7-Difluoromethoxyl-5,4'-di-n-octyl genistein inhibits the stem-like characteristics of gastric cancer stem-like cells and reverses the phenotype of epithelial-mesenchymal transition in gastric cancer cells

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Received January 21, 2016; Accepted March 2, 2016

DOI: 10.3892/or.2016.4848

Abstract. 7-Difluoromethoxyl-5,4'-di-n-octyl genistein (DFOG), a novel synthetic genistein analogue, exerts anti-carcinogenic activity in several types of cancers, including gastric cancer. Accumulating evidence in recent years strongly indicates the existence of cancer stem cells in gastric cancer. The objective of the present study was to investigate whether DFOG inhibits the stemness and reverses the epithelial-mesenchymal transition (EMT) phenotype of gastric cancer stem-like cells (GCSLCs) derived from human gastric cancer SGC-7901 cells and to identify its potential mechanism. Sphere-forming cells (SFCs) from the SGC-7901 cells possessed the properties of GCSLCs. DFOG preferentially inhibited self-renewal, cell migration and cell invasion, and downregulated the expression of stem cell biomarkers in a dose-dependent manner. At the molecular level, these effects were accompanied by the downregulation of forkhead box M1 (FoxM1). Meanwhile, FoxM1 siRNA transfection was able to synergize the inhibition of expression of FoxM1 and Twist1 induced by DFOG in GCSLCs. In addition, we found that DFOG treatment decreased the expression of N-cadherin and increased the expression of E-cadherin. More importantly, FoxM1 siRNA transfection cooperated with DFOG to suppress the self-renewal capacity, cell migration and cell invasion, and downregulated the expression of CD133, CD44, ALDH1, and also regulated the expression of N-cadherin and E-cadherin. These findings showed that DFOG inhibited the stem-like characteristics of GCSLCs and reversed the EMT phenotype by modulation of FoxM1 and further decreased Twist1 expression. Our results provide a further rationale and experimental basis for using DFOG to improve the efficacy of treatment for patients with gastric cancer.

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Key words: 7-difluoromhoxyl-5,4'-di-n-octyl genistein, gastric cancer, cancer stem-like cells, gastric cancer stem-like cells, epithelial-mesenchymal transition, FoxM1, Twist

Introduction

Gastric cancer (GC) is a life-threatening malignant tumor in humans and the second most common cause of cancer-related deaths worldwide (1). Despite the extensive efforts to develop new therapeutic strategies for GC and despite that the incidence rates of GC have noticeably decreased in most countries in the world, it remains the most common cause of cancer-related mortality worldwide, particularly in Eastern Asian countries (2). In China, the incidence of GC ranks second among all types of malignant tumors. GC patients often present with an advanced stage at diagnosis and may also have metastasis when initial symptoms occur. As such tumor recurrence and metastasis impose great difficulty for the prevention and treatment of GC.

Cancer stem cells (CSCs), firstly found in patients with acute myeloid leukemia (3), are a unique subpopulation of cancer cells that have similar characteristics to normal stem cells and display unlimited proliferation potential, self-renewal ability and capability to generate heterogeneous lineages of cancer cells. Numerous studies have demonstrated that CSCs also exist in solid tumors such as breast and brain cancer, glioma and pancreatic cancer (4-8). CSCs were suggested to play a key role in tumor initiation, invasion, metastasis and drug resistance. Currently, CSCs have been proposed as a therapeutic target in the treatment of cancers (9).

There is growing evidence suggesting the existence of gastric cancer stem cells (GCSCs). GCSCs were firstly isolated and identified from human GC cell lines using a defined cell surface marker CD44 in 2009 (10). This study confirmed that $CD44^+$ GC cells exhibit cancer stem cell properties such as self-renewal and high tumorigenicity. GCSCs were success-

fully isolated using CD90 in a previous study (11). In addition, the stem cell markers CD44, CD133 and ALDH1 have been recommended for identifying GCSCs (12,13). Self-renewal and lineage capacity are characteristics of all stem cells. Recently, in order to acquire CSCs, a variety of research methods have been developed based on these features. Liu *et al* (14) obtained GCSCs from human GC cells by cultivating cancer cells in stem-condition culture systems. This method has been used as a representative method by which to obtain GCSCs.

Forkhead box protein M1 (FoxM1), a transcription factor, is an important member of the forkhead transcription family. The International Society for Molecular and Cell Biology and Biotechnology Protocols and Research (ISMCBBPR) recognized Forkhead box protein M1 (FOXM1) as the 2010 Molecule of the Year due to its growing potential as a target for cancer therapies. The FoxM1 transcriptional factor is essential for cell cycle progression and cell survival. Upregulation of FoxM1 has been found in various types of cancers, suggesting that it may be involved in the initiation of human carcinogenesis (15-17). Accumulating evidence suggests that FoxM1 plays an essential role in cancer development and progression by enhancing drug resistance and cancer cell metastasis (18). In addition, alterations in the FoxM1 signaling pathway are reportedly associated with tumorigenesis (19,20). It has been reported that overexpression of FoxM1 leads to epithelial-mesenchymal transition (EMT) by the acquisition of EMT phenotype and downregulation of FoxM1 leads to the inhibition of EMT in GC cell lines (21). Moreover, FoxM1 has been shown to be a key transcription factor in regulating CSC characteristics in several studies (22,23). Thus, FoxM1 may be a new molecular target for discovering tumor therapeutic agents that target CSCs.

The transcription factor Twist is considered as one of the major inducers of the EMT process, and plays a significant role in tumor metastasis through various signal transcription pathways, including Akt, Ras, signal transducer and activator of transcription 3 (STAT3), mitogen-activated protein kinase (MAPK) and Wnt signaling (24,25). Twist is encoded by the Twist1 gene located on human chromosome 7p21 and belongs to the family of basic helix-loop-helix (bHLH) transcription factors (26). Several studies have shown that Twist1 plays an essential role in the regulation of CSC functions and features. For example, overexpression of Twist can facilitate the generation of a breast cancer stem cell phenotype (27). Activation of AKT and β-catenin pathways induced by overexpression of Twist is crucial for the maintenance of the characteristics of breast and cervical CSCs (28).

Genistein, a natural isoflavone, was first isolated from soy products. It has been demonstrated that genistein is a potential chemopreventive agent that inhibits carcinogenesis by mediating multiple regulatory pathways. Our previous studies confirmed that a novel synthetic genistein derivative, 7-difluoromethoxyl-5,4'-di-n-octyl genistein (DFOG), inhibited the growth of GC cells by suppressing FoxM1 (29) and halted the self-renewal of ovarian cancer stem cell by activating Foxo3a (30). In the present study, we investigated the effect of DFOG on gastric cancer stem-like cells (GCSLCs) and its potential mechanism for the first time. The results confirmed that DFOG can attenuate the characteristics of GCSLCs involving the decreased expression level of FoxM1.

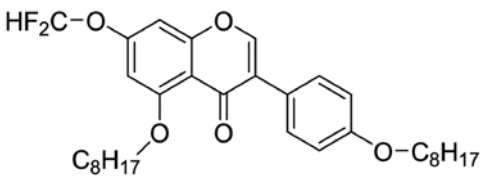

Figure 1. Chemical structure of 7-difluoromethoxyl-5,4'-di-n-octyl genistein (DFOG).

We also evaluated the effects of DFOG on EMT of GCSLCs. The results demonstrated that DFOG was able to reverse the EMT phenotype in GCSLCs by suppressing the expression of Twist1. The present study suggests that DFOG may be a potential therapeutic drug for the treatment of GC by targeting CSCs.

Materials and methods

Cell culture and reagents. Human GC SGC-7901 cells were purchased from the China Center for Type Culture Collection (CCTCC; Wuhan, China) and were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin and 100 μg/ml streptomycin in a humidified incubator containing 5% CO_2 at 37˚C. FBS, trypsin and DMEM were purchased from HyClone (Thermo Scientific, Waltham, MA, USA).

Sphere-forming and self-renewal assay. Parental cells (PCs) were collected and washed to remove serum, and were then suspended in serum-free stem cell conditional medium containing DMEM/F12 (Gibco-Invitrogen, Carlsbad, CA, USA) supplemented with 50X B27 (Invitrogen, Carlsbad, CA, USA), 20 ng/ml EGF, 20 ng/ml bFGF (both from eBioscience, Inc., San Diego, CA, USA), 4 μg/ml insulin, 100 IU/ml penicillin G and 100 μg/ml streptomycin. After that, the cells were plated in ultra-low adherence culture plates (6-wells) at a density of 10,000 cells/ml and maintained in a humidified incubator containing 5% CO_2 at 37˚C. After 5 days of culture, the first generation of sphere-forming cells (SFCs) was obtained after trypsin-EDTA digestion. The first-generation SFCs were further cultured and expanded at a density of 10,000 cells/well in ultra-low adhesion 6-well culture plates to obtain the SFCs.

Scratch assays. The PCs and third-generation SFCs were seeded in 6-well plates at a density of $4x10^5$/well in DMEM supplemented with 10% FBS. When the cells grew to 85% confluency, the wound was generated by scratching the surface of the plates in the central region with a 200 μl pipette tip. Washing was performed 2 times using phosphate-buffered saline (PBS) to remove floating cells and debris. The cells were incubated for 48 h, and were imaged at 0 and 48 h in the same location of the wound, respectively. The numbers of cells in the scratch area were counted, and the migratory rate of the cells was determined in relation to the migratory rate of the PCs considered as the standard migration rate (100%).

Transwell chamber invasion assay in vitro. DMEM (1.0 ml) supplemented with 10% FBS as a chemical inducer was added

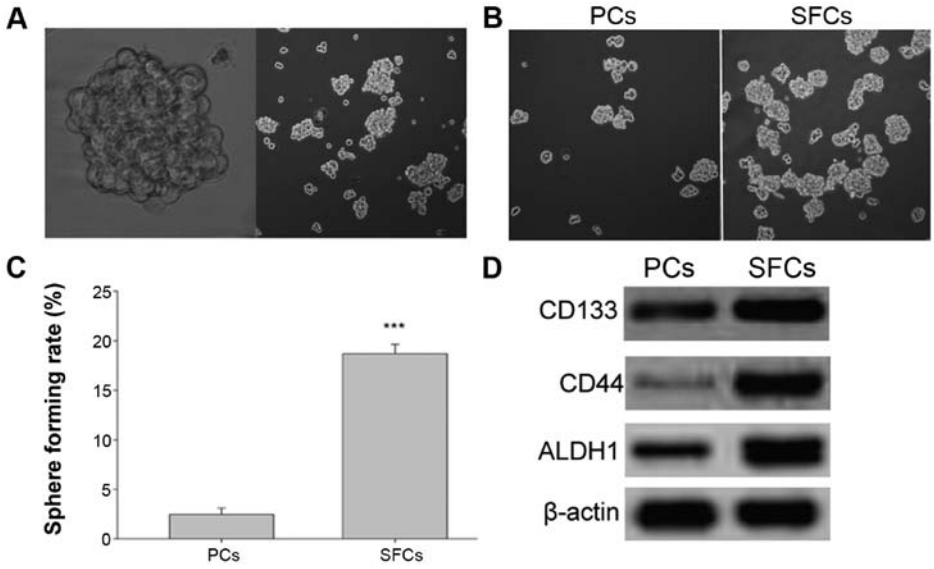

Figure 2. Identification of GCSLCs in the SGC-7901 cell line. (A) Tumor sphere from SGC-7901 cells in the stem cell culture system. (B) The sphere forming ability of parental cells (PCs) and the third-generation sphere-forming cells (SFCs). (C) Statistical analysis of B; each experiment was performed in triplicate. ***P<0.001 vs. the PCs. (D) Western blot analysis showed that protein expression levels of CD133, CD44 and ALDH1 were higher in the SFCs.

to the 24-well cell culture plate which was then embedded in the Transwell chamber. A total of 10,000 PCs or SFCs were plated in the top chamber of the Transwell coated with Matrigel. After being cultured for 24 h, the cells that had not invaded through the pores of the insert were cleared with a sterile cotton swab and discarded. The cells that invaded to the lower chamber were fixed with methanol, stained with crystal violet and counted under an optical microscope with the migration rate of the PCs or SFCs treated with 0.1% dimethyl sulfoxide (DMSO) considered as the standard invasion rate (100%).

Western blot analysis. The cells were washed with PBS once, and lysed in 1 ml lysis enzyme buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.2 mM EDTA, 0.2% NP-40, 10% glycerol, 1 M β-Me, 1 μg/ml Trasylol, 0.5 μg/ml leupeptin, 0.1 mM Na_3VO_4, 0.5 mM 4-NPP, 0.5 mM NaF and protease inhibitors]. The cells were scraped and collected after incubation for 20 min at 4˚C. The lysates was centrifuged at 13,200 rpm for 5 min at 4˚C to prepare whole cell extracts. The Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA) was used to determine the protein content. The proteins were separated and extracted using 10% SDS-polyacrylamide gel electrophoresis, and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membranes were detected using mouse antibodies against CD133 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), CD44, ALDH1 (both from Cell Signaling Technology, Danvers, MA, USA), N-cadherin (Upstate Biotechnology, Inc., Lake Placid, NY, USA), E-cadherin (BD Transduction), FoxM1 (Santa Cruz Biotechnology), Twist1 (Cell Signaling Technology) and β-actin (Sigma, St. Louis, MO, USA), respectively.

Statistical analysis. Data are presented as the mean ± SE (mean ± SD) and were analyzed by SPSS 17.0 statistical software. Multiple comparisons were performed by one-way ANOVA and pair-wise comparison was conducted by the LSD t-test method. The Dunnett's method was used for unequal variances. P<0.05 as considered to indicate a statistically significant result.

Results

Synthesis and identification of DFOG. Compound 1 was synthesized and obtained using methods from the patent application (31) and was identified by a combination of NMR and mass spectral data and by comparison of these to the published literature.

Compound 1, yellow powder; EI-MS, m/z 544.1; ^{1}H NMR (500 MHz, $CDCl_3$): 0.88-0.92 (6H, m), 1.26-1.55 (24H, m), 1.77-1.83 (2H, m), 1.90-1.96 (2H, m), 3.98 (2H, J=6.5 Hz), 4.06 (2H, J=6.5 Hz), 6.53 (1H, d, J=2.0 Hz), 6.65 (1H, t, J=72.5 Hz), 6.69 (1H, d, J=2.0 Hz), 6.94 (2H, d, J=8.5 Hz), 7.44 (2H, d, J=8.5 Hz), 7.77 (1H, s); ^{13}C NMR (125 MHz, $CDCl_3$): 14.1, 22.6, 22.7, 25.8, 26.0, 28.8, 29.1, 29.2, 29.3, 29.4, 29.5, 31.8, 68.0, 69.9, 98.3, 99.1, 112.6, 113.2, 114.4, 115.2, 117.3, 123.6, 126.5, 130.3, 150.3, 154.8, 159.0, 159.1, 161.4 and 175.2. The ^{1}H-NMR data were consistent with the literature (31) and ^{13}C-NMR data were reported for the first time. Thus, compound 1 was identified as 7-difluoromethoxyl-5,4'-di-n-octyl genistein and named DFOG (Fig. 1).

Characteristics of GCSLCs derived from the SGC-7901 cell line. In order to enrich GCSLCs from human GC SGC-7901 cells, a stem cell conditioned medium suspension culture method was used. Under these conditions, the cells grew as non-adherent, three-dimensional sphere clusters. Fig. 2A shows the anchorage-independent spheres that formed in the SGC-7901 cells. After 5 days of incubation, the SFCs from the SGC-7901 cell line were found to generate more and larger spheroid colonies compared with that noted in the PCs (Fig. 2B and C).

Next, western blotting was performed to identify protein expression of the gastric CSC markers [cluster of differentiation CD133, CD44 and aldehyde dehydrogenase 1 (ALDH1)]. The

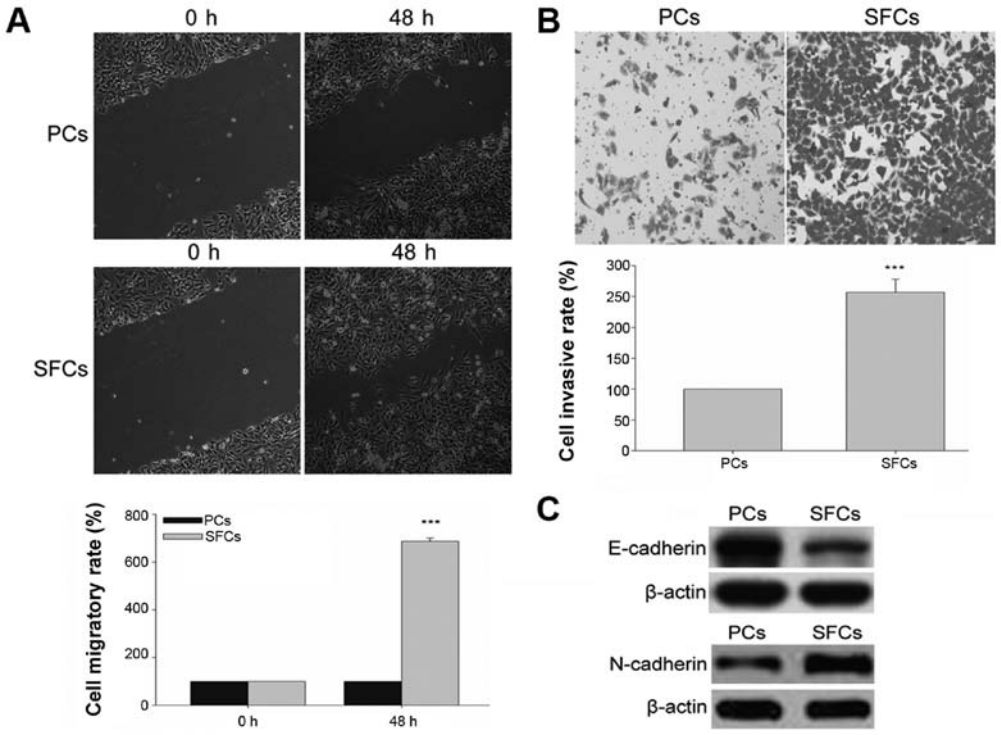

Figure 3. Mesenchymal cell characteristics of the gastric cancer stem-like cells (GCSLCs) derived from the SGC-7901 cells. (A) The cell migration rate of sphere-forming cells (SFCs) from SGC-7901 cells compared with parental cells (PCs) and the statistical analysis. ***P<0.001 vs. the PCs. (B) The invasive ability of the SFCs from SGC-7901 cells compared with PCs and the statistical analysis. ***P<0.001 vs. the PCs. (C) The protein expression levels of N-cadherin and E-cadherin in the SFCs compared with levels in the PCs.

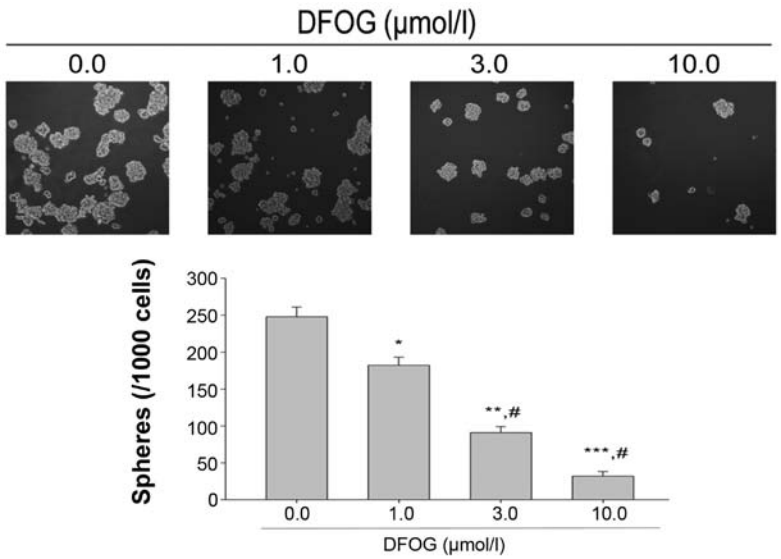

Figure 4. Inhibition of sphere-forming ability by DFOG in gastric cancer stem-like cells (GCSLCs) from SGC-7901 cells and the statistical analysis. *P<0.05 vs. the group treated with 0.1% DMSO; **P<0.01 vs. the group treated with 0.1% DMSO; ***P<0.001 vs. the group treated with 0.1% DMSO; #P<0.05 vs. the group treated with 1.0 μmol/l DFOG.

results showed enrichment of $CD133^+$, $CD44^+$ and ALDH1-high populations in the SFCs derived from the SGC-7901 cells compared with the PCs (Fig. 2D). These results indicated that SFCs from the SGC-7901 cells cultured in stem cell-conditioned medium possessed GCSLC properties.

GCSLCs from the SGC-7901 cell line show mesenchymal cell characteristics. CSCs have higher migratory and invasion capacities, which facilitate metastasis and growth. The migration and invasion capabilities of GCSLCs and PCs were evaluated by scratch method and Transwell chamber invasion assay *in vitro*, respectively. The results demonstrated that GCSLCs showed increased migratory and invasive capabilities than these capacities noted in the PCs (Fig. 3A and B). CSCs are also thought to facilitate metastasis through EMT characteristics related to the mobility of cells. We evaluated

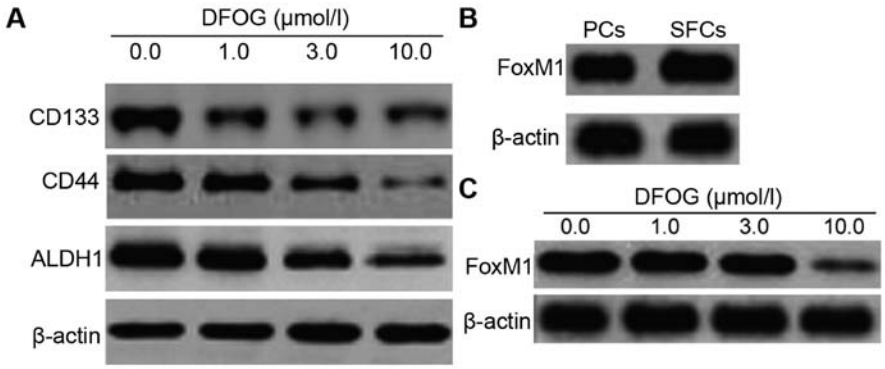

Figure 5. DFOG downregulates the expression of FoxM1 and cancer stem cell (CSC) biomarkers in gastric cancer stem-like cells (GCSLCs) from SGC-7901 cells. (A) DFOG inhibited the expression of markers CD44, CD133 and ALDH1 protein in sphere-forming cells (SFCs). (B) A higher expression level of FoxM1 was noted in the SFCs compared with the parental cells (PCs). (C) DFOG inhibited the expression of FoxM1 in the SFCs.

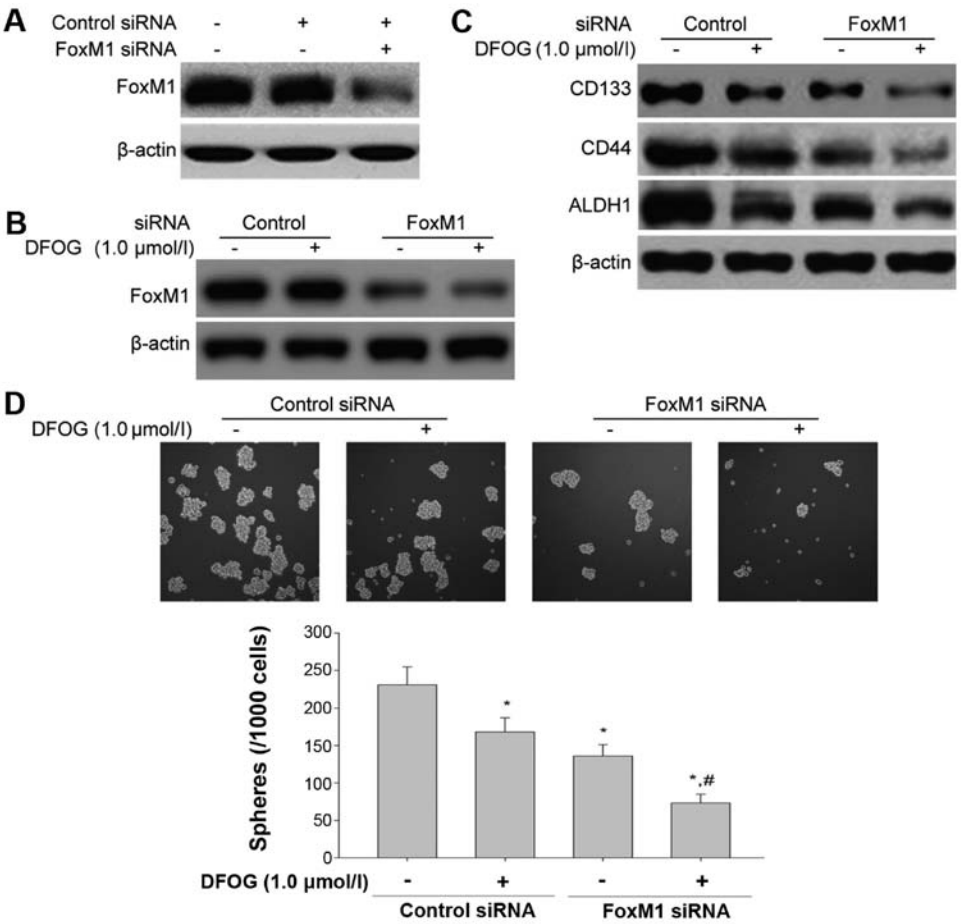

Figure 6. Synergistic effect on gastric cancer stem-like cells (GCSLCs) from SGC-7901 cells by DFOG and transfection of FoxM1 siRNA. (A) The protein expression of FoxM1 in sphere-forming cells (SFCs) was significantly decreased after transfection with FoxM1 siRNA. (B) Synergistic inhibition of expression of FoxM1 in SFCs by DFOG and FoxM1 siRNA transfection. (C) Synergistic inhibition of expression of CD133, CD44 and ALDH1 in SFCs by DFOG and FoxM1 siRNA transfection. (D) Synergistic inhibition of the sphere forming ability of SFCs by DFOG and FoxM1 siRNA transfection and the statistical analysis. *P<0.05 vs. the group treated with 0.1% DMSO; #P<0.05 vs. the group treated with 1.0 μmol/l DFOG alone.

the protein expression of a known mesenchymal phenotype cell biomarker (N-cadherin) and an epithelial phenotype cell biomarker (E-cadherin) by western blot analysis. The results demonstrated that the relative protein level of N-cadherin was highly expressed in the GCSLCs, while that of E-cadherin was weakly expressed (Fig. 3C).

DFOG inhibits the self-renewal of GCSLCs derived from the SGC-7901 cell line. Tumor sphere assay is used to identify stem cells in *in vitro* assays. We examined the tumor sphere formation capacity of SGC-7901 cells following treatment of DFOG. The results showed that DFOG (1.0, 3.0 and 10.0 μmol/l) reduced the number of SFCs derived from the

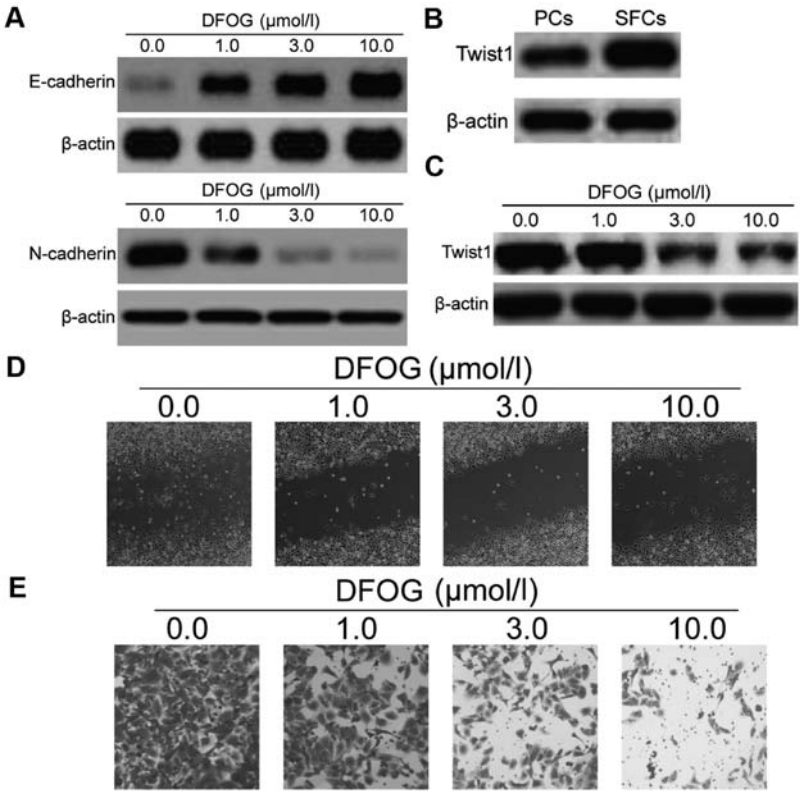

Figure 7. Effects of DFOG on EMT phenotype, migration and invasion capabilities in gastric cancer stem-like cells (GCSLCs) from SGC-7901 cells. (A) The increased expression of E-cadherin and decreased expression of N-cadherin in the sphere-forming cells (SFCs) following treatment with DFOG. (B) Western blot analysis showed that Twist1 was highly expressed in the SFCs. (C) The inhibition of the expression of Twist1 by DFOG in SFCs. (D) Inhibition of the migratory ability by DFOG in SFCs. (E) Inhibition of cell invasive ability by DFOG in SFCs.

SGC-7901 cells in a concentration-dependent manner (Fig. 4), indicating that DFOG is able to preferentially suppress the self-renewal of GCSLCs.

DFOG downregulates the expression of CSC markers and FoxM1 in GCSLCs derived from the SGC-7901 cell line. CD133, CD44 and ALDH1 are used as cancer stem cell markers in many types of tumor cells including GC. To investigate the effect of DFOG on GCSLC surface marker expression including CD44, CD133 and ALDH1, we incubated GCSLCs with DFOG (1.0, 3.0 and 10.0 μmol/l) and DMSO as a control. The expression levels of CD44, CD133 and ALDH1 in the GCSLCs were significantly suppressed following treatment with DFOG compared with the spheres that were untreated (Fig. 5A). A previous study demonstrated that overexpression of FoxM1 led to EMT by the acquisition of the EMT phenotype and downregulation of FoxM1 led to the inhibition of EMT in GC cell lines (21). Therefore, the present study compared the level of FoxM1 protein expression in the PCs and SFCs and evaluated the inhibitory effect of DFOG on FoxM1. The results revealed that FoxM1 expression was higher in the SFCs in comparison with that in the PCs (Fig. 5B). Furthermore, as shown in Fig. 5C, the expression of FoxM1 in the SFCs was significantly reduced by DFOG in a dose-dependent manner.

Transfection of FoxM1 siRNA enhances the inhibitory effects of DFOG on the expression of FoxM1, CSC markers and the self-renewal in GCSLCs derived from the SGC-7901 cell line. We next transfected FoxM1 siRNA into the GCSLCs to confirm the inhibitory effect of DFOG on FoxM1 expression in GC cells and the effect of FoxM1 on the characteristics of GCSLCs. The GCSLCs were transfected with either scramble siRNA or FoxM1 siRNA. The expression levels of FoxM1 and CSC markers including CD133, CD44 and ALDH1 were assessed using western blot analysis. The results showed that the protein expression level of FoxM1 was inhibited following FoxM1 knockdown (Fig. 6A). Furthermore, after transfection of FoxM1 siRNA, the inhibitory effects of DFOG on the expression of FoxM1 and CSC markers (CD133, CD44 and ALDH1) were markedly enhanced compared with the scramble siRNA control group (Fig. 6B and C). The knockdown of FoxM1 also suppressed the sphere-forming capability of the GCSLCs and suppressed the inhibition of the self-renewal of GCSLCs synergistically together with DFOG (Fig. 6D).

DFOG reverses EMT, as well as decreases the migration and invasion of GCSLCs derived from the SGC-7901 cell line. To investigate the effects of DFOG on the EMT process of GCSLCs, the protein levels of E-cadherin, N-cadherin and the Twist1 (EMT-related transcription factors) were measured. The results from the western blot analysis demonstrated that DFOG upregulated the protein level of E-cadherin and downregulated the protein level of N-cadherin (Fig. 7A). The protein expression level of Twist1 in the GCSLCs was higher than that in the PCs (Fig. 7B), while DFOG also dose-dependently suppressed the expression of Twist1 (Fig. 7C). These results clearly demonstrated that DFOG could reverse EMT, relying

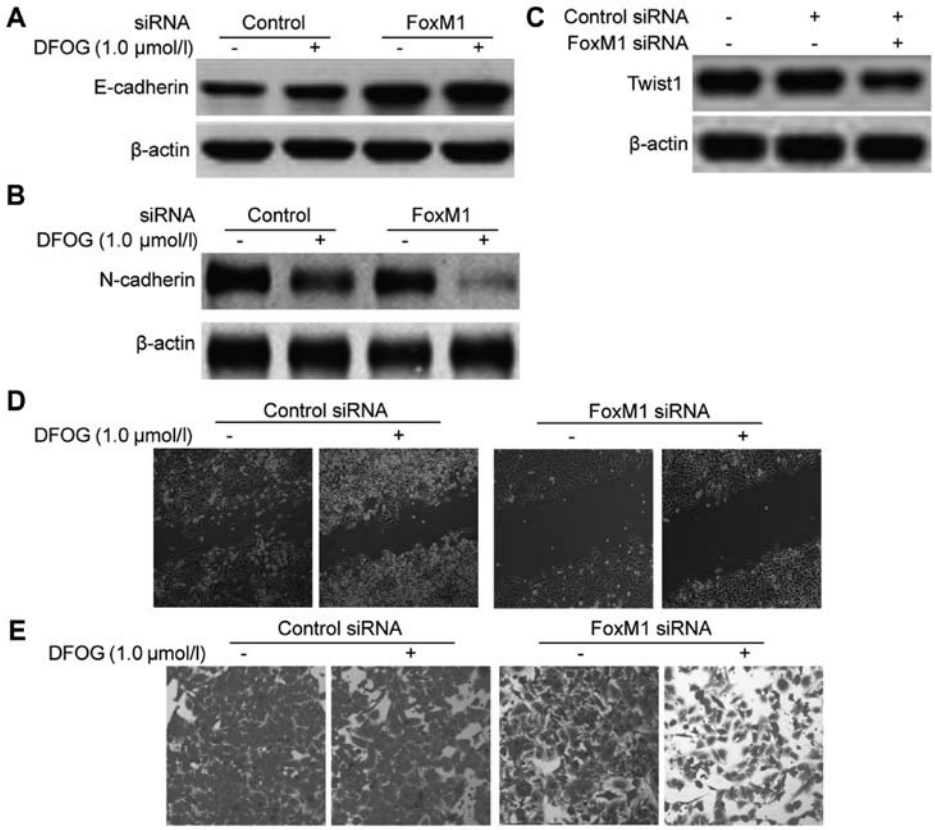

Figure 8. Transfection of FoxM1 siRNA cooperates with DFOG to reverse EMT. (A) The synergistic upregulation of E-cadherin in sphere-forming cells (SFCs) by DFOG and FoxM1 siRNA transfection. (B) Synergistic inhibition of N-cadherin in SFCs by DFOG and FoxM1 siRNA transfection. (C) Synergistic inhibition of Twist1 in SFCs by DFOG and FoxM1 siRNA transfection. (D) Synergistic inhibition of the migratory ability of SFCs by DFOG and FoxM1 siRNA transfection. (E) Synergistic inhibition of the migratory ability of SFCs by DFOG and FoxM1 siRNA transfection.

on inhibition of the EMT phenotypic biomarkers and Twist1. Migration and invasion abilities are important characteristics of CSCs responsible for tumor metastasis and growth. CSCs are assumed to have higher migration capacity than normal cancer cells and Twist1 is closely associated with cancer cell migration and invasion. Functionally, the relative migratory and invasive cell numbers of GCSLCs were significantly decreased when compared to these numbers in the negative control, suggesting that DFOG reduced cell migration and invasion in the GCSLCs (Fig. 7D and E). The above results showed that DFOG inhibited the expression of Twist1, leading to the inhibition of cancer cell migration and invasion.

Transfection of FoxM1 siRNA cooperates with DFOG to reverse EMT. To examine whether forced knockdown of FoxM1 can cooperate with DFOG to reverse EMT, the protein expression of EMT biomarkers (E-cadherin and N-cadherin) and Twist1 was measured by western blot analysis. As compared to the negative control groups, silencing by FoxM1 siRNA increased the protein expression of E-cadherin (Fig. 8A) and decreased the protein expression of N-cadherin and Twist1 (Fig. 8B and C), suggested that knockdown of FoxM1 could reverse EMT. Moreover, transfection of FoxM1 siRNA also enhanced the upregulation of E-cadherin protein expression (Fig. 7A) and the downregulation of N-cadherin and Twist1 protein expression (Fig. 8B and C) caused by DFOG, demonstrating that transfection of FoxM1 siRNA can cooperate with DFOG to reverse the EMT process. Following transfection of FoxM1 siRNA, the influence on the migratory and invasive capabilities of the GCSLCs were evaluated. The results showed that transfection of FoxM1 siRNA suppressed the migration and invasion of GCSLCs and also enhanced the inhibitory effect of DFOG on the migration and invasion of GCSLCs compared with the control group (Fig. 8D and E). These results confirmed that transfection of FoxM1 siRNA and DFOG can synergistically reverse the EMT process of GCSLCs.

Discussion

In the present study, it was confirmed that SFCs derived from the SGC-7901 cell line possessed superactive self-renewal capacity *in vitro* when compared with that noted in the parental cells. It was also found that $CD133^+$, $CD44^+$ and ALDH-high populations were enriched in the tumor spheroid cells from the SGC-7901 cells, which exhibited the characteristics of GCSCs such as invasion capacity and EMT, and were therefore identified as GCSLCs.

Previous studies have confirmed that FoxM1 plays an important role in the regulation of cancer stem cell (CSC) properties and EMT in various types of cancers. Meng *et al* reported that overexpression of FoxM1 promotes EMT and metastasis of hepatocellular carcinoma (32). Miao *et al* reported that downregulation of FoxM1 leads to the inhibition

of EMT in GC cells (21). Bao *et al* discovered that overexpression of FoxM1 led to EMT and a cancer stem cell phenotype in pancreatic cancer cells (22). In our previous study, we found that DFOG inhibited ovarian and GC cell growth by downregulation of FoxM1 (30). The present study uncovered, for the first time, that DFOG can inhibit the function and properties of GCSLCs through downregulation of FoxM1.

Twist1 is reported as one of the major inducers of the EMT process and also acts as an EMT biomarker in CSCs. Ren *et al* demonstrated that overexpression of Twist in HCC cell line SMMC-7721 promoted the generation of a hepatocellular cancer stem cell (HSC) phenotype through upregulation of the expression of the biomarkers CD133 and CD44 (33). He *et al* reported that casticin inhibited EMT in liver CSCs from the SMMC-7721 cell line by downregulating Twist (34). Our results showed that overexpression of Twist protein in GCSLCs were significantly suppressed by exposure to DFOG consistent with the increased expression of E-cadherin and decreased expression of N-cadherin. These results revealed that the EMT process in GCSLCs was suppressed by DFOG. In addition, Qian *et al* reported that Twist1 promoted GC cell proliferation through upregulation of FoxM1, which suggested that Twist1 and FoxM1 can interact with each other to affect the function and properties of CSCs (35). Our current results indicated that DFOG was capable to reverse the EMT phenotype by downregulation of FoxM1 and further downregulation of Twist1 based on different mechanism when compared with that of FoxM1 siRNA.

Overall, our findings further clarify the anticancer effects of 7-difluoromethoxyl-5,4'-di-n-octyl genistein (DFOG), a novel synthetic genistein analogue. DFOG eliminated stem-like characteristics of GCSLCs and reversed EMT, partially due to the downregulation of FoxM1 and EMT-related proteins (Twist1), and attenuated the migratory and invasive abilities of GCSLCs. DFOG may be potentially effective in preventing GC by targeting CSCs.

Acknowledgements

The present study was financially supported by grants from the National Natural Science Foundation of China (nos. 31400311 and 81172375), the Program for Excellent Talents of Hunan Normal University (no. ET1508), the Project of Hunan Provincial Natural Science Foundation (no. 13JJ3061), the Scientific Research Fund of Hunan Normal University (nos. 140668 and 140666), and the Construct Program of the Key Discipline of Basic Medicine in Hunan Province.

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