle
FBs: Fibroblasts
RT: Room temperature

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General

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Ethical Declaration

This research received approval from the Faculty of Science, Mahidol University Biosafety Ethical Committee under the reference Exempt2022-015.

Conflict of Interest

The authors declare that have no conflict of interests.

References

Alkasalias T, Moyano-Galceran L, Arsenian-Henriksson M, et al (2018). Fibroblasts in the Tumor Microenvironment: Shield or Spear?. *Int J Mol Sci*, **19**.

Berumen Sánchez G, Bunn KE, Pua HH, et al (2021). Extracellular vesicles: mediators of intercellular communication in tissue injury and disease. *Cell Commun Signal*, **19**, 104.

Bharti R, Dey G, Mandal M (2016). Cancer development, chemoresistance, epithelial to mesenchymal transition and stem cells: A snapshot of IL-6 mediated involvement. *Cancer Lett*, **375**, 51-61.

Bussard KM, Mutkus L, Stumpf K, et al (2016). Tumor-associated stromal cells as key contributors to the tumor microenvironment. *Breast Cancer Res*, **18**, 84.

Cao LJ, Zhang YJ, Dong SQ, et al (2021). ATAD2 interacts with *C/EBPβ* to promote esophageal squamous cell carcinoma metastasis via TGF-β1/Smad3 signaling. *J Exp Clin Cancer Res*, **40**, 109.

Chen Z, Liu J (2021). MicroRNA-let-7 Targets HMGA2 to Regulate the Proliferation, Migration, and Invasion of Colon Cancer Cell HCT116. *Evid Based Complement Alternat Med*, **2021**, 2134942.

Chen ZY, Wang XY, Yang YM, et al (2022). LncRNA SNHG16 promotes colorectal cancer cell proliferation, migration, and epithelial-mesenchymal transition through miR-124-3p/MCP-1. *Gene Ther*, **29**, 193-205.

Chibaudel B, Tournigand C, Bonnetain F, et al (2015). Therapeutic strategy in unresectable metastatic colorectal cancer: an updated review. *Ther Adv Med Oncol*, **7**, 153-69.

Cui C, Zhang R, Gu F, et al (2022). Plasma CXCL3 Levels Are Associated with Tumor Progression and an Unfavorable Colorectal Cancer Prognosis. *J Immunol Res*, **2022**, 1336509.

Fiori ME, Di Franco S, Villanova L, et al (2019). Cancer-associated fibroblasts as abettors of tumor progression at the crossroads of EMT and therapy resistance. *Mol Cancer*, **18**, 70.

Gieniec KA, Butler LM, Worthley DL, et al (2019). Cancer-associated fibroblasts-heroes or villains?. *Br J Cancer*, **121**, 293-302.

Greten FR, Grivennikov SI (2019). Inflammation and Cancer: Triggers, Mechanisms, and Consequences. *Immunity*, **51**, 27-41.

Gundlach JP, Kerber J, Hendricks A, et al (2022). Paracrine Interaction of Cholangiocellular Carcinoma with Cancer-Associated Fibroblasts and Schwann Cells Impact Cell Migration. *J Clin Med*, **11**.

He C, Wang L, Li L, et al (2021). Extracellular vesicle-orchestrated crosstalk between cancer-associated fibroblasts and tumors. *Transl Oncol*, **14**, 101231.

Henry LR, Lee HO, Lee JS, et al (2007). Clinical implications of fibroblast activation protein in patients with colon cancer. *Clin Cancer Res*, **13**, 1736-41.

Hu B, Ullenbruch MR, Jin H, et al (2007). An essential role for CCAAT/enhancer binding protein beta in bleomycin-induced pulmonary fibrosis. *J Pathol*, **211**, 455-62.

Justus CR, Leffler N, Ruiz-Echevarria M, et al (2014). In vitro cell migration and invasion assays. *J Vis Exp*, **2014**.

Kelley JB, Paschal BM (2019). Fluorescence-based quantification of nucleocytoplasmic transport. *Methods*, **157**, 106-14.

Keum N, Giovannucci E (2019). Global burden of colorectal cancer: emerging trends, risk factors and prevention strategies. *Nat Rev Gastroenterol Hepatol*, **16**, 713-32.

Lei K, Shen Y, He Y, et al (2020). Baicalin Represses *C/EBPβ* via Its Antioxidative Effect in Parkinson's Disease. *Oxid Med Cell Longev*, **2020**, 8951907.

Li C, Teixeira AF, Zhu HJ, et al (2021). Cancer associated-fibroblast-derived exosomes in cancer progression. *Mol Cancer*, **20**, 154.

Li J, Huang L, Zhao H, et al (2020). The Role of Interleukins in Colorectal Cancer. *Int J Biol Sci*, **16**, 2323-39.

Li X, Sun M, Men S, et al (2018). The Inflammatory Transcription Factor *C/EBPβ* Plays a Critical Role in Cardiac Fibroblast Differentiation and a Rat Model of Cardiac Fibrosis Induced by Autoimmune Myocarditis. *Int Heart J*, **59**, 1389-97.

Lim H, Moon A (2016). Inflammatory fibroblasts in cancer. *Arch Pharm Res*, **39**, 1021-31.

Liu X, Huang Z, Zhang Y, et al (2021). Lacidipine Ameliorates the Endothelial Senescence and Inflammatory Injury Through CXCR7/P38/C/EBP-β Signaling Pathway. *Front Cardiovasc Med*, **8**, 692540.

Meng H, Wu J, Huang Q, et al (2019). NEDD9 promotes invasion and migration of colorectal cancer cell line HCT116 via JNK/EMT. *Oncol Lett*, **18**, 4022-9.

Neganova M, Liu J, Aleksandrova Y, et al (2021). Therapeutic Influence on Important Targets Associated with Chronic Inflammation and Oxidative Stress in Cancer Treatment. *Cancers (Basel)*, **13**.

Piersma B, Wouters OY, de Rond S, et al (2017). Ascorbic acid promotes a TGFβ1-induced myofibroblast phenotype switch. *Physiol Rep*, **5**.

Poli V (1998). The role of C/EBP isoforms in the control of inflammatory and native immunity functions. *J Biol Chem*, **273**, 29279-82.

Prasad S, Gupta SC, Tyagi AK (2017). Reactive oxygen species (ROS) and cancer: Role of antioxidative nutraceuticals. *Cancer Lett*, **387**, 95-105.

Regalo G, Förster S, Resende C, et al (2016). *C/EBPβ* regulates homeostatic and oncogenic gastric cell proliferation. *J Mol Med (Berl)*, **94**, 1385-95.

Ribatti D, Tamma R (2019). Giulio Gabbiani and the discovery of myofibroblasts. *Inflamm Res*, **68**, 241-5.

Rybinski B, Franco-Barraza J, Cukierman E (2014). The wound healing, chronic fibrosis, and cancer progression triad. *Physiol Genomics*, **46**, 223-44.

Sahai E, Astsaturov I, Cukierman E, et al (2020). A framework for advancing our understanding of cancer-associated fibroblasts. *Nat Rev Cancer*, **20**, 174-86.

Sampson N, Brunner E, Weber A, et al (2018). Inhibition of Nox4-dependent ROS signaling attenuates prostate fibroblast activation and abrogates stromal-mediated protumorigenic interactions. *Int J Cancer*, **143**, 383-95.

Shiga K, Hara M, Nagasaki T, et al (2015). Cancer-Associated Fibroblasts: Their Characteristics and Their Roles in Tumor Growth. *Cancers (Basel)*, **7**, 2443-58.

Shimura T, Sasatani M, Kawai H, et al (2018). Radiation-Induced Myofibroblasts Promote Tumor Growth via Mitochondrial ROS-Activated TGFβ Signaling. *Mol Cancer Res*, **16**, 1676-86.

Sterken BA, Ackermann T, Müller C, et al (2022). *C/EBPβ* isoform-specific regulation of migration and invasion in triple-negative breast cancer cells. *NPJ Breast Cancer*, **8**, 11.

Tang X, Liang Y, Sun G, et al (2021). UBQLN4 is activated by *C/EBPβ* and exerts oncogenic effects on colorectal cancer via the Wnt/β-catenin signaling pathway. *Cell Death Discov*, **7**, 398.

Tao L, Gu Y, Zheng J, et al (2019). Weichang'an suppressed migration and invasion of HCT116 cells by inhibiting Wnt/β-catenin pathway while upregulating ARHGAP25. *Biotechnol Appl Biochem*, **66**, 787-93.

Tauriello DV, Calon A, Lonardo E, et al (2017). Determinants of metastatic competency in colorectal cancer. *Mol Oncol*, **11**, 97-119.

Tejada S, Batle JM, Ferrer MD, et al (2019). Therapeutic Effects of Hyperbaric Oxygen in the Process of Wound Healing. *Curr Pharm Des*, **25**, 1682-93.

Toullec A, Gerald D, Despouy G, et al (2010). Oxidative stress promotes myofibroblast differentiation and tumour spreading. *EMBO Mol Med*, **2**, 211-30.

Vanoni S, Tsai YT, Waddell A, et al (2017). Myeloid-derived NF-κB negative regulation of PU.1 and c/EBP-β-driven pro-inflammatory cytokine production restrains LPS-induced shock. *Innate Immun*, **23**, 175-87.

Vokurka M, Lacina L, Brábek J, et al (2022). Cancer-Associated Fibroblasts Influence the Biological Properties of Malignant Tumours via Paracrine Secretion and Exosome Production. *Int J Mol Sci*, **23**.

Wang D, Cabalag CS, Clemons NJ, et al (2021). Cyclooxygenases and Prostaglandins in Tumor Immunology and Microenvironment of Gastrointestinal Cancer. *Gastroenterology*, **161**, 1813-29.

Wikberg ML, Edin S, Lundberg IV, et al (2013). High intratumoral expression of fibroblast activation protein (FAP) in colon cancer is associated with poorer patient prognosis. *Tumour Biol*, **34**, 1013-20.

Wu T, Dai Y (2017). Tumor microenvironment and therapeutic response. *Cancer Lett*, **387**, 61-8.

Zhang D, Li D, Shen L, et al (2020). Exosomes derived from Piwil2-induced cancer stem cells transform fibroblasts into cancer-associated fibroblasts. *Oncol Rep*, **43**, 1125-32.