RESEARCH ARTICLE

Secretomes of Primary Cancer-associated Fibroblasts Upregulate the Expression of Stemness Markers in HT-29 Human Colorectal Carcinoma Cells

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Abstract

BACKGROUND: Cancer-associated fibroblast (CAF) is the most abundant tumor stroma. Our previous study has demonstrated that the secretomes of CAF isolated from colorectal carcinoma (CRC) patients could induce epithelial-mesenchymal transition in the HT-29 CRC cell line. However, the role of CAF secretomes in CRC stemness is needed to be further investigated. Therefore, the present study aimed to investigate the effect of CAF secretomes from CRC patients on the expression of stemness markers in HT-29 CRC cells in comparison with the secretomes from normal fibroblasts.

METHODS: Fibroblasts were isolated from tumor (CAF) and their counterpart non-tumor (NF) areas of three CRC patients undergone surgical resection. Normal preputium fibroblasts (PF) were isolated during circumcision of three healthy boys aged 8 years. All fibroblasts were grown in free-serum culture medium for 24 hours to collect 50% (v/v) conditioned medium (CM). Then, CM was supplemented to HT-29 CRC cells for 72 hours. The effects of CAF- and NF-CM on the mRNA expression of CD44, CD133, OCT4, and ALDH1A1 were analysed using qRT-PCR. Cells proliferation was measured using the trypan blue exclusion assay.

RESULTS: Supplementation of CAF-CM (50% v/v) significantly increased CD44, CD133, OCT4, and ALDH1A1 mRNA expressions compared to that of NF-CM and control without supplementation but had no effect on the proliferation of HT-29 cells.

CONCLUSION: CAF secretomes from CRC patients upregulate the expression of CRC stemness.

KEYWORDS: cancer-associated fibroblasts, ALDH1A1, OCT4, CD44, CD133, colorectal carcinoma

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Introduction

Colorectal carcinoma (CRC) is a malignant tumor arising from the colon and rectum epithelium with a high mortality rate. Previous studies have indicated that therapy resistance, recurrence, and aggressiveness of CRC are related to the presence of cell subpopulations in tumor mass that sharing similar properties with normal stem cells, such as self-renewal, differentiation, chemoresistance, and metastatic potential, referred to cancer stem cells (CSCs). (1,2) In CRC, the presence of CSCs can be determined through the expression of CD44 and CD133 stemness markers which are associated with poor prognosis.(1,3) Furthermore, the expression of aldehyde dehydrogenase (ALDH), particularly its isotype ALDH1A1, may be used for identifying and isolating human colonic stem cells.(4) Several transcription factors including Octamer-binding transcription factor 4 (OCT4), Tír na nÓg (NANOG), sex determining region Y-box 2 (SOX2), Kruppel-like factor 4

(KLF4), and cellular Myelocytomatosis (c-Myc) have been reported to be essential for maintaining stemness properties such as self-renewal and cell pluripotency.(5)

Recent studies have suggested that some cancer cells acquire stemness properties through epithelial-mesenchymal transition (EMT).(6,7) EMT processes facilitate the invasiveness and high motility of cancer cells enabling them to disseminate to adjacent tissue and distant organs. The abundance of cancer-associated fibroblasts (CAF) in the CRC microenvironment plays a critical role to induce EMT phenotype.(7,8) The heterogeneity and complexity of CAF are represented by their secretomes that mediate the crosstalk between CAF and cancer cells via paracrine signaling to promote aggressiveness and stemness properties of cancer stem cells.(9) Our previous study has demonstrated that the secretomes of CAF isolated from CRC patients induced epithelial-mesenchymal transition in HT-29 human CRC cell line.(10)

Secreted factors of CAFs have been implicated in the generation of the CSC niche and promote stemness in various cancers. CAF secretomes induced NANOG expression and promote lung cancer stemness by activating IGF-II/IGF1R signaling.(11) Other study showed that CAF-released factors sustained stemness properties in Head and Neck Squamous Carcinoma Cells by increasing ALDH1, NANOG, SOX2, and OCT4 expression.(12) In colorectal cancer, CAF secretomes upregulated TIAM1 expression to increase stemness in CRC cell lines.(13) Moreover, CAF secreted exosomal lncRNA H19 that promote stemness through sphere formation in CRC cell lines.(14) In that study, the expressions of stemness markers, such as OCT4, CD44, and CD133, were assessed on the SW480 cells overexpressed with lncRNA H19 rather than supplemented with CAF or NF secretome. However, the comparison effect between tumor and normal fibroblasts on the stemness properties of CRC has not yet been explored.

The present study aimed to investigate the effect of primary CAF secretomes from CRC patients on the expression of stemness markers in HT-29 CRC cells in comparison with the secretomes from normal fibroblasts. In addition to CAF and NF isolated from CRC patients, we also used normal preputium fibroblasts isolated during circumcision of three healthy young boys. Understanding the presence of CSCs is important to elaborate the aggressiveness and progressiveness of CRC. Further studies on the CAF secretomes-containing factors are required for novel anticancer targeted to eliminate CSC populations efficiently.

Methods

Primary Culture of Fibroblasts

In this present study, primary fibroblasts were obtained and isolated from tumor and their counterpart nontumor areas of three colorectal adenocarcinoma patients undergone surgical resection without prior radiotherapy or chemotherapy at Cipto Mangunkusumo General Hospital, Jakarta, as described previously.(15) Normal preputium fibroblasts (PF) were isolated from preputium tissues during circumcision of three boys aged 8-10 years. The ethical clearance and written informed consent have been approved by the Ethics Committee of the Faculty Medicine, Universitas Indonesia (No. 433/UN2.F1/ETIK/2017) based on the Declaration of Helsinki for ethical principles using human specimens.

All fibroblasts were cultured using the FGM-2 Bullet kit (Lonza, Basel, Switzerland) medium and fibroblast growth was maintained under standard cultured condition (5%CO_2, 20%O_2 at 37°C). All fibroblasts have been determined for CAF markers in our previous study showing positive expression for α-SMA and vimentin, but negative expression of E-cadherin (in press).

Conditioned Medium of Fibroblasts

To prepare conditioned medium from tumor fibroblasts (CAF-CM), nontumor fibroblasts (NF-CM), and preputium fibroblasts (PF-CM), about $2x10^5$ fibroblasts were seeded into a six-well plate in 1 mL of Fibroblasts Basal Medium (FBM) supplemented with FGM-2 bullet-kit (Lonza). After the growth of fibroblasts reaches 80-90% confluence, cells were washed with PBS three times and then given 2 mL of free-serum FBM in each well. Following 24 h cell culture without serum, conditioned medium (CM) from CAF, NF, and FP were collected, then filtered with a 0.22 µm syringe filter and preserved at -80ºC for further study.

HT-29 Human Colorectal Carcinoma Cell Culture

The human colorectal carcinoma cell lines HT-29 (ATCC® HTB-38) were seeded in a twelve-well plate with cell density about $1x10^5$/well and grown in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 1% Penicillin–streptomycin (Gibco, Thermo Fisher Scientific, Inc.) and 1% Amphotericin B (Gibco, Thermo Fisher Scientific, Inc.) under standardized cell culture condition (37ºC, humid atmosphere, CO_2 5%), as described previously in our previous report. After 24 hours, culture medium was

replaced with 50% (v/v) CAF-CM and 50% (v/v) NF-CM, and 50% (v/v) PF-CM, respectively.

Determination of Cell Proliferation

After 72 hours treatment with CAF-CM, NF-CM, and PF-CM HT-29 cells were harvested. Cell proliferation was determined using a trypan blue exclusion assay (Gibco, Thermo Fisher Scientific, Inc.), as described previously. (16) The number of live cells was estimated using a Luna™ automated cell counter (Logos Biosystems, Anyang, Gyeonggi, Korea) by comparing the percentage of live cells compared with control.

Total RNA Isolation and qRT-PCR

Total RNA of HT-29 post cultured with fibroblast CM was isolated using Tripure isolation reagents® (Roche, Basel, Switzerland) according to the manufacturer's instructions. Quantitative reverse transcriptase of polymerase chain reaction was performed using Bioline SensiFASTTMSYBR®No-ROX One-Step Kit manufacturer instructions. The experiment repeated three times. Primers used for qRT-PCR analysis are list in Table 1.(16,17) The data were then analyzed using the ΔΔCq method and normalized to the internal control 18S rRNA gene.

Statistical Analysis

Data were presented as the mean±standard error of the mean. Statistical analysis was performed using SPSS 20 (IBM Cooperation, Armonk, NY, USA) and Student's t-test to compare the differences between treatment with CAF-CM, NF-CM, and PF-CM to HT-29 colorectal carcinoma cells. $p<0.01$ is considered significant statistically.

Results

Effect of CAF Secretomes on mRNA Expression of CD44, CD133, OCT4 and ALDH1A1 in HT-29 Cells

In this study, we focused on studying the upstream regulation of gene expression at the level of mRNA synthesis in human HT-29 CRC cells in order to analyze the effect of CAF secretomes supplementation compared to the secretomes from NFs and from normal preputium. Our parallel study has demonstrated that fibroblasts from tumor (CAF) express significantly higher α-SMA mRNA compared to their counterpart from nontumor area (NF).(10) Figure 1 shows that the 50% (v/v) CAF-CM supplementation to HT-29 cells significantly increased the expression of stemness markers, CD44, CD133, ALDH1A1, and OCT4, compared to cells without supplementation and to that with NF-CM and PF-CM supplementation. Interestingly, the NF-CM supplementation also increased significantly the expressions of ALDH1A1 and OCT4 compared to cells without supplementation and PF-CM supplementation. However, the p value was significantly lower than that of CAF-CM supplementation. Referring to our parallel data on α-SMA expression (10), we also correlated the α-SMA and stemness marker mRNA expression levels and found a significant positive correlation between the mRNA expression of α-SMA and CD44 ($r=0.758$; $p<0.05$), CD133 ($r=0.903$; $p<0.01$), ALDH1A1 ($r=0.881$; $p<0.01$), and OCT4 ($r=0.966$; $p<0.01$), respectively.

Effect of CAF Secretomes on HT-29 Cell Proliferation

Following 72 hours supplementation with CAF-, NF-, and PF-CM, the morphology of HT-29 cells revealed no visible changes on the shape, cytoplasm, and nucleus of HT-29 cells compared to control cells without any supplementation (Figure 2). To assess the effect of CAF-CM and NF-CM supplementation on HT-29 cell proliferation, we determined the viable cell count using trypan blue exclusion assay. The results showed that the viable cell count after CAF-CM supplementation was not statistically different with that after NF-CM supplementation or without any supplementation (Figure 3). These data suggest that the supplementation of CAF secretomes did not affect HT-29 cell proliferation.

Discussion

The presence of CAF as the most abundant cell population in CRC stroma has been suggested to be associated with

Table 1. Primer sequences for mRNA expression analysis using qRT-PCR.

Gene	Forward Primer	Reverse Primer	Product Size (bp)
CD44[14]	CTGCTACCAGAGACCAAGACA	ATGTGTCAGTTGTAGCGAGGTG	361
CD133	CACTACCAAGGACAAGGCGT	TCCTTGATCGCTGTTGCCAT	135
ALDH1A1[13]	GGAGGAAACCCTGCCTCTTTT	TTGGAAGATAGGGCCTGCAC	117
OCT4[13]	GAGGAGTCCCAGGACATCAAA	AGCTTCCTCCACCCACTTCT	234
18S rRNA[13]	AAACGGCTACCACATCCAAG	CCTCCAATGGATCCTCGTTA	155

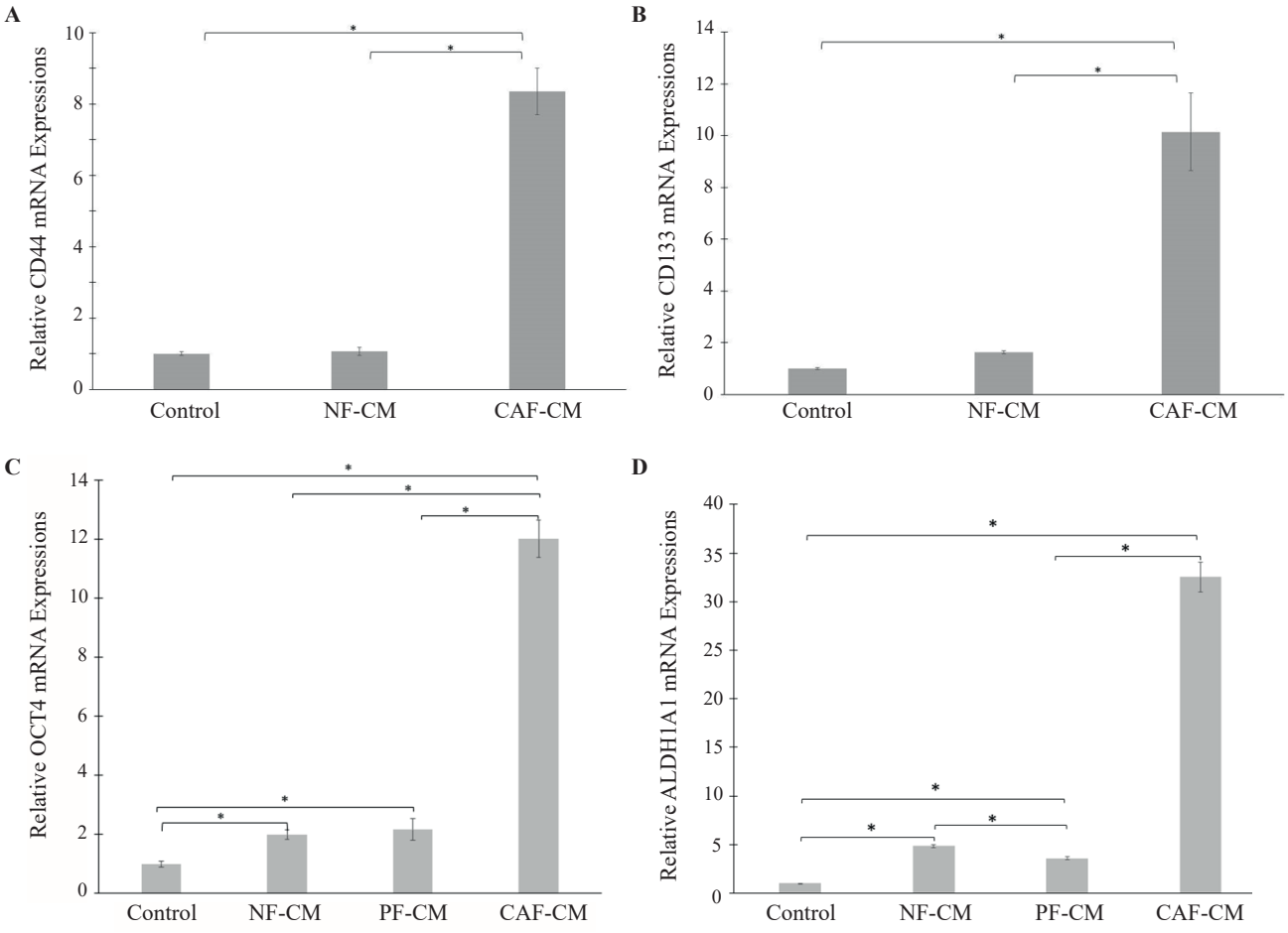

Figure 1. The effect of fibroblast secretomes on the stemness markers in HT-29 colorectal carcinoma cells line. The mRNA relative expression levels of CD44 (A), CD133 (B), OCT4 (C), and ALDH1A1 (D) in HT-29 colorectal cells line after supplemented with 50% v/v of CAF-CM, NF-CM, and PF-CM were measured using qRT-PCR and analyzed using the Livak formula. Data were normalized to 18S rRNA and control medium (cells without treatment) Data were presented in mean ± standard error of the mean. Statistical analysis was performed using a student's t-test independent assay. *Significant difference at $p<0.01$.

prognosis, progression, and aggressiveness of cancer. (8,18,19) We have also indicated the role of CAFs in EMT processes due to their association with tumor budding grade in CRC.(18) CAFs are activated due to reciprocal communications with cancer cells mediated by secretomes in a paracrine manner.(20) However, relatively little is known about the occurrence of CAF activation in human CRC microenvironment. In our parallel study, we indicated that fibroblasts isolated from the tumor area have significantly elevated expression of α-SMA, a myofibroblast marker, compared to their counterpart isolated from nontumor area at least five centimeters distal to the outer margin of the tumor area.(10) In addition, fibroblasts from the tumor also exhibited significantly higher expression of vimentin, the mesenchymal cytoskeletal marker, compared to that from nontumor area. Therefore, we confirmed that fibroblasts from the tumor area used in this study represent CAFs, whereas those from nontumor area are NFs.

Our result revealed that the CAF-CM significantly increased the mRNA expressions of CD44, CD133, ALDH1A1, and OCT4 in HT-29 CRC cells compared to their counterpart NF-CM as well as to PF-CM. The mRNA expression of CD44, CD133, ALDH1A1, or OCT4 in HT-29 cells following CAF- and NF-CM supplementation has a significantly strong correlation with the α-SMA mRNA expression of fibroblasts from tumor and nontumor areas, indicating that activated CAFs upregulate the stemness marker expression through complex pathways that need to be further investigated. One of the mechanisms is through the activation of Wnt/β-catenin signalling pathway.(14)

Our parallel study using the same CRC specimens has demonstrated that CAF-CM enhanced the expression of vimentin mRNA, indicating the increase of EMT in CRC. (10) Therefore, we suggest that the increase of stemness marker expression in this study might be correlated with the EMT process induced by CAF-CM supplementation.

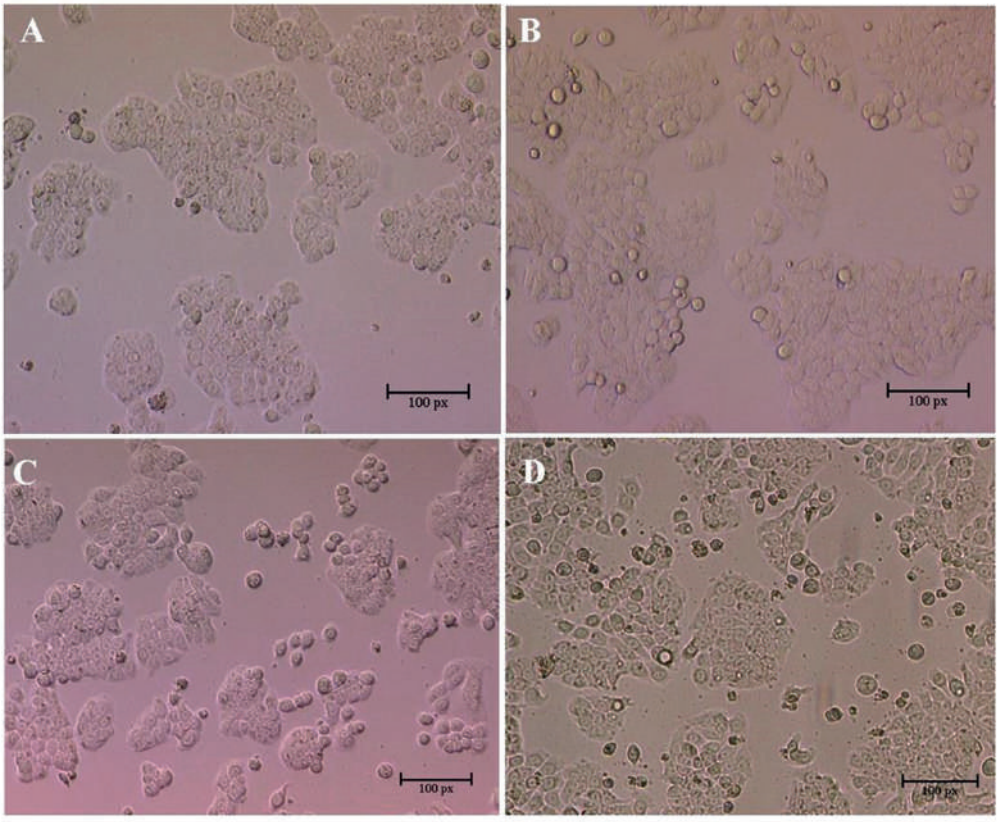

Figure 2. Morphology of HT-29 colorectal cells after CAF-CM, NF-CM, and PF-CM supplementation for 72 hours. Cell morphology without supplementation (A), after supplementation with NF-CM (B), CAF-CM (C), or with PF-CM (D), was visualized under an inverted microscope, magnification 200x. Scale bar: 100px.

However, further experiments are needed to elaborate on the causal relationship between EMT and stemness properties of cancer cells.

Mesenchymal and stemness properties in cancer cells are the feature of cancer stem cell subpopulation within the tumor mass that are responsible for metastasis and resistance of chemotherapy.(21) Epithelial to mesenchymal transition (EMT) facilitate cancer cells to gain mesenchymal phenotypes like cancer stem cells which are characterized by decreased expression of E-cadherin levels, metastatic capability, and invasiveness.(21) There is an integrated mechanism in the regulation of EMT and stemness involving epigenetics, tumor microenvironment, and dedifferentiation. Through EMT process, the terminally differentiated epithelial cells regain some properties of pluripotent cells.(22) Other studies reported that CAFs were involved in regulating plasticity and maintaining stemness through the secretion of the hepatocyte growth factor (HGF) in metastatic CRC which induced EMT.(10) CAFs also promote aggressive phenotypes of breast cancer cells through EMT induced by paracrine TGF-β1 signaling. (23) CAFs increase aggressiveness in breast cancer cells by inducing the expression of stemness marker (OCT3/4, NANOG, and Sox2) and phenotypes of EMT (Snail and Zeb).(24)

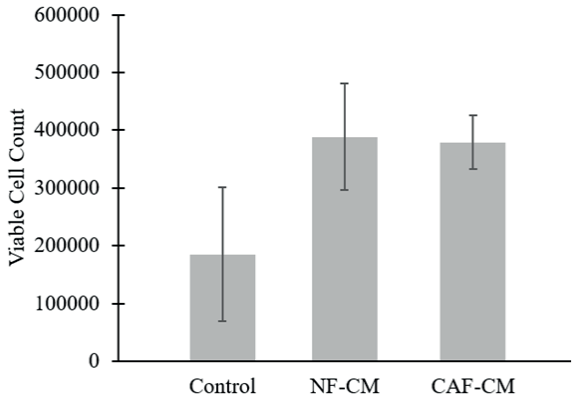

Figure 3. HT-29 colorectal carcinoma cells proliferation. HT-29 cells were treated with conditioned medium from CAF and NT for 72 hours. Cell proliferation was determined by performing a trypan blue exclusion assay. Data were presented in mean±standard error of the mean. Statistical analysis was performed using a student's t-test independent assay. There were no significant differences between all groups.

Interestingly, we found that NF secretomes also significantly increased ALDH1A1 and OCT4 expressions in HT-29 cells compared to the PF secretomes and control

cells, although at lesser levels when the cells were induced by CAF secretomes. This might be due to the presence of a small amount of CAF population in nontumor area of CRC as detected by α-SMA expression.(18) Therefore, we suggest that the fibroblasts in nontumor area have also the CAF properties, although less than in tumor area but more than in HT-29 epithelial cancer cells, that could also upregulate the OCT4 and ALDH1A1 expression. This is also the case for the PF secretomes which affected higher ALDH1A1 expression in HT-29 cells compared to control. We assume that preputium still contains high amount of myofibroblasts which have similar properties with CAFs. The presence of CAF population needs to be further investigated to determine the wide range of CRC microenvironment that can still affect the aggressiveness of CRC to be considered for tumor resection.

Secretomes consist of a subset of proteins and metabolites secreted by cells that play a critical role in mediating cellular interaction.(25) In the tumor microenvironment, secretomes derived from both cancer cells and cancer-related stromal cells contain growth factors, cytokines, and microRNAs that mediate cellular interaction by autocrine or paracrine manner, which could affect progressivity and aggressivity of cancer.(25) Our previous studies reported that secretomes of adipose and umbilical cord-derived stem cells could significantly increase ALDH1A1 expression in breast cancer stem cells (BCSCs).(26) Moreover, we have also demonstrated that secretomes of mesenchymal stem cells from human exfoliated deciduous teeth (SHED) contained TGF-β1 that upregulates the expression level of stemness markers such as OCT4 and ALDH1A1 in $ALDH^+$ breast CSCs.(27) TGF-β1-containing secretomes from primary CAF of urinary bladder cancer patients may induce EMT in bladder cancer cells.(28) Fibroblast growth factor-1(FGF-1) is high increased factor in CAF-CM and promotes proliferation, migration, and invasion in ovarian cancer cells.(29) Our parallel study has recently reported that hepatocyte growth factor in CAF secretomes promote EMT by activating the HGF/c-MET pathway.(10) Hence, we recommend further analysis to identify the components of CAF secretomes involved specifically in the signaling pathway of CRC stemness.

In this study, we could not found any significant differences between the effects of CAF- and NF-CM on HT-29 cell proliferation. This result is contrary to the previous study reported that CAFs could significantly increase cell proliferation compared to their paired nontumor fibroblasts under the co-culture system of breast cancer cells.(30) This might be due to the heterogeneity of CAFs depending on the intrinsic factors (differences in cellular phenotypes) and extrinsic factors (tumor type, stage, therapy, and spatial distribution).(21) Furthermore, the distance of tumor and nontumor sampling area, culture method, and preparation of CM could contribute to differences in both results.

Conclusion

We conclude that the CAF secretomes from CRC patients upregulate the mRNA expression of CRC stemness. Further studies are required to identify the components of CAF secretomes involved specifically in the signaling pathway of CRC stemness in order to develop novel anticancer targeted to CSCs.

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