

Deferoxamine enhances cell migration and invasion through promotion of HIF-1 α expression and epithelial-mesenchymal transition in colorectal cancer

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Abstract. Deferoxamine (DFX), a metal chelator, has been previously reported to induce hypoxia and hypoxia-inducible factor-1 α (HIF-1 α) expression. HIF-1 α is a common inducer of epithelial-mesenchymal transition (EMT) in many solid tumors. However, the effect of DFX on cancer metastasis and the related mechanisms are not well established. In the present study, we aimed to ascertain whether DFX enhances EMT and cancer metastasis in colorectal cancer. After confirmation of DFX-inducing HIF-1 α expression, we examined the effect of DFX on cell adhesion, migration and invasion abilities and found a positive effect on the above functions. Consequently, cell morphology, cell growth and expression of EMT markers were assessed in cells with or without DFX treatment. We found that cells exposed to DFX were more isolated. They were spindle-shaped and looked similar to fibroblast-like cells, accompanied by increased anchorage-independent growth. DFX-treated cells expressed E-cadherin and plakoglobin at a higher level, and vimentin and N-cadherin at a lower level, when compared with these levels in control cells. Furthermore, the expression of E-cadherin in the cell membrane was markedly decreased in DFX-treated cells. These results suggest that DFX promotes cancer migration and invasion via a process consistent with EMT in colorectal cancer.

Introduction

Mounting evidence indicates that iron plays a crucial role in malignant cell proliferation (1,2). A dysregulated iron metabo-

lism reveals a poor outcome in breast cancer and esophageal adenocarcinoma patients (3,4). Deferoxamine (DFX), a high affinity iron chelator, is known to upregulate inflammatory mediators and it has been shown to have significant and selective antitumor activity, although the mechanisms of action remain to be completely elucidated (5-7). DFX has the ability of hypoxia mimetics to induce a hypoxic state which results in the upregulation or stabilization of hypoxia-inducible factor-1 (HIF-1) (8,9). HIF-1 is a nuclear protein involved in the transcriptional activation of a broad array of genes that activate mitogenic, pro-invasive, pro-angiogenic and pro-metastatic genes (10-12). Hence, DFX-induced hypoxia is a complicated event, which may affect various pathways and gene expression through the regulation of HIF-1 expression.

Epithelial-mesenchymal transition (EMT) is a critical process for tumor invasion and metastasis (13-16). It is characterized by loss of cell polarity and cell adhesion, repression of E-cadherin expression and increased cell motility. At the same time, the traits of epithelial cells such as long-lasting morphological and molecular changes are altered to mesenchymal characteristics, such as fibroblastic morphology, increased vimentin and N-cadherin expression, enhanced migration and invasion capacities, thus causing cancer cell metastasis (14,17). It is important to note that the stabilization and activation of HIF-1 are the most important mechanisms that promotes metastasis and thereby, increase tumor aggressiveness (18,19). For example, hypoxia or HIF-1 overexpression induces EMT and metastatic phenotypes through the direct activation of Twist in head and neck squamous cell carcinoma (HNSCC) (20). Hypoxia-induced EMT may be enhanced by the formation of the HIF-1/ β -catenin complex, which increases the transcriptional activity of HIF-1 α and consequently facilitates EMT under hypoxic conditions in hepatocellular carcinoma (HCC) (21).

In the present study, we treated colorectal cancer (CRC) cells with DFX to create a DFX-induced hypoxic cell model, and then explored EMT-associated features, such as the expression of EMT-associated molecules, the cellular location of E-cadherin and vimentin, cell morphological changes, adhesion, migration and invasion abilities. Our study showed that DFX treatment increased HIF-1 α expression and enhanced

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EMT. Thus, we reported the potential mechanism underlying DFX-induced hypoxia on cell migration and invasion in CRC cells.

Materials and methods

Cell culture and cell treatments. HT29 and HCT116 cells were grown in RPMI-1640 (Gibco-BRL) supplemented with 10% fetal bovine serum (FBS) as we previously described (22). For DFX treatments, we utilized concentrations of 0, 50, 100 and 200 μM respectively, of DFX supplemented with 10% FBS. Forty-eight hours later, cell morphological changes were observed or cell lysates were extracted to perform western blot analysis.

Western blotting, immunofluorescence assays and antibodies. Western blotting and immunofluorescence assays were carried out as previously described (23). Antibodies to HIF-1 α (Novus Biologicals, Littleton, CO, USA), E-cadherin and vimentin were applied in the immunofluorescence staining (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Vimentin (prediluted), plakoglobin, N-cadherin and glyceraldehyde-3-phosphate dehydrogenase (Abcam, Cambridge, UK) were all commercial products.

Hematoxylin and eosin (H&E) staining. Exponentially growing cells were seeded in coverslips and then exposed to 100 μM of DFX. Forty-eight hours later, coverslips were fixed in 4% paraformaldehyde for 15 min, washed in phosphate-buffered saline (PBS) and running water, followed by incubation with hematoxylin solution for 5 min and washed under running water. Subsequently, the coverslips were dipped in acid-ethanol, washed again, and then stained in eosin-ethanol (1% eosin in 80% ethanol) for 3 min, subjected to sequential dehydration and mounted. The coverslips were observed under an Olympus microscope (Olympus Optical Co., Ltd., Tokyo, Japan).

Immunofluorescence. Cells growing on coverslips were fixed with 4% paraformaldehyde at room temperature for 15 min, washed with PBS and permeabilized with 0.1% Triton X-100 for 5 min. After blocking with 1% bovine serum albumin (BSA) for 30 min, cells were incubated with E-cadherin and vimentin antibodies for 2 h. After washing with PBS, IgG Texas Red- (TR-) and IgG-FITC-labeled secondary antibodies were added and incubated for another 1 h. Cells were then rinsed with PBS followed by Hoechst 33258 staining to reveal nuclei. Fluorescence images were captured and analyzed using the Olympus CKX41 fluorescence microscope (Olympus Optical Co., Ltd.).

Adhesion assay. Substrates for adhesion assays included 25 $\mu\text{g}/\text{ml}$ fibronectin or 10 mg/ml heat-inactivated BSA. After overnight coating, wells were rinsed and blocked with 10 mg/ml heat-inactivated BSA as the negative control. Cells were treated with or without 100 μM DFX for 48 h, harvested, resuspended in serum-free medium, and 5×10^4 live cells were seeded into single wells of a 96-well plate. Cells were then incubated for 2 h at 37°C. Subsequently, the detached cells were washed away, and attached cells were determined by MTT using a microplate reader.

Migration, invasion and wound healing assays. Cell migration and invasion assays were performed using Transwell inserts (BD Biosciences, Bedford, MA, USA) in a similar manner. The difference was that the upper surface of the chambers in the invasion assay was pre-coated with Matrigel (BD Biosciences), while not in the migration assay. Standard protocol was followed. In brief, cells were pre-treated with or without 100 μM DFX for 24 h. Then cells were digested, washed and seeded into the inner chamber (5,000 cells/chamber) in medium without FBS. RPMI-1640 plus 15% FBS was added to the lower chamber, and the plate was incubated for 24 h. Cells on the upper surface of the filter were scraped away with a rubber scraper, while cells on the lower surface were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet, and then counted under a light microscope. For the wound healing assay, DFX-treated cells were seeded in 6-well plates. After scratching the monolayer, cells were washed with PBS, cultured in RPMI-1640 plus 10% FBS, and photographed under a x10 objective lens at the indicated time points. All of the above assays were carried out in duplicate, and the results presented are the means of 5 random fields from each well.

Cell growth in soft agar. Cells that were treated with or without DFX (100 μM) were harvested and pipetted well to become a single-cell suspension, and then mixed with regular medium containing 0.7% agar resulting in a final agar concentration of 0.35%. The above cell suspensions were immediately plated in 6-well plates coated with 0.6% agar in regular medium and cultured for 14 days. Tumor cell colonies were observed and counted under a dissecting microscope.

Statistical methods. Statistical analysis was carried out using SPSS 13.0 for Windows (SPSS, Inc.). Quantitative variables were analyzed using the t-test. $P < 0.05$ was considered to indicate a statistically significant result.

Results

DFX treatment induces HIF-1 α expression. To select a suitable cell candidate to induce HIF-1 α expression and EMT, we first detected the endogenous expression level of HIF-1 α in 6 colorectal cancer cell lines. As shown in Fig. 1A, the level was much lower in the HT29 and HCT116 cells when compared with that in the SW480, SW620, SW1116 and DLD1 cells. Therefore, HT29 and HCT116 cells were applied in our further experiments.

Next, we incubated HT29 and HCT116 cells with DFX at doses of 0, 50, 100 and 200 μM , respectively, for 48 h, to determine the maximum efficacy at inducing EMT in both cell lines. Treatment with DFX resulted in marked HIF-1 α expression in a dose-dependent manner in the range of 0 to 100 μM as shown in Fig. 1B. No significant difference between cells was noted at 100 and 200 μM . Thus, the concentration of 100 μM was used in the following experiments.

DFX treatment enhances cell adhesion, migration and invasion abilities. Considering that the adhesion of cancer cells to the extracellular matrix (ECM) is the first step in tumor metastasis (24,25), we first evaluated the effect of DFX on cell adhesion. HT29 and HCT116 cells with or without DFX

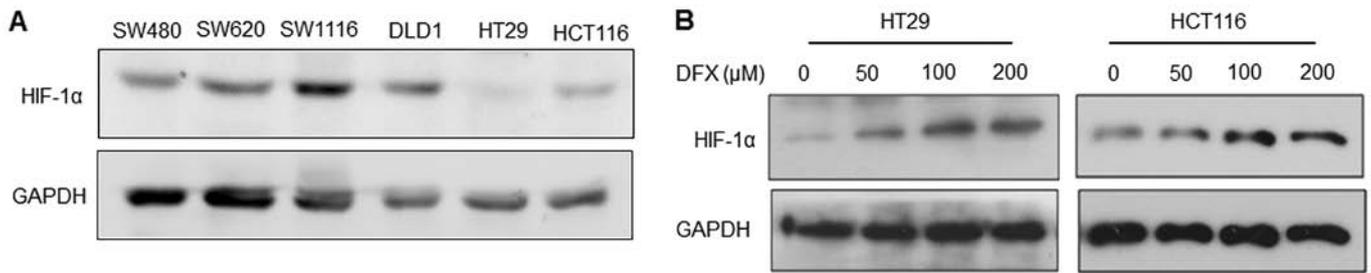


Figure 1. Induction of HIF-1 α -expression by DFX in CRC cell lines. (A) Protein expression of HIF-1 α in the indicated 6 cell lines was detected by western blotting. GAPDH was loaded as the internal control. (B) HT29 and HCT116 cells were stimulated with DFX (50, 100 and 200 μ M) or were mock treated. Proteins were loaded 48 h later and analyzed by western blotting for HIF-1 α expression. GAPDH was used as a loading control. HIF-1 α , hypoxia-inducible factor-1 α ; DFX, deferoxamine; CRC, colorectal cancer; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

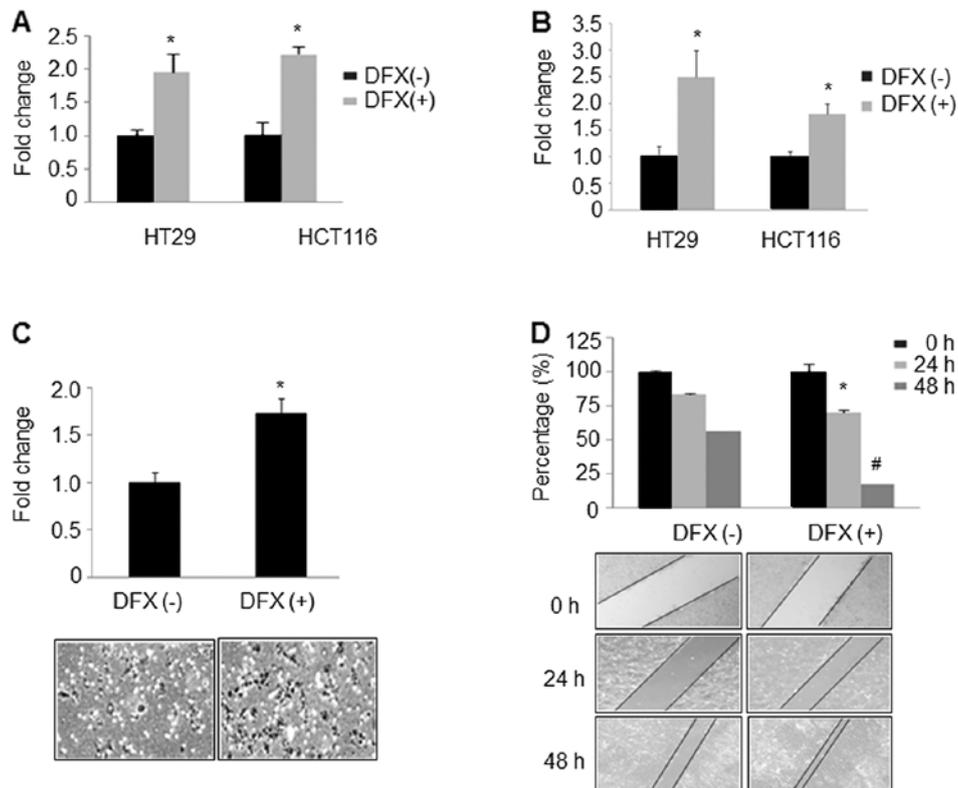


Figure 2. Functional assays of DFX in HT29 and HCT116 cells. (A) Cells were exposed to DFX (100 μ M) or were mock treated for 48 h. Single-cell suspensions were respectively seeded into fibronectin-coated 96-well plates (5×10^4 /well) for 2 h followed by quantitative assessment of cell viability by MTT assay. * $P < 0.05$ between comparison groups. (B and C) Migration and invasion assays were performed using Transwell inserts without or with Matrigel. The same number of cells with or without DFX treatment was seeded into the upper compartments of Transwell inserts with FBS-free medium, and the lower compartments were filled with 10% FBS medium. Quantitative analysis was carried out to determine the relative numbers of cells that crossed the pore filter of the inserts to the bottom medium. (D) Cells treated with DFX were wounded followed by further culturing for another 48 h, then the wound areas were observed at the indicated time points. The relative wound area was calculated (wound area at 24 or 48 h/wound area of 0 h), which was then normalized to that of the DFX-free cells. Data shown are representative of 3 independent experiments; means \pm SD. * $P < 0.05$ comparing with 24 h and # $P < 0.05$ comparing with 48 h without DFX treatment. DFX, deferoxamine; FBS, fetal bovine serum.

treatment were incubated with one major ECM component in fibronectin-coated 96-well plates for 2 h. We found that DFX treatment increased cancer cell adhesion to fibronectin by 1.96- and 2.23-fold in HT29 and HCT116 cells, respectively, when compared to the cells without DFX treatment (Fig. 2A). Next, we detected the effect of DFX on cell migration and invasion. As shown in Fig. 2B and C, DFX significantly increased migration and invasion of HT29 (2.5 ± 0.5 vs. 1 ± 0.2 ; $P < 0.05$) and HCT116 cells (1.8 ± 0.2 vs. 1 ± 0.1 , $P < 0.05$), when compared with the untreated control cells. Similarly, the wound healing

assay also indicated that DFX significantly induced cell migration at 24 and 48 h, respectively (Fig. 2D, $P < 0.05$). Collectively, these observations indicate that DFX induced cell adhesion, migration and invasion abilities in HT29 and HCT116 CRC cells.

DFX treatment results in altered cell morphology and cell growth. Morphological change plays an important role in many cellular processes such as migration, differentiation and apoptosis (22,23,26). We then investigated whether the

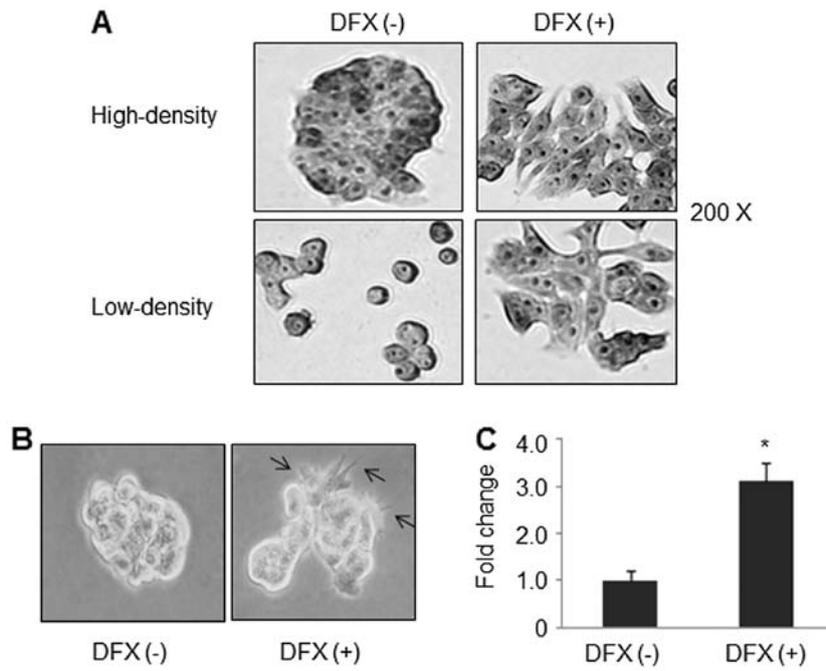


Figure 3. Effect of DFX on cell morphology and cell growth. (A) Representative examples of light microscopic images of H&E-stained HT29 cells without or with DFX treatment in high- and low-density fields, respectively. Original magnification, x200. Colony formation assay was performed by observing (B) sphere morphology and (C) calculating the number of spheres. Arrows indicate finger-like protrusions in B. (* $P < 0.05$). The samples were measured in triplicates, and the experiment was independently performed 3 times. DFX, deferoxamine; H&E, hematoxylin and eosin.

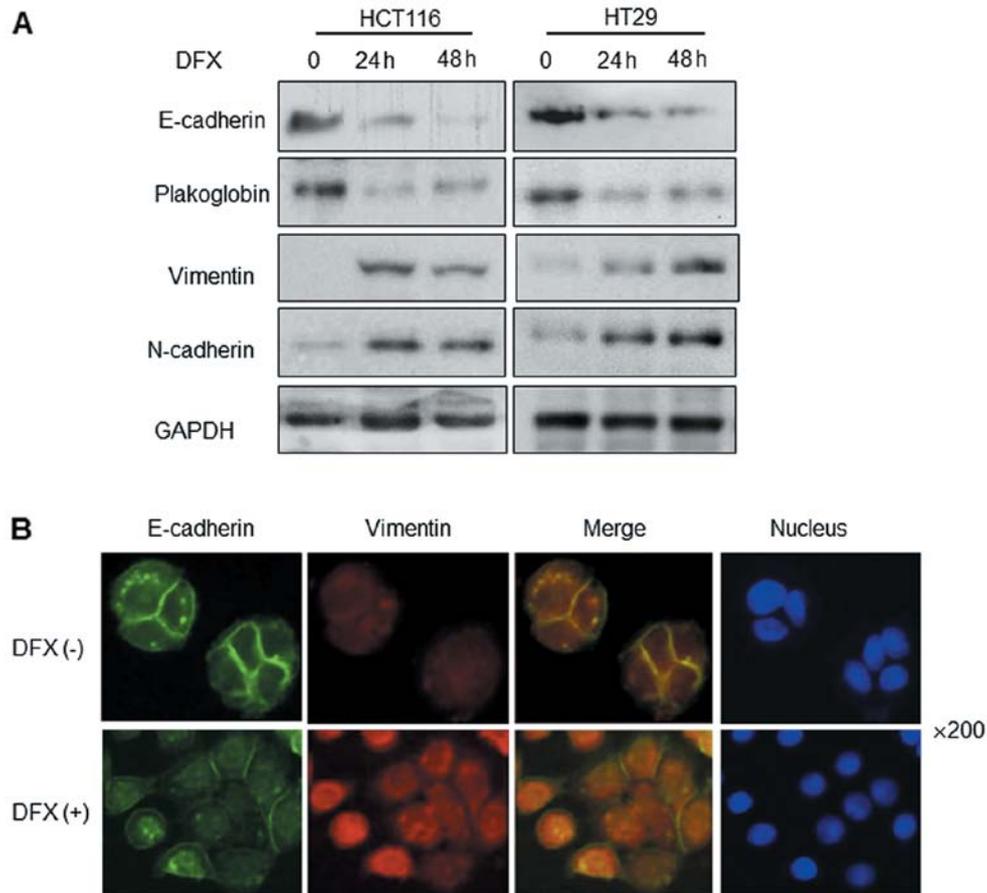


Figure 4. DFX alters the expression and localization of EMT markers. (A) HT29 and HCT116 cells were exposed to DFX for 24 or 48 h, respectively. E-cadherin, plakoglobin, vimentin and N-cadherin expression was detected by western blot analysis with GAPDH used as the internal control. (B) Membrane-associated E-cadherin and vimentin were assessed with the corresponding primary antibodies, and Texas Red- or FITC-labeled secondary antibodies. Cell nuclei are shown by Hoechst 33258 staining. Representative images of immunofluorescence staining are shown at an original magnification of x200. DFX, deferoxamine; EMT, epithelial-mesenchymal transition; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

increased motility and invasive ability of CRC cells was coupled with any morphological change. H&E staining results revealed that treatment with DFX resulted in dramatic morphological alterations in HT29 cells at both high-density and low-density as shown in Fig. 3A. In fact, cells treated with DFX displayed flattened, spread morphology. Cells became more isolated and looked like spindle-shaped and fibroblast-like cells. In contrast the untreated HT29 cells were much more round and had very tight connections. Similar results were found in soft agar assay experiments. As shown in Fig. 3B, cell-cell contacts through the 'pseudopod' were present in DFX-treated cells. The edges of these cells commonly showed several finger-like protrusions which may aid in cell motility. In contrast, cells without DFX treatment had a clear boundary and resembled scattered spheres. At the same time, DFX significantly increased anchorage-independent cell growth by 3.1-fold (Fig. 3C).

Effect of DFX on EMT markers. In addition to alterations in the functions (adhesion, migration and invasion) and cell morphology, the altered expression of EMT markers is another typical feature of EMT and cancer metastasis. We evaluated the effect of DFX on EMT epithelial markers (E-cadherin and plakoglobin) and mesenchymal markers (vimentin and N-cadherin). Western blot analysis indicated that E-cadherin and plakoglobin expression was significantly decreased, while vimentin and N-cadherin expression was increased after DFX treatment for 24 or 48 h (Fig. 4A). As a critical adhesion molecular, E-cadherin is reported as the most important epithelial marker of EMT. We then observed the expression and localization of E-cadherin and vimentin in cells with or without DFX treatment by immunofluorescence staining. As shown in Fig. 4B, the expression of E-cadherin in the cell membrane was markedly decreased in the DFX-treated HT29 cells, which indicated lower cell adhesion ability. At the same time, vimentin expression was upregulated. Furthermore, DFX-treated cells were able to spread much wider on coverslips which was consistent with the H&E staining results (Fig. 3A). These findings suggest that DFX treatment promotes EMT progression in CRC.

Discussion

Accumulating evidence indicates that hypoxia or overexpression of HIF-1 α are linked to the genesis, progression and metastasis of human cancers, suggesting HIF-1 α as an emerging biomarker for cancer prognosis and as a promising therapeutic target (27-29). Using a DFX-induced hypoxia model, we revealed that DFX promoted HIF-1 α expression and enhanced cell adhesion, migration and invasion abilities in CRC cells, through modulating cell morphology and expression of EMT markers.

Hypoxia is a major pathophysiological condition for the induction of angiogenesis, which is a common and crucial aspect of cell growth in solid tumors (10-12,30). DFX-induced hypoxia is a complicated event, which may affect a variety of cellular pathways and gene expression. Particularly, DFX is capable of inducing the activation of HIF-1 α and NF- κ B (31). HIF-1 α represents the most important transcription factor regulating gene expression under hypoxic conditions. It acts by

binding to hypoxia-responsive elements (HREs) in promoters, thereby regulating gene expression and playing a central role in cancer tumors. Yang *et al* (20) reported that HIF-1 α promotes tumor progression, EMT and metastasis by direct regulation of Twist, a key transcriptional regulator of EMT. In the present study, we found that DFX increased the HIF-1 α protein level in a dose-dependent manner, which may be one of the mechanisms of DFX-induced cell migration and invasion.

As an important adhesion molecule, membrane E-cadherin is an important hallmark of EMT (15,19,32). We demonstrated that incubation of HT29 cells with DFX led to the loss of E-cadherin in the cell membrane. Furthermore, the expression of EMT mesenchymal marker, vimentin, was increased following DFX treatment, which are both consistent with the EMT event.

In conclusion, the present study demonstrated that DFX increased HIF-1 α protein, decreased membrane-associated E-cadherin expression and increased vimentin expression. Consequently, less E-cadherin combines with other adhesion molecules on the membrane to decrease cell-cell contact and increase migration and invasion. At the same time, due to the transition from an epithelial-like to a mesenchymal-like cell morphology, cell motility was facilitated. These findings explain, in part, the marked role of DFX in colorectal cancer metastasis.

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