RESEARCH ARTICLE

Down-regulation of FRα Inhibits Proliferation and Promotes Apoptosis of Cervical Cancer Cells *in Vitro*

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Abstract

Folate receptor alpha (FRα) mediates folate uptake by endocytosis, and while folate is essential to DNA methylation and synthesis and may have an important role in proliferating cells. FRα is known to be expressed in rapidly proliferating cells, including many cancer cell lines, but there has been no systematic assessment of expression in cervical cancer cell lines. The aim of the present study was to evaluate the effects of FRα on proliferation and apoptosis of cervical cells and correlation mechanism. In this study, we investigated the biological function of FRα in Hela cells using RNA interference. Cell proliferation was evaluated by Cell Counting Kit-8 (CCK8) assay, while cell cycling and apoptosis were assessed by flow cytometry, mRNA levels by real time-PCR and protein levels of FRα, c-Fos and c-Jun by Western blotting. The results revealed that FRα was highly expressed in Hela cells and its silencing with a small interfering RNA (siRNA) inhibited cell proliferation and induced cell apoptosis, arresting the cell cycle in G0/G1 stages while decreasing the proportion in S and G2/M stages, and suppressed the expression levels of c-Fos and c-Jun. In conclusion, the results of this study indicated that FRα down-regulation might be capable of suppressing cervical cancer cell proliferation and promoting apoptosis. It suggested that FRα might be a novel therapeutic target for cervical cancer.

Keywords: Folate receptor alpha - proliferation - cell cycle - apoptosis - c-Fos - c-Jun

Introduction

Cervical cancer is the second most common malignancy in women in the world and it remains a leading cause of cancer-related death of women. Persistent infection of human papillomavirus (HPV) is considered as the primary risk factor in the development of cervical cancer (zur Hausen, 2002; Zhang et al., 2013). However, only HPV infection may not induce cervical cancer, many cofactors of cervical cancer have been established for female with persistent infection. In the other hand, though recently two prophylactic vaccines against the high-risk strains of HPV, mainly targeting HPV-16 and 18 types, have been developed and approved in more than 100 countries in the world, they are not yet widely used in developing countries (Liu et al., 2013). Surgery is still the primary therapeutic approach for early-stage cervical cancer patients while radiotherapy and chemotherapy for the advanced stages. So to investigate the novel biotherapeutics target has been concerned by many scholars.

Folate receptor alpha (FRα), called as folate binding protein (FBP), is a membrane-bound protein linked to cell surfaces via a glycosylphosphatydilinositol (GPI) anchor, which has a high affinity to mediate folate uptake

by endocytosis (Zhao et al., 2013). Folate is a basic component involved in the one-carbon transfer reactions that are essential for DNA synthesis and replication, cell division, growth, survival (Leung et al., 2013), and particularly for rapidly dividing cells, which was demonstrated in our previous study of cervical cancer cells (Jin-Tao et al., 2014). Interestingly, comparing with normal tissues and cells, there was a higher expression in various tumors of epithelial origin, including ovarian (Kalli et al., 2008), breast (O'Shannessy et al., 2012), lung (Bremer et al., 2013), colorectal cancers (Shia et al., 2008), and cervical cancer (Pillai et al., 2003). The characterization that FRα is high expressed in tumor tissues and high affinity to folate has been extensively exploited in therapy (Armstrong et al., 2013) and diagnosis (Wood, 2012). Many reports showed that folic acid conjugated with various pharmaceuticals such as pemetrexed, cisplatin and cetuximab (Schmid-Bindert et al., 2013) had been successfully delivered to cancer cells expressing FRα, and reduced toxic side effects of these pharmaceuticals on nontarget tissues. Besides, the folic acid conjugated imaging agents such as radiopharmaceutical agent of 99mTechnetium (Fisher et al., 2008) and magnetic resonance imaging contrast

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agent of Gadolinium-tetraazacyclododecane tetraacetic acid (Kalber et al., 2011) were also exploited for cancer diagnosis.

In view of the high affinity of FRα to folate which is the basic component of cell metabolism and DNA repair, especially for rapidly dividing cancer cells, it can be speculated that FRα may play an important role in promoting tumor growth. In fact, the speculation was proved by previous literatures (Yao et al., 2009). Activating protein 1 (AP-1) is a transcription factor composed of proteins containing a leucine zipper domain, which is very important for dimerization and DNA binding. The c-Fos and c-Jun are the major subunits of AP-1 proteins and they usually play a key role as dimeric form, involving in cell proliferation and apoptosis (Turpaev, 2006). We deduced that down-regulation of FRα might inhibit tumor cell proliferation by regulating the activation of AP-1. Most researches indicated that down-regulation of FRα could restrain tumor cell proliferation *in vitro* or tumor growth *in vivo* (Yao et al., 2009). However, whether it has the same effects on cervical cancer has been not reported.

In the present study, we attempted to address whether down-regulating FRα inhibits the proliferation and as well as if the suppression resulting from the regulating for the expression of c-Fos and c-Jun. So we measured cell proliferation ability by CCK8 assay, and tested the expression levels of FRα and cell cycle and apoptosis by flow cytometry, and the levels of c-Fos and c-Jun by western blot in Hela cells with the highest FRα expression. All the experiments had been performed in three groups including control, transfected with negative small interfering RNA (siRNA) sequence, and transfected with the efficient FRα siRNA sequence groups.

Materials and Methods

Cell culture

Hela cells, human cervical cancer cell line, were purchased from National institute of experimental cell resources (Beijing, China). Cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum and 100 units/ml of penicillin and 100 units/ml of streptomycin at 37°C in an atmosphere containing 5% CO_2.

siRNA design and siRNA interference experiment

According to the principles of siRNA design, three duplexes of specific siRNA sequences targeting for FRα (GenBank Accession NG_015863, Gene ID: 2348), including siRNA sequence 1 (GGACUGAGCUUCUCAAUGUTT (132-152)), siRNA sequence 2 (GAUGUUUCCUACCUAUAUATT (272–292)) and siRNA sequence 3 (CCACUGUUCUGUGCAAUGATT (597–617)), and negative siRNA (sense 5' to 3': UUCUCCGAACGUGUCACGUTT) were synthesized by Shanghai Genepharma RNAi company (Shanghai, China) using software provided at http://www.ambion.com/techlib/misc/siRNA _design.html. A FAM termination signal was introduced at the 5'-end of the oligo.

Hela cells cultured in a 24-well plate were transfected with the siRNA oligo sequences by using X-tremeGENE siRNA Transfection Reagent which was obtained from Roche Applied Science (Cat.No.04476093001) (Indianapolis, USA). In the present study, Hela cells were individed into three experiment groups as control, negative siRNA group and positive siRNA group. After incubation for 48 h, the transfection efficiency and the suppression efficiency were detected under fluorescence microscope and using flow cytometry.

Detection of mRNA expression levels by Real-Time PCR (RT-PCR)

The mRNA expression levels of the three group cells were determined by RT-PCR, using the following forward primers (F) and reversed primers (R), respectively. FRα: F: 5'-AAAGAGGACTGTGAGCAATGGT-3', R: 5'-GTGTGGGGAAGTAGAAATGGAA-3' (Sangon Biotech, Shanghai, China). β-actin: F: 5'-AGCGAGCATCCCCCAAAGTT-3', R: 5'-GGGCACGAAGGCTCATCATT-3' (Takara, Japan). Cycling was carried out at 95°C for 5 min followed by 40 cycles with amplification steps at 95°C for 10 s, then with annealing steps at 60°C for 30 s. Ct values were used as the results of the qPCR. The comparative Ct method was used for every cDNA sample with the ratio of FRα and β-actin expression level (2-ΔΔ Ct). In this study, after transfection for 48h, the expression levels of mRNA of Hela cells were assessed by RT-PCR Detection System (Applied Biosystems 7500 Instrument, ABI, USA), using the QuantiFast SYBR Green PCR Kit (QIAGEN, Düsseldorf, German).

Investigation of protein expression levels by flow cytometry and western blot

After incubation with the antibody against FRα (Monoclonal Anti-human FOLR1-Phycoerythrin, FAB5646P, R&D), the cell suspension were made to determine FRα protein expression levels of the three group cells by flow cytometry (Becton Dickinson Oxford, U.K). Meanwhile, we evaluated protein expression levels of c-Fos and c-Jun by western blot. The antibodies againsted c-Fos (c-Fos: ab134122; phosphorylated c-Fos: ab17933; Abcam plc) and c-Jun (c-Jun: ab32137; phosphorylated c-Jun: ab30620; Abcam plc) were adopted in the study.

Evaluation of cell proliferation ability by Cell Counting Kit-8 (CCK8)

Cell proliferation ability was evaluated by CCK8 (Dojindo Laboratories, Shanghai, China) assay. The cells were respectively transfected with the selected positive siRNA sequence and negative siRNA sequence, and then were cultured for 12, 24, 48 and 72 h. At each time point, 10 µl CCK8 stock solution were added to each well of three groups and the plates were further incubated for 2 h at 37°C. Then the absorbance was detected at 450 nm in a microplate reader (ST-360, KHB, Shanghai, China).

Detection of cell cycle and apoptosis

The positive and negative FRα siRNA oligo sequences were transfected into cells, respectively. The three group cells were harvested using 0.25% trypsin, and then were added to 1 ml 70% pre-cooling ethanol overnight at 4°C. Next day, The cells centrifugated were treated with 100
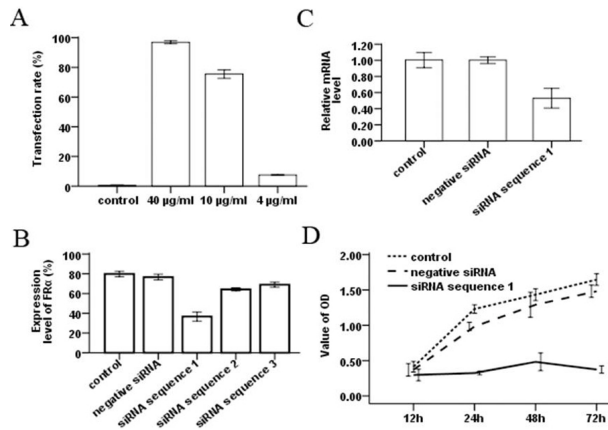
Figure 1. Down-regulation of FRα and the Changes of Proliferation Ability of Hela Cells. (A) The transfection efficiency after transfection using siRNA oligo at different concentration for 48 h. (B)The expression level of FRα in Hela cells by siRNA interference. (C) The relative mRNA level of FRα in Hela cells after interfered with siRNA. (D) Effects of down-regulation of FRα on Hela cells proliferation. Shown are mean values of triplicate assays ± SD.

µl RNase at 37ºC for 30 min and then stained with 400 µl PI (KGA511, KeyGEN Biotech, Nanjing, China) for 30 min. Lastly, cell cycle was detected by flow cytometry. In this study, an Annexin V-FITC/PI apoptosis detection kit (KGA108, KeyGEN Biotech, Nanjing, China) was used to observe early apoptosis.

Statistical analysis

All experiments were independently repeated three times. All quantitative data were presented as the mean ± standard deviation (SD). SPSS 16.0 software package was used for statistical analysis. ANOVA analysis was used to compare means between three groups, and Post Hoc multiple comparisons was used for any two groups. Differences were regarded as statistically significant when $p<0.05$.

Results

Down-regulated expression of FRα in Hela cells

Hela cells were transfected with the FAM stained siRNA oligo (40 µg/ml, 10 µg/ml, 4 µg/ml). The transfection efficiency was determined under the inverted microscope and by flow cytometry. The results exhibited that more than 90% cells were labeled with green fluorescence at 48 h after transfection with the concentration of 40 µg/ml under the inverted microscope. Further, the transfection rate were tested by flow cytometry, 40 µg/ml group also showed the highest rate (95%), which had a significant difference in comparison to the other concentration groups (Figure 1A). This result demonstrated that the Hela cells could be successfully transfected with siRNA oligo at the dosage of 40 µg/ml, therefore, the concentration should be selected for the following experiments.

Then, we selected the desired siRNA sequence, which could down-regulate significantly the expression of FRα. As shown in Figure 1B, the inhibition rate of siRNA sequence1 was higher than that of sequence2 and sequence3. Further, we detected mRNA expression of FRα
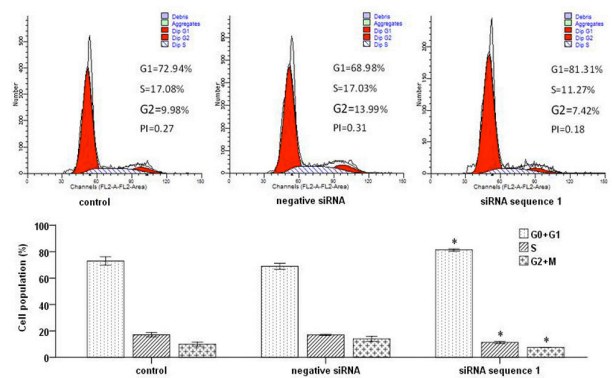

Figure 2. Effects of Down-regulation of FRα on Cell Cycle. The cell cycle of Hela cells in three groups were analyzed by flow cytometery and the cell population at different stages were calculated. Shown are mean values of triplicate assays ± SD. (*$p<0.05$)

by RT-PCR after these Hela cells were transfected with siRNA sequence1 at the dosage of 40 µg/ml. The result showed that mRNA expression levels of FRα were also decreased dominantly by siRNA sequence1 (Figure 1C).

The down-regulation of FRα inhibited the proliferation of Hela cells

CCK8 assays were used to determine the effects of reduced FRα expresssion on Hela cell growth rate. Hela cells were divided into three groups including control, negative siRNA group and siRNA sequence 1 group, the inhibition rate of which were detected in the stages of post-transfection 12 h, 24 h, 48 h and 72 h. The result showed that the inhibition rate of siRNA sequence 1 group at different time points were 43.9%, 71.5%, 72.7% and 80.5%, respectively (Figure 1D). Comparing with the inhibition rates of the other groups, the differences of the inhibition rate in the stages of post-transfection 24 h, 48 h and 72 h were significantly. However, there was no significant difference between control and negative siRNA group.

The effects of down-regulation of FRα on cell cycle and apoptosis of Hela cells

We studied the effects of down-regulation of FRα on cell cycle and apoptosis of Hela cells by flow cytometry. In comparison to the control and negative siRNA group, the proportions of S and G2/M period frequency of the Hela cell in siRNA sequence 1 group were significantly decreased (*$p<0.05$). In contrast, the proportion of G0/G1 stage was significantly increased (*$p<0.05$). However, in comparison to control, there was no significant difference in negative siRNA group (Figure 2). As shown in Figure 3, the apoptosis ratio of Hela cells was increased (*$p<0.05$) prominently in siRNA sequence 1 group.

The effects of down-regulation of FRα on the expression of c-Fos and c-Jun

In order to investigate whether the phenomenon that the down-regulation of FRα suppressed cell proliferation was attributed to decreased activation of AP-1 or not, we tested the expression of c-Fos and c-Jun by western blot after transfection with siRNA for 48 h. The result displayed
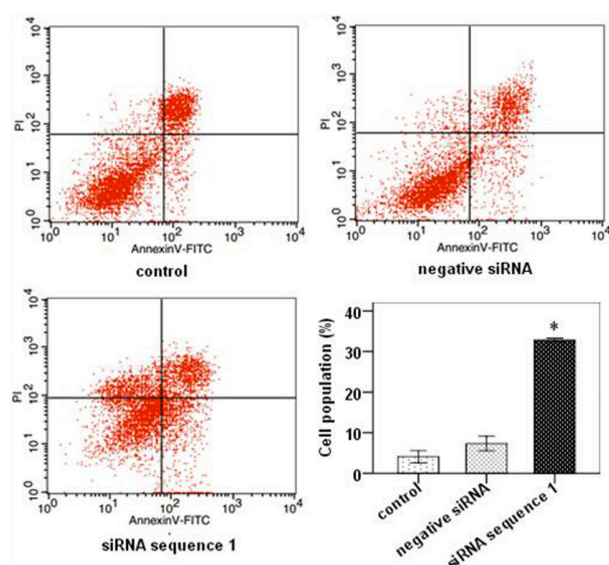
Figure 3. Effects of Down-regulation of FRα on Cell Apoptosis. Cell apoptosis of Hela cells silenced FRα in three groups were investigated by flow cytomery, analyzing their early cell apoptosis. Shown are mean values of triplicate assays ± SD (*p<0.05)
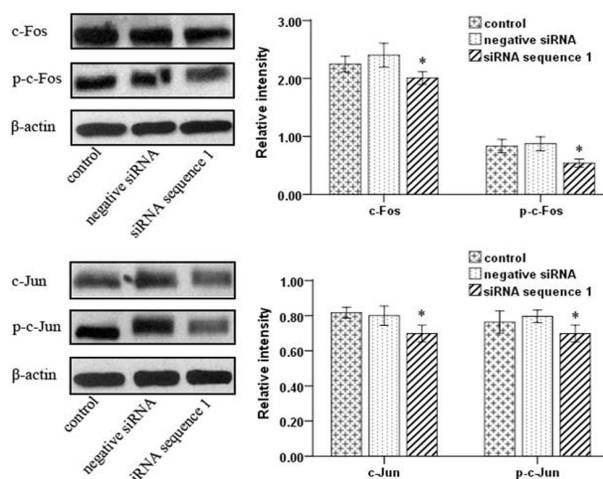

Figure 4. Changes of the Expression Levels of AP-1 Members. The expression of AP-1 members, including c-Fos, p-c-Fos, c-Jun and p-c-Jun, were tested by western blot after transfection with siRNA for 48 h. Shown are mean values of triplicate assays ± SD (*p<0.05)

that the expression levels of c-Fos, phosphorylated c-Fos (p-c-Fos), c-Jun and phosphorylated c-Jun (p-c-Jun) in siRNA sequence 1 group discreased compared with control and negative siRNA group (Figure 4).

Discussion

To explore therapeutic targets and look for molecular mechanisms for cancer has been increasingly focused by more and more scholars. FRα, as a single-chain GPI-anchored membrane protein, is high expressed in many kinds of carcinoma tissues, and correlated with tumor progression (Siu et al., 2012). In virtue of these characteristics, a variety of FRα targeted therapies have shown prospects in preclinical and clinical tests (Schmid-Bindert et al., 2013). Nevertheless, there was a little study on the effect of FRα in cervical cancer. In this study, we

analyzed the affection of the expression level of FRα on the proliferation and development of Hela cells.

Previous studies have showed that many cancer cell lines had a high expression of FRα (Jhaveri et al., 2004). Most epidemiologic researches reported that there was a negative correlation between folate intake and the development and progression of various tumors (Pericleous et al., 2013). Decreased folate intake might induce the development and progression of tumors. However, not all studies show the same results, recently a matched case-control study showed no correlation between folate intake and breast cancer (Liu et al., 2013). About cervical cancer, our previous study demonstrated that the growth of cervical cancer cells was suppressed with the folate concentration increasing (Ding et al., 2013). FRα, as a main carrier transporting folate by endocytosis, had been regulated by folate level, which had been reported in a vitro study (Sadasivan et al., 2002). Take account of the above findings, we deduced that increasing expression of FRα was a regulation mechanism in the development and progression of tumors. In our study, we demonstrated that Hela cells had high expression levels of FRα. However, silencing FRα by siRNA suppressed the cell proliferation, which might be involved in two regulatory mechanisms. On the one hand, decreasing folate intake resulting from the down-regulation of FRα might cause uracil incorporation into DNA and subsequent chromosome breakage (Crott et al., 2001), ultimately leading to proliferation suppression. On the other hand, the down-regulation of FRα might regulate the expression of some oncogenes, and then restrain the proliferation of Hela cells. Therefore, down-regulating the expression of FRα may be a strategic treatment for cervical carcinoma.

Folate palys an important role in the sythesis of DNA during the process of cell proliferation, so the changes of folate level may affect cell cycle and apoptosis. G1, S and G2/M transitions are the important checkpoionts during cell cycle progression. In the tumor development progression, to escape the cell cycle arrest is the most common phenomenon. Few studies have reported the role of FRα in cell cycle of cancer cells (Crott et al., 2008), but no previous study reported it in cervical carcinoma cells. In the different types of cancer cells, apoptosis can be induced through different signaling pathways, concluding caspase-mediated common pathway (Ray et al., 2008), mitochondria-mediated pathway (Jin et al., 2007), death receptor-mediated pathway (Pallepati et al., 2011), and regulation of apoptosis-related proteins (Cakir et al., 2011), and so on. In the present study, we found that down-regulating the expression of FRα made cell cycle of Hela cells to be arrested in G0/G1 stage, which was associated with the quiescence status of cells suffering from no stimulation and associated with the senescence of cells (Scicchitano et al., 1997), and promoted the apoptosis of Hela cells. Our results confirmed that down-regulating the expression of FRα decreased folate intake, restrained the sythesis of DNA, arrested the cell cycle in G0/G1 stage, and finally suppressed the proliferation of Hela cells.

AP-1 plays important role in cell proliferation and cell cycle regulation, which independently had been shown to develop carcinogenesis in a variety of tissues

(Milde-Langosch, 2005). The fact that a significant overexpression of constitutively active AP-1 family members in cervical precancer and cancer tissues had also been reported (Prusty et al., 2005). Inhibition of AP-1 was also accompanied by changes in the composition of their DNA-binding complex (Mahata et al., 2011). We further looked into the effect of down-regulated FRα on AP-1 family members in cervical cancer cells, HeLa harboring HR-HPV18 infection. Our results indicated that c-Fos and c-Jun expression levels were decreased in FRα silenced Hela cells, especially the phosphorylated proteins. These observations suggested that down-regulation of FRα might decrease the activation of AP-1 by suppressing the expression of c-Fos and c-Jun, and then restrain the proliferation of Hela cells.

In conclusion, down-regulating the expression of FRα in Hela cells could arrest the cell cycle in G0/G1 stage and promote apoptosis, and finally suppress the proliferation. Decresed c-Fos and c-Jun expression levels resulting from down-regulating the expression of FRα, which might be responsible for changes in gene expression of some stemness factors that be correlated with AP-1 gene expression levels (Apostolou et al., 2013). All of these findings indicated that decreasing FRα levels might be responsible for the inhibition of cancer cell proliferation, suggesting that FRα might be as a new target for the therapy of cervical cancer.

Acknowledgements

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