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Original Article

Fusobacterium nucleatum produces cancer stem cell characteristics via EMT-resembling variations

Qin Wang[1*], Chen Yu[2*], Chao Yue[3], Xin Liu[4]

[1]Department of Clinical Medicine, Jiangsu Health Vocational College, Nanjing, China; [2]Department of Integrated Traditional Chinese and Western Medicine, Jiangsu Cancer Hospital & Jiangsu Institute of Cancer Research & The Affiliated Cancer Hospital of Nanjing Medical University, Nanjing, China; [3]Department of General Surgery, Jiangsu Cancer Hospital & Jiangsu Institute of Cancer Research & The Affiliated Cancer Hospital of Nanjing Medical University, Nanjing, China; [4]Department of Prevention and Control for Occupational Disease, Jiangsu Provincial Center for Disease Prevention and Control, Nanjing, China. [*]Equal contributors.

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Abstract: Objective: To explore the involvement of epithelial-mesenchymal transition (EMT) and cancer stem cell (CSC) characteristics induced by *Fusobacterium nucleatum (Fn)* in colorectal cancer (CRC) in vitro. Methods: SW480 and HCT116 cells were co-cultivated with *Fn*. Western blot (WB) and real-time PCR were used for detecting EMT markers' expression. CSC-resembling phenotypes were observed through migration, intrusion, and spherical colony formation assays. Flow cytometry was employed for sorting, based on the expression of CD44. Results: It was displayed that *Fn* infection was responsible for an EMT phenotype associated with an increase in mesenchymal markers (Snail1, Vimentin, and ZEB1) as well as CD44 expression. *Fn* treatment induced stronger expressions of such markers when MOI increased. Furthermore, infection resulted in augmented migration, intrusion, and tumorsphere formation capacities. Cell classification implicated that mere $CD44^{high}$ cells exhibited CSC characteristics and mesenchymal phenotype (MP) in vitro, accompanied with augmented tumor-causing capacity over $CD44^{low}$ cells. Finally, we demonstrated IL-6/STAT3 pathway was involved in EMT-CSC-resembling behavior of CRC cells. Conclusion: All of these data suggest that *Fn* reveals CSC-resembling characteristics through activating IL-6/STAT3 and eliciting EMT-resembling variations in colorectal epithelial cells (CECs).

Keywords: *Fusobacterium nucleatum* (*Fn*), colorectal cancer (CRC), epithelial-mesenchymal transition (EMT), cancer stem cell (CSC)

Introduction

CRC is the 3rd common malignancies and the 4th prevalent causative factor of cancer-related death worldwide. Five-year survival rate almost reaches 65% [1]. CRC is a complicated disease affected by genetic and environmental factors. The majority of CRC cases are intermittent. Many studies have implied that intestinal microbes assume an essential part in CRC progression [2, 3].

More than one thousand diverse microbes suppress human gastrointestinal tract with high heterogeneity [4]. The gut microbes (GMs) are important to normal physical processes at homeostasis state, but likewise are connected with many diseases such as CRC and obesity [5-7]. The bacteria density in the large intestine (approximately 10^{12} cells/ml) is much larger than that in the small intestine (approximately 10^2 cells/ml). More and more evidence confirmed that GMs are relevant to CRC, which were characterized by decreased probiotics and opportunistic pathogen growth, like *Campylobacter, E.coli, Enterococcus, Anaerotruncus,* and *Collinsella*, displaying these bacteria's latent part in CRC progression [8, 9]. Further, *Fn* abundance in CRC was dramatically higher against that in the control group [10]. Other studies likewise demonstrated that *Fn*, together with several Gram-negative bacteria, such as *Streptococcus* and *Campylobacter spp.*, promoted CRC occurrence [11, 12].

Fn, an opportunistic symbiotic anaerobe, participated in diverse periodontitis. Furthermore, it is a particularly common extra oral infection type. More and more evidence displayed that *Fn* levels in tumor tissues and stool samples

from CRC patients are obviously increased against those in normal controls [13]. Kostic et al. reported that *Fn* in Apc (Min/+) mice accelerated CRC occurrence [14]. Rubinstein et al. confirmed that *Fn* triggered tumor cells in CRC to grow through acting on β-catenin signaling and elicited oncogene expression through FadA adhesion virulence factor (VF) [15]. Collectively, those studies showed that *Fn* assumes a crucial part in initiating CRC and accelerating tumor cells' growth, which confirmed that *Fn* is a causative factor instead of a outcome of CRC.

Recently, EMT has attracted much attention concerning metastatic dissemination. EMT is considered as an early event in metastasis, which participates in tumor cells' migration and intrusion [16]. The latest evidence likewise displays that cells which receive EMT exhibit stem cell-resembling characteristics [17, 18]. Particularly, Mani et al. indicated that EMT suppression in breast epithelial cells (BECs) produced a $CD44^+/CD24^-$ cell subpopulation with breast CSCs-resembling phenotype and characteristics [17]. CSCs possess a capacity to induce tumor and retain tumor self-renewal. Various cell surface markers have already been depicted and characterized in CSCs among varied cancers. It's reported that CD44 was a CSCs marker of some solid tumors, which are not confined to head and neck, breast and pancreas cancers [19]. As for CRC, CD44 has likewise been confirmed to be a classic marker, while the part performed by *Fn* in CSC occurrence remained to be investigated [20]. Hence, the study was directed toward delving into it in EMT and colorectal CSCs occurrence.

Materials and methods

Bacterial strains and culture conditions

Fusobacterium nucleatum ATCC25586 was purchased from ATCC (Manassas, VA, USA). Fn culture and co-culture assays were conducted as depicted before [21]. The number of Fn was quantified as described by Gendron et al. [22]. Fn was grown in BHI broth for 48 h. Before incubation with eukaryotic cells, BHI broth was removed by low-speed centrifugation and replaced with suitable antibiotic-free medium. Co-cultures were conducted at MOI of 10, 100, 500, respectively for 24 h in a damp 5% CO_2 condition at 37°C prior to analysis.

CRC cell culture

The colon cancer epithelial cell lines SW-480 and HCT116 were grown at 37°C and 5% CO_2 in the appropriate medium [23, 24].

Flow cytometry (FC) analysis

Cells returned to the original state and were subjected to staining with CD44-APC antibody (1:25) (10^5 cells per condition) in PBS, BSA (0.5%), and EDTA (2 mmol/L). FC was conducted through DIVA and FACScan software. Cells were subjected to dual CD44 and DAPI staining (exclusive of positive dead cells), and classified for their CD44 expression levels indicated on flow cytometer.

Migration and intrusion assays

Cells returned to the normal state and were put in the upper side of Transwell insert in 24-well plates (8-mm) (5×10^4 cells per condition) with medium added FBS (5%). In intrusion assay, inserts were pre-covered with COL I (50 ng/ml) at 37°C for 40 min. The inserts were cultivated at 37°C for 18 h, followed by fixation in cold methanol and hematoxylin staining, as depicted before [25]. Cells passing through inserts' lower side were quantified in 5 distinct randomly selected regions of each insert via light microscopy.

Spherical colony formation

Cells returned to the original state and were put in 96-well plates without adhesion (covered with polyHEMA solution (10%) in anhydrous ethanol and dried at 56°C overnight) (500 cells), followed by culture at 37°C for 5 d in a non-serum medium comprised of DMEM-F12 Glutamax added glucose (0.3%), N2-added 100× (1:100), EGF (0.02 mg/ml), basic-FGF (0.01 mg/ml), amphotericin B (2.5 mg/ml), gentamicin (5 mg/ml), as well as penicillin (50 IU/m). The density of spheroids was calculated.

RNA isolation and qRT-PCR

Total RNAs were isolated with Trizol and quantified by their A_{260}. 1 μg of total RNAs was retrotranscribed through Quantification RT kit as the guidance's provided by manufacturer. qPCR was conducted through StepOne plus real-time PCR instruments and specific primers at 0.3

μM. All used primers were obtained from Sigma. The operating procedures were summarized below: denaturation at 95°C initially for 10 min then for 60 s, annealing at 60°C for 20 s, and extension at 56°C for 1 min.

Western blotting

Western blotting was carried out essentially as described [25]. Proteins were resolved typically by either 12.5% acrylamide or gradient acrylamide (4-15%) SDS-PAGE gels. Proteins were transferred to a PVDF membrane through a semi-dry blotting apparatus. The blots were blocked with 5% dry milk without fat or 3% BSA, in PBS, followed by probing of the membrane with the appropriate primary antibody at the recommended dilution, which then was incubated overnight at 4°C. Blots were washed several times with PBS containing 0.05% v/v Tween-20, subsequently cultivated with appropriate secondary antibody at the recommended dilution for 1 h at room temperature. After washing, antibody reactivity was visualized using enhanced chemiluminescence (Thermo ECL, Shanghai, China). Briefly, PVDF membrane were immersed for 1 min in 50 ml of Tris buffer (50 mM, pH8.8) containing luminol (1.25 mM), iodophenol (400 μM), and hydrogen peroxide (0.01%, v/v). Finally the blot was exposed to Tanon 5200 (Tanon, Shanghai, China) for various periods of time to capture the image.

Statistical analysis

Statistical analyses were carried out through 18.0 SPSS. Pearson's chi-square test was used for comparing categorical variables, while student's t-test was applied to evaluate the difference in continuous variables between the groups. All values are expressed as the mean ± standard deviation (SD) of the mean. Two-sided *p*-values were calculated for all tests and a *p*-value of less than 0.05 was considered statistically significant.

Results

Infecting colon epithelial cells with Fn produces cells with mesenchymal stem cell (MSC) characteristics through epithelial-mesenchymal-resembling transition

To confirm if *Fn* infection was able to produce cells with CSC characteristics through epithelial-mesenchymal-resembling transition, co-culture assays with colon cancer epithelial cells were conducted and demonstrated on SW-480 and HCT116. As expected, *Fn* infection (at MOI of 100:1 and 500:1) elicited morphological variations, featuring polygon loss, cell cluster disruption, and the emergence of lathy cells with membrane ruffles, displaying a MP.

qPCR displayed a marked increase in mesenchymal markers, together with CD44 in SW-480 and HCT116 cell lines. Cells treated with *Fn* at MOI of 500:1 induced stronger Snail1, Vimentin, ZEB1, and CD44 expression when compared with the treatment with MOI of 10:1 and 100:1 ($P<0.05$, **Figure 1A-D**).

To further examine the effect of *Fn* on colonic epithelial cells, western blotting analyses were performed and revealed an increase expression of Snail1, Vimentin, ZEB1, and CD44. Also, cell treatment with *Fn* at an MOI of 500:1 induced stronger expression of those markers when compared with the treatment with MOI of 10:1 and 100:1 (**Figure 1E**).

Cells infected by Fn possess CSC characteristics

CSCs express specific markers and feature self-renewal and tumor-producing capacities as well as augmented migration and intrusion potency. The tumor sphere assay is one of the most frequently used and is ideal to identify CSC characteristics.

The spheroid-forming capacity of colonic cells was investigated in vitro in culture conditions without adhesion. SW-480 and HCT116 belong to tumor cells. While they do not exhibit obvious tumor-causing characteristics in basic conditions, they have a limited capacity of naturally forming tumors following culture. However, *Fn* infection resulted in the marked elevation of in spheroid formation, with similar results obtained in SW-480 and HT29 cell lines. *Fn* at MOI of 500:1 induced stronger ability to form tumorspheres when compared with the treatment with MOI of 10:1 and 100:1 ($P<0.05$, **Figure 2A**).

Experiments were conducted to assess migration and intrusion characteristics. *Fn* infection significantly stimulated migration and intrusion characteristics compared to the basic levels indicated in control. Also, cell treatment with *Fn* at MOI of 500:1 induced stronger invasive behavior ($P<0.05$, **Figure 2B**, **2C**).

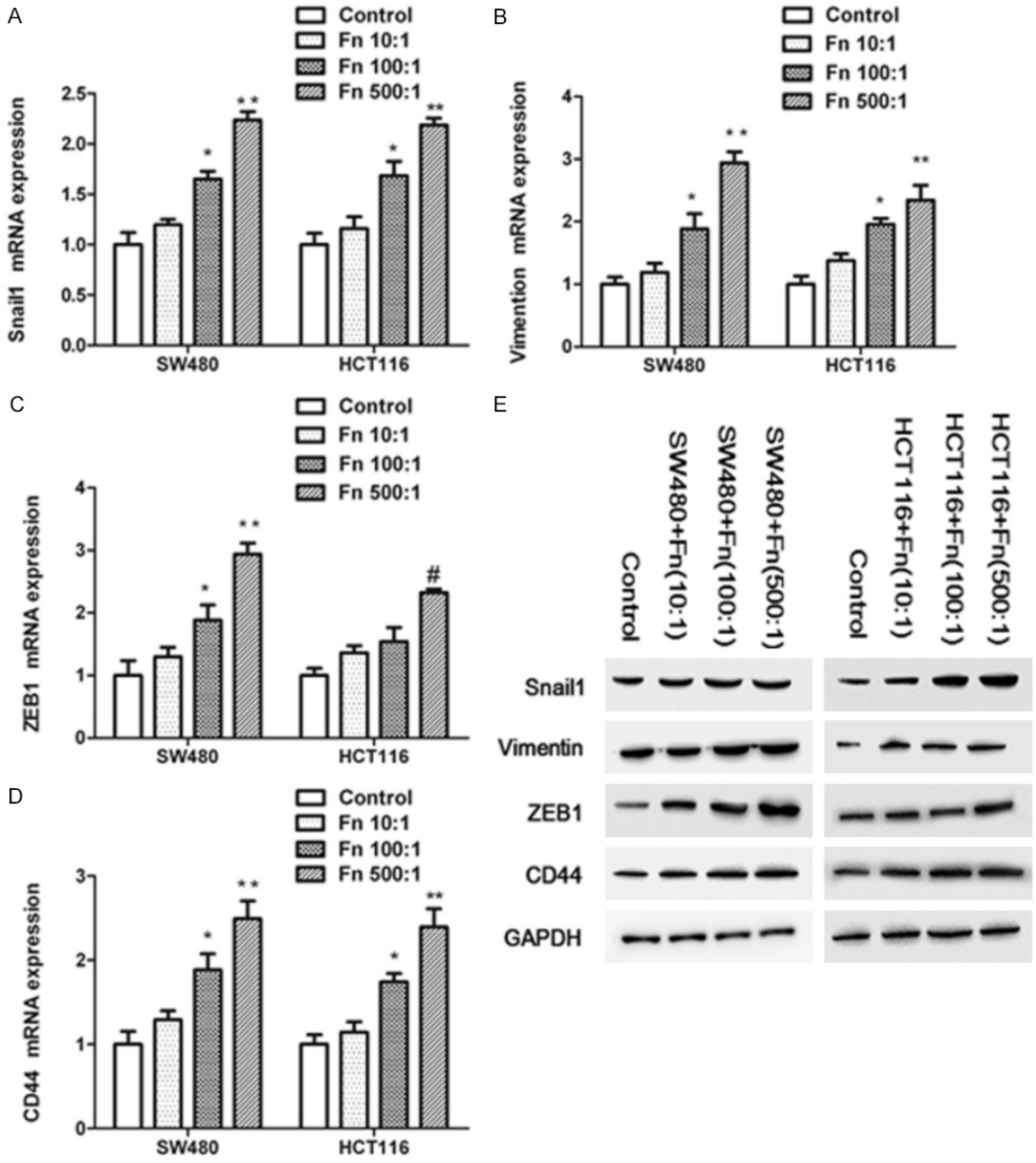

Figure 1. *Fn* Infection triggers an EMT-resembling phenotype and CD44 expression in SW480 and HCT116 cells. Those cells were exposed to *Fn* (MOI 10:1, 100:1 and 500:1) for 24 h. A-D. Relative expression levels of mRNA encoding mesenchymal markers and CD44 in infected cells determined by RT-qPCR (n=3 respectively, **P*<0.05 vs *Fn* (MOI 10:1 and 500:1) and control groups, ** *P*<0.01 vs *Fn* (MOI 10:1) and control groups, # *P*<0.05 vs *Fn* (MOI 10:1) and control groups). E. WB was conducted to examine the expression levels of snail1, vimentin, ZEB1 and CD44 in SW480 and HCT116 cells.

$CD44^{high}$ cells isolated from Fn-infected cells exhibit MSC characteristics

Cell classification via FC was conducted on *Fn*-infected SW-480 and HCT116 cells at MOI of 500:1 to disassociate $CD44^{high}$ and $CD44^{low}$ cells.

The $CD44^{high}$ cells formed more tumorspheres (**Figure 3A**) and displayed stronger migration and intrusion abilities over $CD44^{low}$ (**Figure 3B**, **3C**). The same $CD44^{high}$ classified cells (*Fn* infected) had an increased mesenchymal marker and CD44 levels relative to the $CD44^{low}$ cells (**Figure 3D-F**). The findings collectively confirm

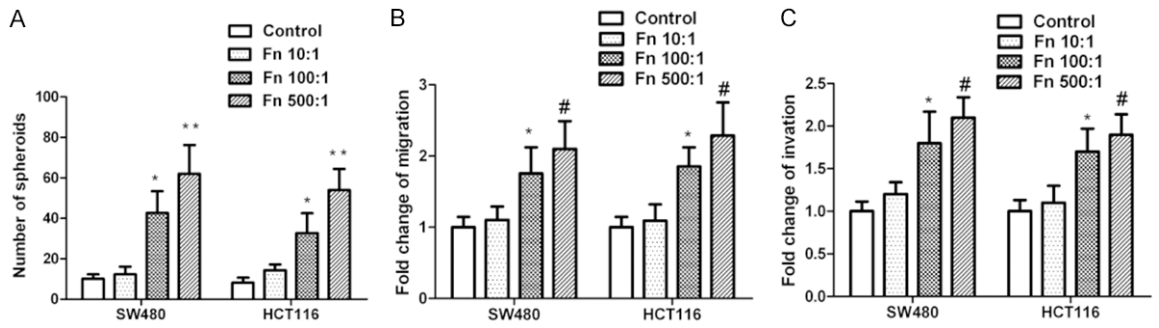

Figure 2. *Fn* infection elicits migration, intrusion, and tumorsphere formation. SW480 and HCT116 cells were exposed to *Fn* for 24 h at MOI of 10:1, 100:1 and 500:1, respectively, followed by recovery and inoculation in culture without adhesion for spheroid formation (A) or used for migration and intrusion assays (B, C). (A) Spheroids' absolute number quantification 5 d later. (B) Migration quantification 18 h later. (C) Intrusion quantification 18 h later. (n=3 respectively, * P<0.05 vs *Fn* (MOI 10:1 and 500:1) and control groups, # P<0.05 vs *Fn* (MOI 10:1) and control groups).

that CD44^{high} cells elicited by *Fn* infection displayed MSC characteristics.

IL-6/STAT3 signal participated in Fn-induced EMT-CSC like changes

To determine the molecular mechanism on which *Fn* infection depended, western blot was employed to analyze IL-6/STAT3 pathway. The elevated levels of IL-6, pSTAT3, as well as downstream VEGF, survivin and Bcl-2 confirmed that *Fn* treatment could activate IL-6/STAT3 signaling pathway (**Figure 4A**). After blockage of IL-6/STAT3 signal, the levels of EMT markers Snail1, Vimentin, ZEB1, and CSC markers SOX2, OCT4, c-MYC, as well as ABCG2 decreased (**Figure 4B**), suggesting that IL-6/STAT3 signaling pathway can regulate EMT-like effect and CSC formation in colorectal cancer cells.

Discussion

Reportedly, high levels of *Fn* may be connected to the poor outcomes of CRC. Li et al. showed that lymph node metastases lie in 52/88 subjects with a high *Fn* abundance and in 0/13 with a low *Fn* abundance, indicating the *Fn* enrichment is relevant to CRC progression and metastasis [26]. Furthermore, several studies have found that *Fn* DNA load is related to higher cancer-relevant mortality and *Fn* DNA was possibly a latent unfavorable prognosis indicator [27]. *Fn* was accumulated in microbiota that could stick to and intrude human epithelial and endothelial cells [28]. Nowadays, a couple of studies have displayed that *Fn* is a nosogenic bacterium which enhances CRC occurrence [15, 29]. Some studies displayed that its VF had a close correlation with colorectal lesions. *Fn* has been confirmed to be able to intrude human epithelial cells, act on β-catenin signaling, elicit oncogene expression and accelerate CRC cells' growth [29]. Another VF, an auto transporter protein, Fap2, could enhance CRC progression through suppressing the activities of immune cells [30]. However, numerous diverse risk factors existed in CRC and the relationship of *Fn* and CRC remains to be investigated. Since EMT is a stage that precedes carcinogenesis, it was of great interest to examine the ability of *Fn* to influence EMT to drive inflammation and malignant cell transformation that enables neoplastic progression. The work was aimed at exploring the carcinogenic potential of *Fn* to induce EMT-like behavior and the emergence of colorectal CSCs.

EMT has gained much attention concerning metastatic dissemination. It is considered an early event in metastasis, which participates in tumor cells' migration and intrusion. It is associated with the modification of gene expression involved in inhibiting epithelial phenotype (EP) and activating MP [31]. In addition, EMT induces cell proliferation that mimics the progression of invasive CRC, which correlates with CRC progression in humans [32]. Such processes can be observed in other cancer including gastric cancer, breast cancer, and cholangiocarcinoma [33-35]. Also, deregulation of tumor suppressor, epigenetic reprograming, and the activation of CSC associated pathways elicited by bacteria, may be the causative factor of cancer

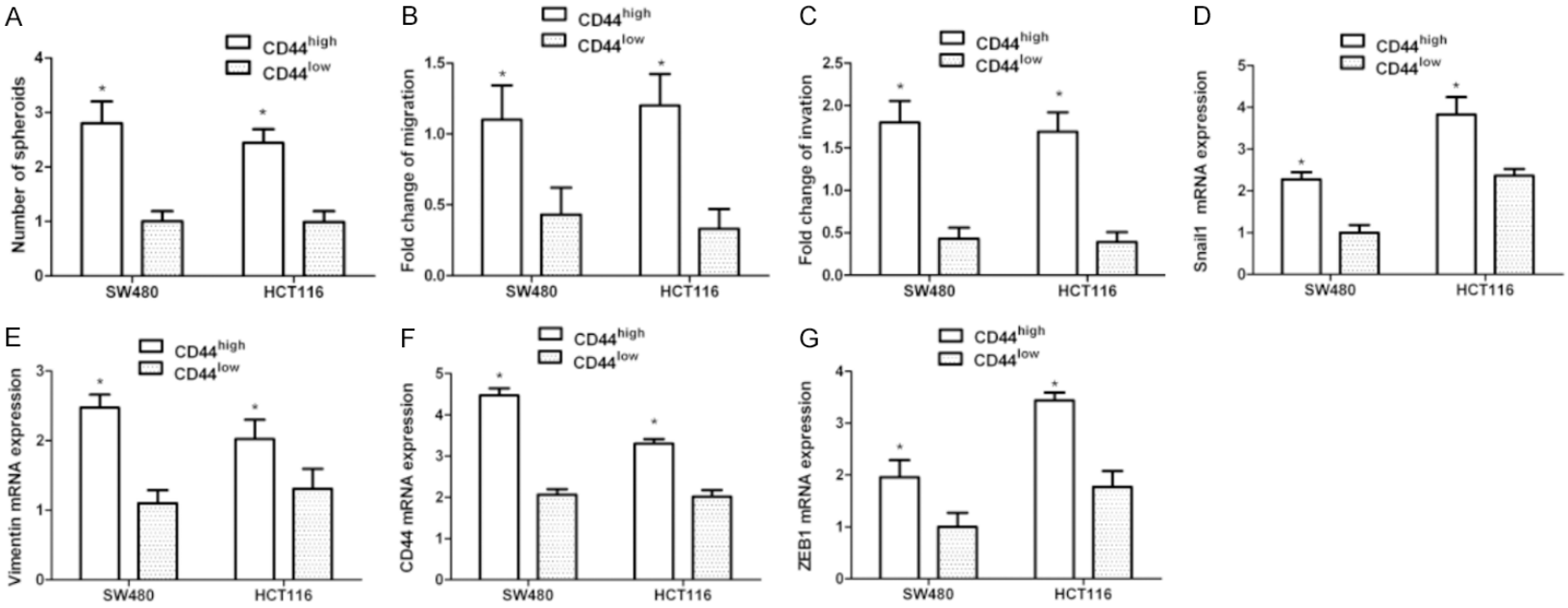

Figure 3. The CD44^{high} cells produced by *Fn* infection shared EMT and CSC characteristics. SW480 and HCT116 cells were co-cultivated with *Fn* at MOI of 500:1 for 1 d, and then stained with DAPI, and CD44-APC antibodies, along with DAPI-negative/CD44^{high} or CD44^{low} cells were classified through FACS as depicted. A. Spheroids' quantification following 5-day culture of classified cells in conditions without adhesion. B. Migration quantification 18 h later. C. Intrusion quantification 18 h later. D-G. Relative expression levels of mRNA encoding mesenchymal markers, CD44 in CD44^{high} vs CD44^{low} classified cells detected through RT-qPCR. Data were expressed as the average proportion of CD44^{high}/CD44^{low} cells. (n=4 respectively, **P*<0.05 vs CD44^{low} groups).

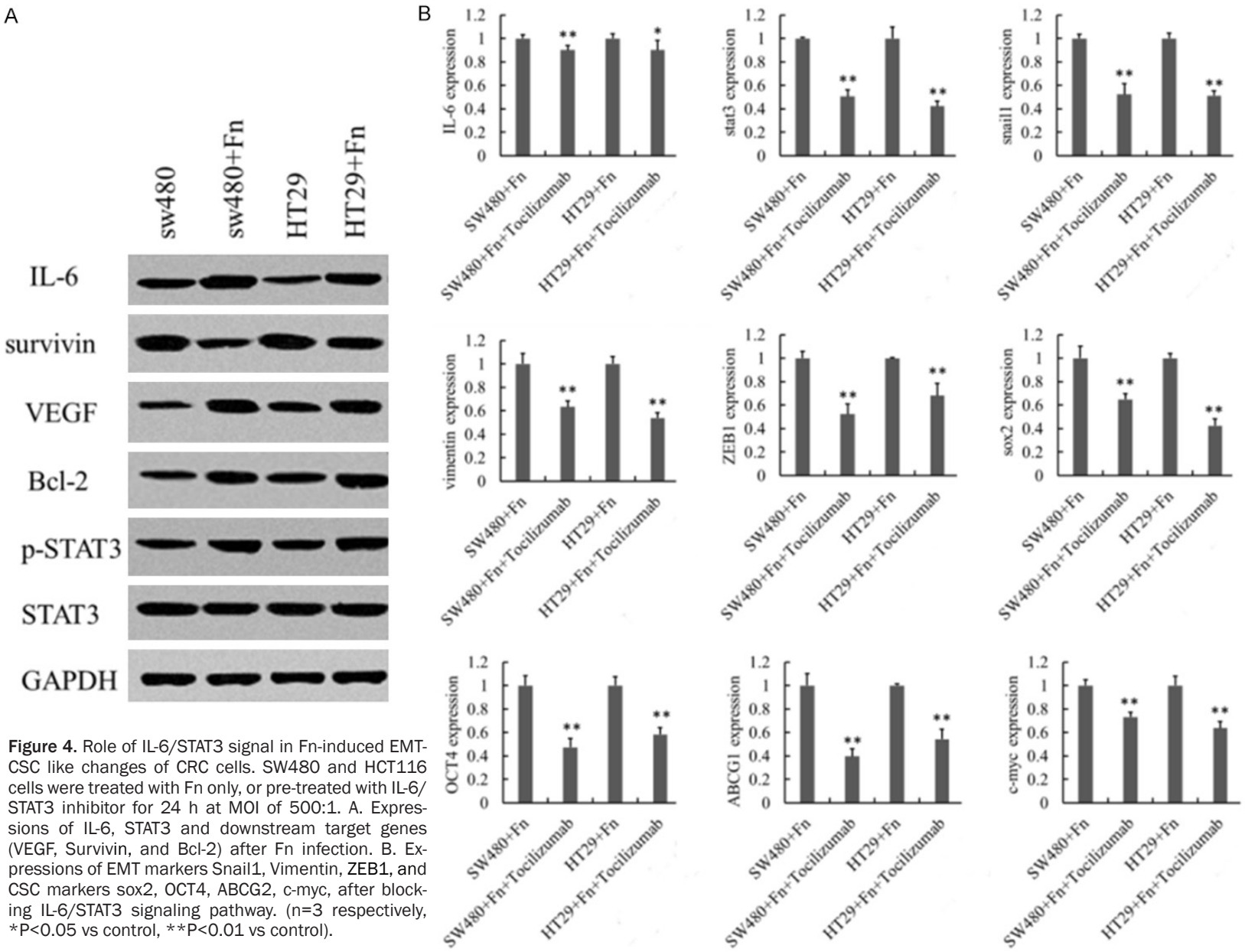

Figure 4. Role of IL-6/STAT3 signal in Fn-induced EMT-CSC like changes of CRC cells. SW480 and HCT116 cells were treated with Fn only, or pre-treated with IL-6/STAT3 inhibitor for 24 h at MOI of 500:1. A. Expressions of IL-6, STAT3 and downstream target genes (VEGF, Survivin, and Bcl-2) after Fn infection. B. Expressions of EMT markers Snail1, Vimentin, ZEB1, and CSC markers sox2, OCT4, ABCG2, c-myc, after blocking IL-6/STAT3 signaling pathway. (n=3 respectively, *P<0.05 vs control, **P<0.01 vs control).

occurrence and progression [36]. Lately, *Fn* has been reported to be able to elicit a EMT-resembling process, our principal objective was to investigate if *Fn* participated in CSC occurrence through EMT [37]. In consequence, CRC cells naturally tend to cover EMT. We found that *Fn*-infected SW480 and HCT116 cells were diverse and consisted of cells that preserved a polygonal phenotype and harbored 'hummingbird' phenotype. *Fn* infection triggered a notable improvement in mesenchymal markers, as previously stated [37]. The findings demonstrated that *Fn* infection influences epithelial differentiation.

A close link between CSCs and EMT-relevant gene expression has been confirmed recently. EMT transcriptional mediators snail or twist in BECs exhibited a stem cell-relevant $CD44^+/CD24^-$ expression way and a 30-fold augmented sphere formation against control cells [17]. CD44, a cell adhesion molecule, can accomplish cell-ECM and cell-cell interactions by connecting with corresponding major ligand, hyaluronic acid. $CD44^+$ CRC cells possess stronger colony-forming and tumor-causing capacities relative to $CD44^-$ cells. Further, mere $CD44^+$, while not confined to $CD44^-$ CRC cells can keep the morphological and phenotypic features of tumor lesions where they originated after serial transplantations [38]. CD44 has already been identified as CSCs markers of colon cancers. It is likewise seductive that *Fn*-infected cells have a pretty high CD44 expression. Also, cells infected by *Fn* possessed CSCs characteristics and shared characteristics of high tumorigenic and migration or intrusion. Furthermore, the findings indicated that the $CD44^{high}$ cell subpopulation triggered by *Fn* infection exhibited CSC-resembling characteristics regarding phenotype along with migration, intrusion, and tumor-forming capacities against their $CD44^{low}$ counterparts. Seductively, the identical $CD44^{high}$ infected cells displayed increased mesenchymal marker levels than the $CD44^{low}$ cells. At last, we suggested IL-6/STAT3 pathway may be implicated in EMT behavior as well as CSC-resembling changes of CRC cells. *Fn* probably assumes a part in EMT-CSC cross talk in CRC progression.

In brief, we first reported that *Fn* infection can trigger EMT-resembling actions and the appearance of $CD44^{high}$ cells with CSC characteristics via IL-6/STAT3 signal. These findings, together with additional literatures, enhance the knowledge of the cellular mechanisms concerning cancer-causing behaviors in the course of *Fn* infection.

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Disclosure of conflict of interest

None.

Address correspondence to: Chao Yue, Department of General Surgery, Jiangsu Cancer Hospital & Jiangsu Institute of Cancer Research & The Affiliated Cancer Hospital of Nanjing Medical University, 42 Baiziting Road, Nanjing 210029, Jiangsu, China. E-mail: chaoyue0402@gmail.com; Xin Liu, Department of Prevention and Control for Occupational Disease, Jiangsu Provincial Center for Disease Prevention and Control, 172 Jiangsu Road, Nanjing 210009, China. E-mail: liux@jscdc.cn

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