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Effect of FAK, DLC-1 gene expression on OVCAR-3 proliferation

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Abstract The study investigates the effect of FAK, DLC-1 on OVCAR-3 proliferation. FAK gene siRNA vector recombinant plasmid was constructed using RNA interference technique. FAK gene-transfected OVCAR-3 cells, OVCAR-3 cells with DLC-1 gene expression, and OVCAR-3 cells with simultaneous expression of DLC-1 and FAK genes were obtained using gene transfection technology. In addition, siRNA control group and blank control were also given. Effect of FAK, DLC-1 gene expression on OVCAR-3 proliferation was examined by FCM and Cell Counting Kit-8 (CCK-8) methods. Results showed that DLC-1 gene high expression and FAK gene silencing, single silencing FAK gene, and single DLC-1 gene high expression in OVCAR-3 cells may decrease S and G2/M phase proportion of the cell cycle. Moreover, DLC-1 gene high expression and FAK gene silencing in OVCAR-3 cells can display the most significant effect. This confirmed that DLC-1 gene high expression and FAK gene silencing may significantly inhibit the OVCAR-3 cells proliferation. CCK-8 analysis showed that silence FAK gene exprssion or/and increasing DLC-1 gene expression may decrease OVCAR-3 growth rate. Moreover, simultaneous silence the exprssion of FAK gene and high expression of DLC-1 gene can display the most significant effect on OVCAR-3 growth. It can be concluded that downregulation of FAK gene expression or/and upregulation of DLC-1 gene expression can all inhibit the OVCAR-3 growth. Moreover, DLC-1 gene expression and FAK gene silencing can display the most marked inhibitory effect on the OVCAR-3 growth.

Keywords FAK · DLC-1 · OVCAR-3 · Transfection · RNA interference · Cell proliferation · Cell cycle

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Introduction

Ovarian cancer is one of malignant tumors in female genital system, but is the leading cause of death from gynecological cancer in the world [1]. Due to its tendency to spread into the peritoneal cavity, the bowel, and the bladder and the relatively asymptomatic progression of ovarian cancer, the majority of cancer patients present with advanced disease and have a poor long-term prognosis. Ovarian cancer generally responds well to platinum and taxane-based therapies, but the incidence of recurrence is high, and oftentimes ovarian tumors become resistant to these therapies [2, 3]. As a result of these and other factors, there is a continued need for new modalities of treatment in order to decrease recurrence and improve the long-term prospects of survival for ovarian cancer patients. Despite improvements in the application of aggressive cytoreductive surgery and combination chemotherapy, ovarian cancer has the most unfavorable prognosis due to its insidious onset, diagnosis at late stage, dissemination, relapse, and tendency to develop chemotherapy resistance.

One of the many mechanisms for tumor cells to survive cell–matrix detachment during metastasis, i.e., avoid

anoikis, is by the overexpression of focal adhesion kinase (FAK), which has been shown in several tumor types [4–8]. These include cancers of the breast and ovary; and FAK amplification or overexpression is correlated with poor prognosis in these two diseases [9, 10]. Further, perturbation of FAK using genetic, RNA interference or dominant negative approaches attenuate tumor formation and metastasis in animal models of breast and ovarian cancer [10–12]. FAK also functions in angiogenesis and is overexpressed in endothelial cells isolated from ovarian cancers [13, 14].

The deleted in liver cancer (DLC-1) gene was isolated from a primary human hepatocellular carcinoma [15]. The human DLC-1 gene encodes a 1,091 amino acid protein that is highly homologous to the rat p122-RhoGAP [16, 17]. Rho family GTPases play important roles in the regulation of a variety of cellular processes including cell proliferation, gene expression, cytoskeletal organization, cell adhesion to extracellular matrix, and have been implicated in oncogenic transformation and cancer progression. Recent evidence has suggested that DLC-1 meets several criteria of a tumor suppressor gene. It is frequently inactivated due to genomic deletion or promoter hypermethylation in transformed cells, and its overexpression can result in the inhibition of in vitro colony formation, cell migration, and the suppression of tumor formation in immunocompromised mice [18, 19].

Here, we have studied the effect of FAK and DLC-1 gene expression on human ovarian carcinoma cells (OVCAR-3) growth and cycle.

Materials and method

Cell cycle analysis

Raw instrument files from method development were analyzed using FlowJo 7.5.3 to determine the percentage of cells in G2/M and positive for MPM2 [20]. The Watson (Pragmatic) model was used to compute the cell cycle data. Cellular aggregates and doublets were gated out by the FL-2 area versus FL-2 width discrimination. For the validation studies, analysis of MPM2 was consistent with method development, while cell cycle analysis was done using ModFit LT 3.2 by application of a diploid tetraploid model with apoptosis and auto debris options turned on and auto aggregates option turned off. Aggregates were excluded by FL-3 area versus FL-3 width discrimination. An example of the staining pattern for Draq5 and MPM2 is shown in Fig. 1. The mean, standard deviation (SD), and % coefficient of variation (%CV) were calculated using Excel 2003 (Microsoft). Simple ligand binding calculations were done with SigmaPlot 11.0.

Cell culture and treatments

Cells were kept in 10 ml dishes at 5 % CO_2 in Dulbecco's Modified Eagle Medium (1,000 mg/ml glucose, 110 mg/ml pyruvate, and 580 mg/ml glutamine) supplemented with 10 % fetal bovine serum, 1 % non-essential amino acids, 100 μ/ml penicillin, and 100 lg/ml streptomycin [21]. Cells were seeded in 96-well plates for treatments, except for the experiments using the culture system that was carried out in 12-well microplates with transwell inserts as described below. Treatments were carried out in the same standard incubation conditions, and were initiated by replacing the medium with fresh medium containing the hydrolysates at a concentration of 10 % (v/v).

Statistical analysis

Statistical analysis was carried out using SPSS version 10.0 for Windows software (SPSS, Chicago, IL, USA). Group differences were analyzed using parametric statistical methods, paired independent sample *t*-tests following one-way ANOVA. Results were presented as mean ± SD. $p < 0.05$ was considered statistically significant.

Results and discussion

Cell cycle analysis

Small interfering RNA (siRNA), sometimes known as short interfering RNA or silencing RNA, is a class of double-stranded RNA molecules, 20–25 nucleotides in length. siRNA plays many roles, but its most notable is in the RNA interference (RNAi) pathway, where it interferes with the expression of specific genes with complementary nucleotide sequence. siRNA also acts in RNAi-related pathways, e.g., as an antiviral mechanism or in shaping the chromatin structure of a genome. The complexity of these pathways is only now being elucidated [22].

FAK is a nonreceptor tyrosine kinase that is activated by integrin clustering. There are limited data regarding the functional role of FAK in ovarian cancer migration and invasion. As mentioned earlier, overexpression of FAK appears to promote the activation of NFκB and to enhance the expression of Xiap. Interestingly, FAK contains a consensus D-X-X-D caspase-3 cleavage site at amino acid 772. While caspase-3-mediated cleavage of FAK has been demonstrated in several studies, the exact consequences of this event are unclear. Cleavage of FAK at this site results in the separation of the C-terminal focal adhesion targeting sequence from the kinase domain. It is possible that this cleaved FAK may be ineffective in transmitting survival

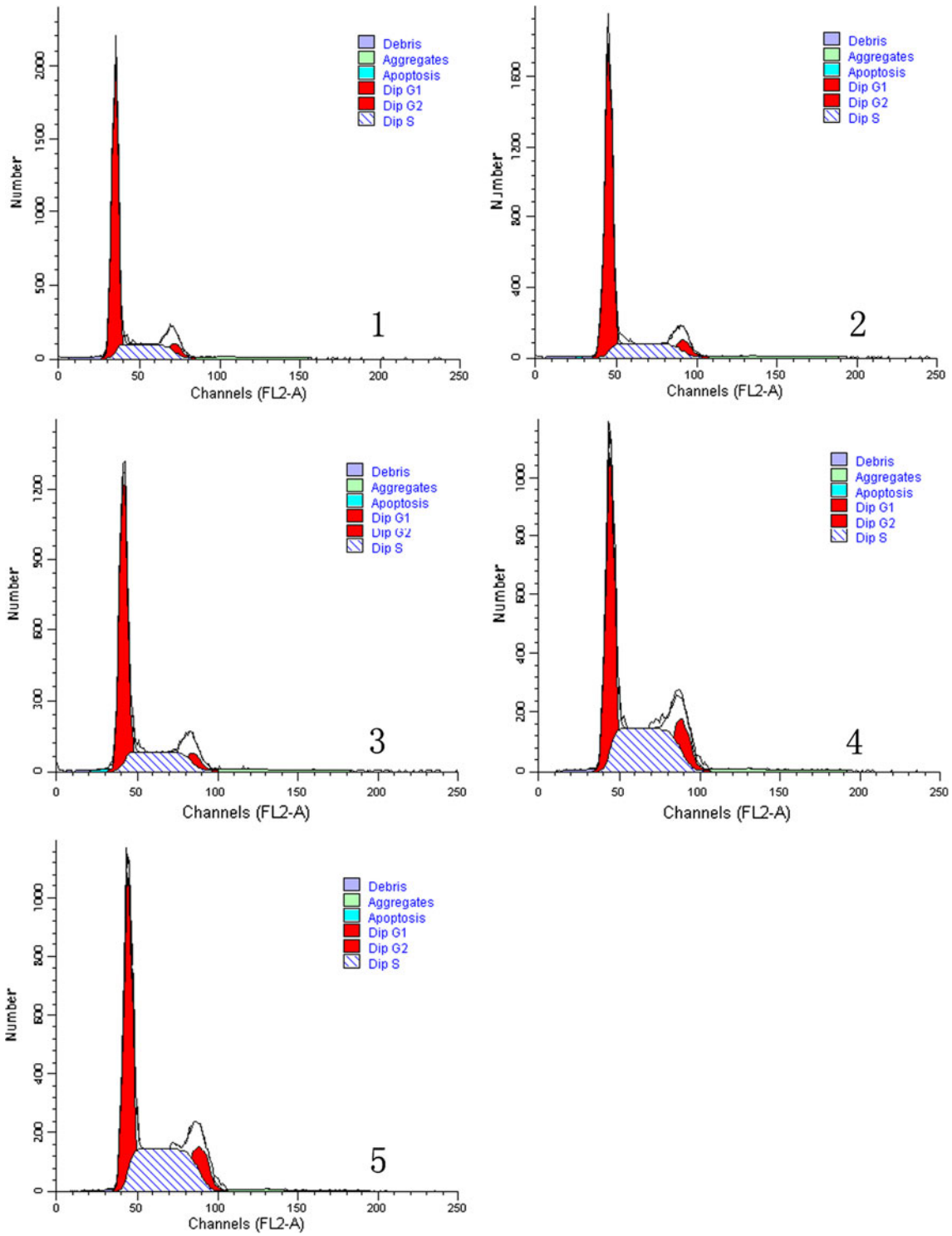

Fig. 1 Cell cycle proportional analysis in different groups. *1* Cell cycle proportional analysis in FAK+DLC-1 group, *2* cell cycle proportional analysis in FAK group, *3* cell cycle proportional analysis in DLC-1 group DLC-1, *4* cell cycle proportional analysis in siRNA group, *5* cell cycle proportional analysis in blank control group

signals, or may actively promote cell cycle arrest and/or apoptosis [23].

DLC-1 is considered as a kind of important tumor inhibitory factor, gene heterozygous lost or DNA methylation can cause DLC-1 expression decreasing or deletion in liver cancer, lung cancer, prostate cancer and nasopharyngeal carcinoma cancer tissues [24–26]. Research also shows that DLC-1 protein has RhoGAP activity, START structure

Table 1 Cell cycle proportional distribution in different groups

Group	*n*	G_0–G_1 (%)	S (%)	G_2–M (%)
DLC-1+FAK	5	71.22 ± 2.27*	22.15 ± 0.46*	6.63 ± 0.13*
FAK	5	70.04 ± 3.03*	21.38 ± 0.67*	8.58 ± 0.19*,#
DLC-1	5	64.47 ± 2.56*	25.72 ± 0.71*	9.81 ± 0.26*,#
siRNA control	5	46.27 ± 1.97	39.07 ± 0.79	14.66 ± 0.31
Blank control	5	48.09 ± 2.06	38.21 ± 0.86	13.7 ± 0.35

* $p < 0.01$, compared with siRNA control group or blank control group; # $p < 0.01$, compared with DLC-1+FAK group

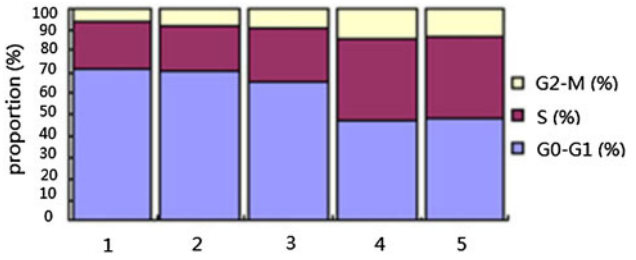

Fig. 2 Cell cycle proportional distribution in different groups

domain and elastin combined activity, its most important functions is to guide DLC-1 to stress fiber and focal adhesion [27–29], to maintain the cell shape and endow cells tenacity and strength, which affect the cytoskeleton building, cells movement and migration, DLC-1 is of great important to the tumor invasion and metastasis.

Cell cycle proportion in FAK+DLC-1 group, FAK group, DLC-1 group, siRNA control group and blank control group was presented in Table 1 and Fig. 1. There wasn't significant ($p > 0.05$) difference in S, G2/M and G0/G1 proportion between blank control group and siRNA group. Compared with two control groups, S, G2/M proportion in FAK+DLC-1 group, FAK group and DLC-1 group significantly ($p < 0.01$) decreased, and G0/G1 proportion ($p < 0.01$) increased. There was also significant ($p < 0.01$) difference in S, G2/M and G0/G1 proportion between FAK+DLC-1 group and FAK group or DLC-1 group. This indicated that both DLC-1 and silence FAK expression, DLC-1 expression, silence FAK expression may decrease S, G2/M proportion, and increase G0/G1 proportion. Moreover, the effect in DLC-1+FAK group is most significant (Fig. 2).

Cell growth curve

In our previous study, we examine expression of DLC-1 protein in normal ovarian tissue and in epithelial ovarian cancer tissues, and expression of p-FAK protein in normal ovarian tissue and in epithelial ovarian cancer tissues. In epithelial ovarian cancer tissues, strong positive expression of p-FAK proteins was detected. Cytoplasm in cancer cells showed tan or brown. In normal ovarian tissue, negative or weakly positive expression of p-FAK proteins was

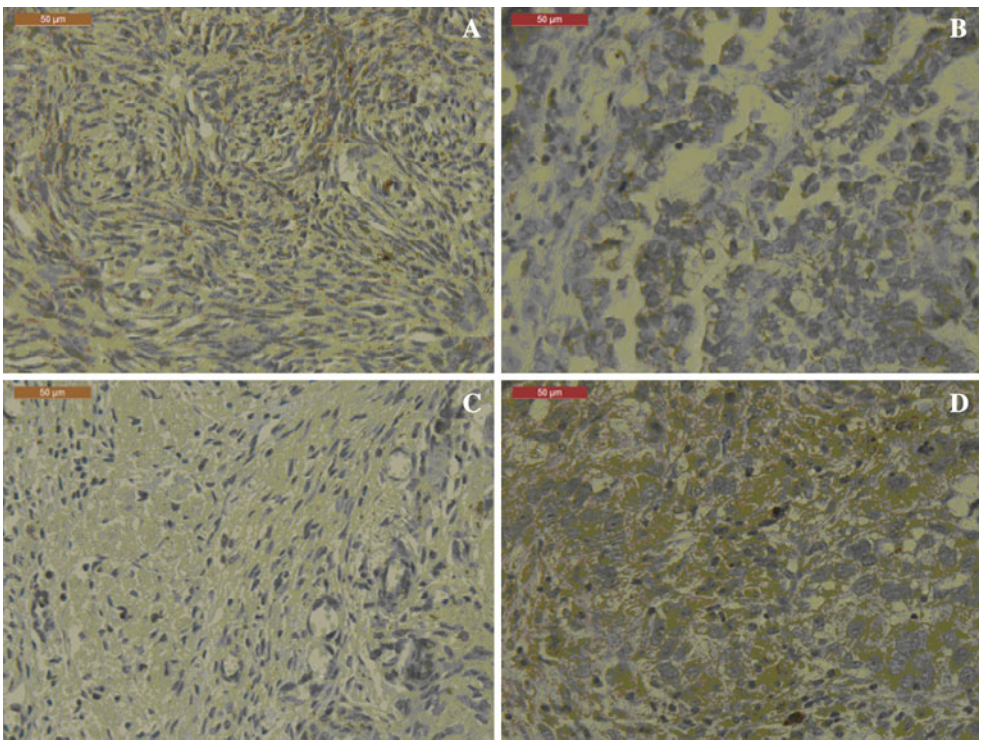

Fig. 3 Expression of DLC-1 protein in normal ovarian tissue (×400) (**a**) and in epithelial ovarian cancer tissues (×400) (**b**); Expression of p-FAK protein in normal ovarian tissue (×400) (**c**) and in epithelial ovarian cancer tissues (×400) (**d**)

Table 2 The absorbance value (A) at different time of groups

Time	FAK+DLC-1 group	FAK group	DLC-1 group	siRNA control group	Blank control group
1d	0.213 ± 0.018	0.231 ± 0.021	0.212 ± 0.019	0.205 ± 0.019	0.222 ± 0.018
2d	0.337 ± 0.041*	0.333 ± 0.037*	0.422 ± 0.038*	0.532 ± 0.058	0.551 ± 0.061
3d	0.525 ± 0.059*	0.571 ± 0.062*	0.624 ± 0.072*	0.804 ± 0.102	0.936 ± 0.11
4d	0.703 ± 0.082*	0.765 ± 0.085*	0.877 ± 0.089*	1.187 ± 0.099	1.207 ± 0.121
5d	0.737 ± 0.094*	0.844 ± 0.091*	0.983 ± 0.081*	1.423 ± 0.131	1.498 ± 0.117

* $p < 0.01$, compared with siRNA control group and blank control group

detected. Cytoplasm showed pale yellow, in normal ovarian tissue, strong positive expression of DLC-1 proteins was detected. Cytoplasm showed tan or brown (Fig. 3).

Inhibitory effects of DLC-1 on tumor cell migration were previously reported in human liver cancer cell lines [28]. In addition, Goodison et al. [30] reported that the restoration of DLC-1 in metastatic sublines of breast cancer cells resulted in the inhibition of migration and invasion in vitro and a significant reduction in the ability of forming pulmonary metastases in athymic mice. Previous studies demonstrated that in breast cancer, FAK gene is amplified and its protein is overexpressed [5, 31]. FAK is activated by multiple agonists and is implicated in differentiation, proliferation, apoptosis and in the invasive properties of cancer cells [6, 32–34]. In addition, FAK has been implicated in the regulation of cell migration, because FAK deficient cells migrate poorly in response to chemotactic and haptotactic signals [35–37].

In the present study, logarithmic growth phase cells in different groups were collected. Then, cells (5×10^3) were seeded in 96-well cell culture plate. Within 5 days of incubation, the absorbance value (A) in wells of different groups was determined using Cell Counting Kit-8 (Table 2). Cells growth curve of different groups was drawn in Fig. 4. Statistical analysis showed that there wasn't significant ($p > 0.05$) difference in the absorbance value (A) between blank control and siRNA control groups. Compared with two control groups, the absorbance value (A) in FAK+DLC-1 group, FAK group and DLC-1 group was markedly ($p < 0.01$) decreased at the 2nd, 3rd, 4th and 5th day. In addition, there was significant ($p < 0.01$) difference in the absorbance value (A) between FAK+DLC-1 group and FAK group or DLC-1 group. The expression of DLC-1 gene and/or silence the expression of FAK gene in OVCAR-3 cells may decrease OVCAR-3 cells growth. Moreover, the effect in silence FAK gene simultaneous high expression of DLC-1 gene group was the most significant (Table 2).

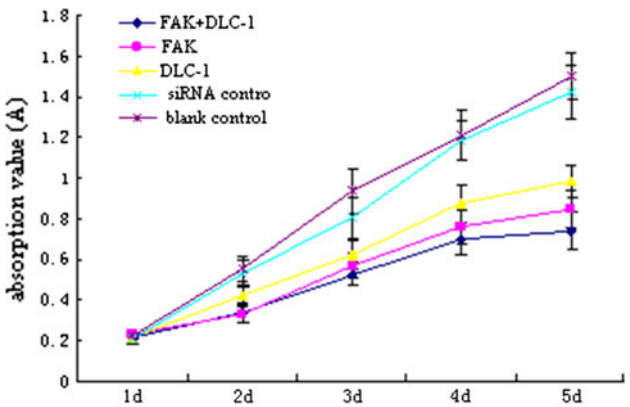

Fig. 4 Cells growth in different groups

Conclusion

The present study demonstrated that FAK and DLC-1 play a crucial role in the arrest of cell proliferation of human OVCAR-3 by regulating the expression of FAK, and DLC-1. Therefore, FAK and DLC-1 may be a potential tumor suppressor gene and could be a potentially effective target gene for preventing the growth and invasion of ovarian cancer cells.

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