

Original Article**FHL2 Antagonizes Id1-Promoted Proliferation and Invasive Capacity of Human MCF-7 Breast Cancer Cells**

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ABSTRACT

Objective: FHL2 was previously identified to be a novel interacting factor of Id family proteins. The aim of this study was to investigate, the effects of FHL2 on Id1-mediated transcriptional regulation activity and its oncogenic activity in human breast cancer cells.

Methods: Cell transfection was performed by Superfect reagent. Id1 stably overexpressed MCF-7 cells was cloned by G418 screening. The protein level of Id1 was detected by western blot analysis. Dual relative luciferase assays were used to measure the effect of E47-mediated transcriptional activity in MCF-7 human breast cancer cells. MTT assay was used to measure cell proliferation. Transwell assay was used to measure the invasive capacity of MCF-7 cancer cells.

Results: The basic helix-loop-helix (bHLH) factor E47-mediated transcription activity was markedly repressed by Id1 in MCF-7 cells. This Id1-mediated repression was effectively antagonized by FHL2 transduction. Overexpression of Id1 markedly promoted the proliferation rate and invasive capacity of MCF-7 cells; however, these effects induced by Id1 were significantly suppressed by overexpression of FHL2 in cells.

Conclusion: FHL2 can inhibit the proliferation and invasiveness of human breast cancer cells by repressing the functional activity of Id1. These findings provide the basis for further investigating the functional roles of FHL2-Id1 signaling in the carcinogenesis and development of human breast cancer.

Key words: FHL2; Id1; Repressor; MCF-7; Proliferation; Invasiveness

INTRODUCTION

Inhibitor of differentiation (Id) proteins belong to helix-loop-helix (HLH) transcription factors. They do not bind to DNA because of lack of a DNA binding domain, but they form heterodimers with basic HLH factors, such as E proteins (E47, E2-2 and HEB), and inactivate their functions by inhibiting their binding to DNA^[1, 2]. Id proteins are key regulators in

differentiation, cell cycle control, and cell lineage commitment in both vertebrates and invertebrates^[3, 4]. They display overlapping but distinct expression patterns in a variety cell types and tissues. Elevated levels of Id proteins have been reported in diverse human tumor types^[5, 6]. Id1 is frequently overexpressed in a subset of human breast cancers and the high level of Id1 is associated with disease severity and poor prognosis^[7, 8]. By fueling several key features of tumor progression, including deregulated proliferation, invasiveness, angiogenesis and metastasis, Id1 contributes to multiple steps of tumorigenesis.

The LIM-only protein FHL2 (four-and-a-half- LIM-only protein 2) interacts with a

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broad variety of transcription factors, and its expression is often deregulated in various types of cancers^[9, 10]. The function of FHL2 in cancer is particularly intriguing, since it may act as an oncoprotein or as a tumor suppressor in a tissue-dependent fashion. The dual nature of FHL2 is reflected by the finding that it functions as an activator or a repressor of its interacting transcription factors depending on the cell type. FHL2 is overexpressed in almost all human mammary carcinoma samples but not in normal breast tissues, and only low levels of FHL2 expression are present in premalignant ductal carcinoma in situ (DCIS)^[11, 12]. So far, the FHL2 function in breast cancer cells is still unclear.

In our previous study, we demonstrated that FHL2 is a novel interacting factor of Id protein family, including Id1^[13]. By interacting with Id2, FHL2 significantly repressed its oncogenic activity in human neuroblastoma cells^[13]. In this study, we revealed that FHL2 can effectively repress Id1-promoted cell proliferation and invasion of human MCF-7 breast cancer cells. These data suggest that FHL2 may repress the progression of human breast cancer cells by antagonizing the oncogenic activity of Id1.

MATERIALS AND METHODS

Plasmids

Human E47 gene expression vector pcDNA3-E47 and 5×E-Box-driven luciferase reporter plasmid 5×E-Box-Luc were kindly provided by Prof Iavarone from Columbia University Medical Center. The full-length human Id1 was amplified by PCR from human endometrium cDNA library and was subsequently cloned into pcDNA3.1 expression vector through *Bam*H I/*Eco*R I digestion sites as described previously^[13]. The pRL-SV40 reporter vector was purchased from Promega (Madison, WI). The FHL2 expression vector pcDNA3.1-FHL2 was described previously^[13].

Cell Culture and Transfection

Human MCF-7 breast cancer cells and human embryonic kidney 293T cells were originally obtained from American Type Culture Collection (ATCC, Rockville, MD, USA) and were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10%–15% FBS (Hyclone, UT), 100 U/ml

penicillin G and 100 g/ml streptomycin in a humid atmosphere with 5% CO₂ at 37°C. For MCF-7 cell culture, 0.01 mg/ml bovine insulin (Invitrogen) was supplemented.

MCF-7 cells were seeded in 60-mm culture dishes before transfection. When the cell confluence reached to 40%–60%, the cells were stably transfected with 5 µg pcDNA3.1-Id1 by use of the Superfect transfection reagent (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The empty vector was used as the negative control. Two days post-transfection, the MCF-7 cells were treated with 800 µg/ml G418 (Gibco, NY) for 10–14 d and then were continuously cultured with 200 µg/ml G418.

Luciferase Assays

Cells at 50% confluence in 35-mm dishes were transfected by use of Superfect reagent. An amount of 0.4 µg of the luciferase reporter construct 5×E-box-Luc was cotransfected with 0.4 µg of pcDNA3-E47, pcDNA3.1-Id1 and/or its mutant construct pcDNA3.1-Id1-del and/or 0.2–0.4 µg of pcDNA3.1-FHL2. pRL-SV40 was used as an inner control (1 ng/well). Cell extracts were prepared 42 h after transfection, and the luciferase activity was measured by the Dual-Luciferase Reporter Assay System (Promega) as described previously^[14]. All experiments were performed in triplicate and repeated three times.

Cell Proliferation Assay

For cell proliferation assay, cells were plated onto 24-well plates at 1×10^4 cells/well in culture medium supplemented with 10% FBS. After incubation for 12 h, the medium was replaced with fresh medium containing 1% FBS and 200 g/ml G418. The medium was changed every 2 d. After washing with PBS two times, the cells were trypsinized and then counted by the Trypan Blue exclusion method using a hemocytometer every 24 h for 7 d. Each experiment was performed in triplicate and repeated on three occasions.

Transwell Assays (Boyden Chamber Invasion Assays)

Invasion assays were carried out in modified Boyden chambers with 8 µm pore filter inserts for 24-well plates (BD Transduction) as

described^[15]. Briefly, the surfaces of the filters were coated with 15 μ l ice-cold Matrigel (15 mg/ml protein; BD Transduction) for 60 min at room temperature. Uniformity of the coating was checked by coomassie blue staining and low-power microscopy observation. The lower chamber was filled with medium containing 10% serum. Fibronectin (16 μ g/chamber) was added as the chemoattractant to the lower chamber. Cells (1×10^5 cells/well) were washed with $1 \times$ PBS twice, re-suspended in 200 μ l of serum-free medium and then transferred into the upper chamber. After 24 h of incubation, the filter was gently removed from the chamber, the cells on the upper surface were removed by wiping with a cotton swab, and cells that had invaded to the lower surface areas were fixed, stained with hematoxylin and eosin (H&E) and counted in 15 randomly selected fields on microscope ($\times 100$). Experiments were performed independently at least three times.

Western Blot

The expression of Id1 and β -actin proteins was examined by Western blot analysis as described previously^[14]. Antibodies were polyclonal rabbit anti-Id1 (1:400) and anti- β -actin (Santa Cruz Biotechnology). Blots were probed with the primary antibodies, washed and then incubated with horseradish peroxidase-labeled secondary antibodies (Santa Cruz Biotechnology), and the binding was detected using enhanced chemiluminescence.

Statistical Analysis

Experiments were performed in triplicate and repeated at least thrice, and the results were expressed as the $\bar{x} \pm s_{\bar{x}}$. Statistical analysis was performed using Chess software. Paired Student's t-tests or two-way ANOVA followed by the Student-Newman-Keuls test were used where applicable to assess significant differences between groups. $P < 0.05$ was considered statistically significant.

RESULTS

FHL2 Antagonizes the Inhibition of E-box-mediated Transcription by Id1, but not by Id1-del

Previously, we demonstrated the physical

interactions of FHL2 with the four members of Id protein family^[13]. Further mutation analysis revealed that this interaction is mediated by the conserved N-terminal region of Ids^[13]. To further explore the possible significance of the observed FHL2-Id1 interaction, we performed luciferase reporter assays with five multimerized E-boxes driving the expression of luciferase (5 \times E-box-Luc). Transfection of MCF-7 cells with reporter alone or reporter plus FHL2 expression vector revealed background luciferase activity (Figure 1A, Lane 1 and 2). Cotransfection of E-box-Luc plus E47 significantly activated the system in cells (Figure 1A, Lane 3). Given that E47/Id1 heterodimers lose the ability to bind to the E-box, Id1 cotransfection resulted in a significant repression of the E47-activated luciferase activity (Figure 1A, Lane 5); however, this repression was markedly antagonized by FHL2 in a dose-dependent manner (Figure 1A, Lanes 6–8). FHL2 slightly increased E47 activation of the reporter (Figure 1A, Lane 4), which might be due to FHL2 inhibition of the endogenous Id activities. Similar results were obtained in 293T cells (Figure 1B), which suggests that the inhibitory effect of FHL2 on Id1 activity in this assay is independent of other tissue-specific cofactors. To further confirm whether this repression was mediated by the direct interaction of FHL2 and Id1, we used the Id1-del expression vector, an 11-aa deleted (from 34 to 44 aa) Id1 mutant that cannot interact with FHL2 as described previously. As expected, Id1-del also significantly inhibited E47-mediated reporter activity as did the wild-type Id1 (Figure 1C, Lane 6); however, unlike Id1 repression (Figure 1C, Lane 4), Id1-del repression was not antagonized by FHL2 (Figure 1C, Lane 7). Taken together, these data strongly indicate that FHL2 can potently repress Id1 activity by directly associating with it, thus restoring the transcription programme driven by E47.

FHL2 Suppresses Id1-promoted Proliferation of MCF-7 Cells

Previous data suggested that the expression level of Id1 was positively correlated with the proliferation rate of human breast cancer cells^[7]. To investigate whether the FHL2-Id1 association can repress Id1-promoted cell proliferation, we stably transfected Id1 into MCF-7 cells and performed cell proliferation assays. As shown in

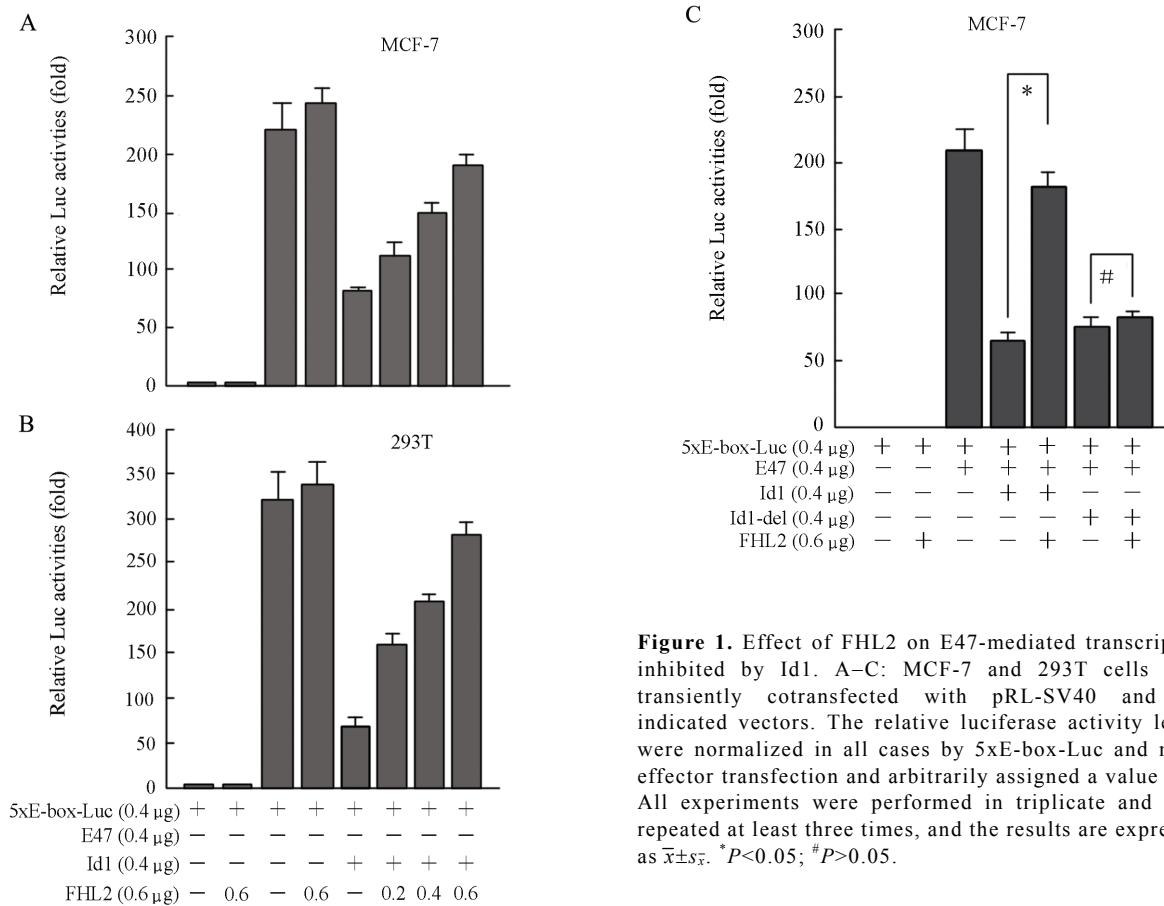


Figure 1. Effect of FHL2 on E47-mediated transcription inhibited by Id1. A–C: MCF-7 and 293T cells were transiently cotransfected with pRL-SV40 and the indicated vectors. The relative luciferase activity levels were normalized in all cases by 5xE-box-Luc and mock effector transfection and arbitrarily assigned a value of 1. All experiments were performed in triplicate and were repeated at least three times, and the results are expressed as $\bar{x} \pm s_{\bar{x}}$. * $P < 0.05$; # $P > 0.05$.

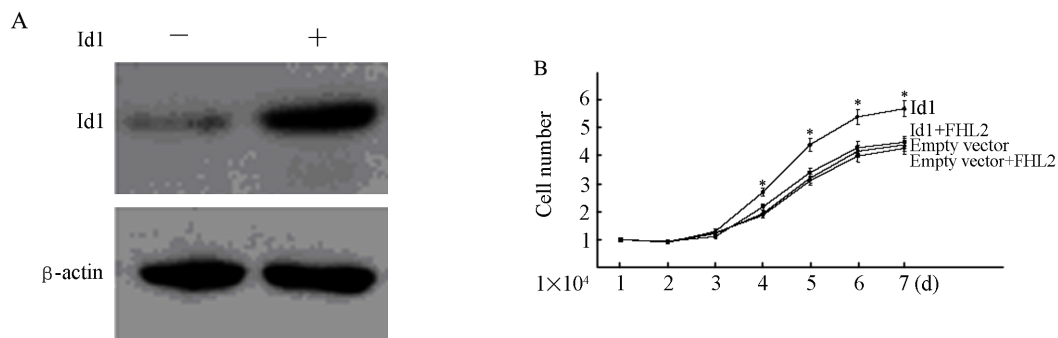


Figure 2. Effect of FHL2 on Id1-promoted cell proliferation. A: MCF-7 cells were stably transfected with Id1 expression vector or empty vector and were screened by G418. Cell lysates were extracted and were subjected to immunoblotting analysis. β -actin was used as the loading control. B: MCF-7 cells stably expressing ectopic Id1 or empty vector were transiently transfected with either FHL2 or empty vector. Cell proliferation assays were performed as described in “Material and Methods”. All experiments were performed in triplicate and were repeated at least three times, and the results are expressed as $\bar{x} \pm s_{\bar{x}}$. * $P < 0.05$.

Figure 2A, transfection of pcDNA3.1-Id1 significantly increased the expression level of Id1 protein in cells compared with the mock

transfection. Overexpression of Id1 markedly promoted cell proliferation (Figure 2B). On the fourth day of observation, the number of cells

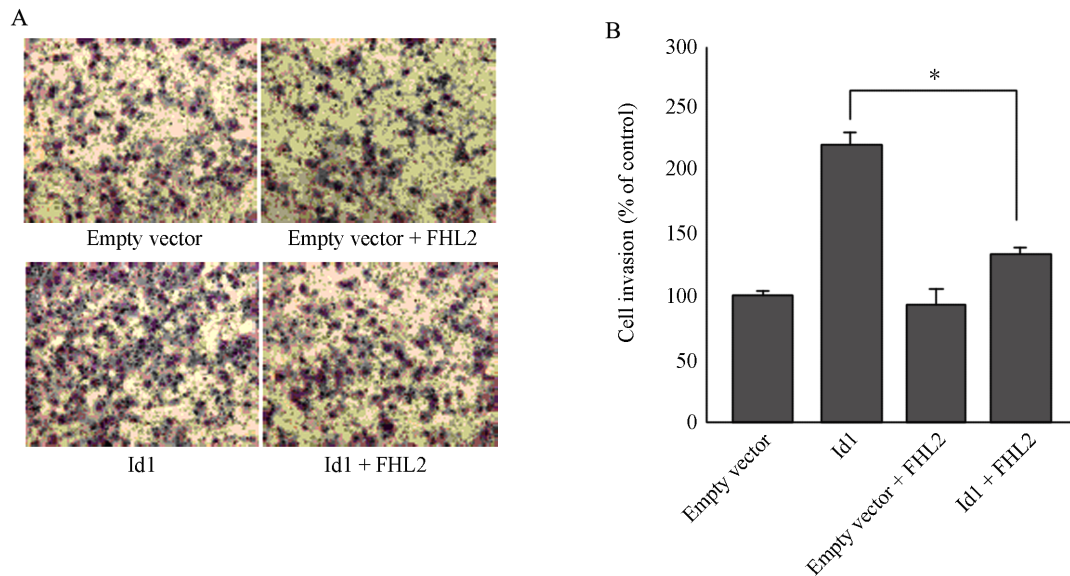


Figure 3. Effect of FHL2 on Id1-promoted cell invasion. A: MCF-7 cells stably expressing ectopic Id1 or empty vector were transiently transfected with either FHL2 or empty vector. Transwell assays were performed as described in “Materials and Methods”. This panel shows the representative pictures. B: As described in (A), each experiment was performed in triplicate and was repeated three times. Data are expressed as the percentage of the control cells ($\bar{x} \pm s_{\bar{x}}$, the bottom panel). * $P < 0.05$.

transfected with Id1 was about 1.5-fold greater than the mock-transfected cells. Transiently transfection of FHL2 into mock-transfected cells appeared to slightly inhibit cell proliferation, but no significant difference was observed. However, transfection of FHL2 in Id1-overexpressed cells significantly inhibited cell proliferation rate. These data demonstrated that Id1-promoted cell proliferation can be suppressed by FHL2.

FHL2 Suppresses Id1-enhanced invasiveness of MCF-7 Cells

Accumulated evidence demonstrated that Id1 overexpression is positively linked to invasive growth of primary human breast cancer cells and is a key initiating factor of the enhanced invasive phenotype of human breast cancer cell lines^[7, 8]. To investigate whether FHL2 is an effective factor to antagonize Id1-enhanced cell invasion, a modified Boyden chamber assay (Transwell assay) was carried out to determine the ability of cells to invade through biological matrices *in vitro*. The relevance of this assay for cell invasion and for *in vivo* malignancy has been documented

extensively^[16]. MCF-7 is a poorly invasive cell line, but its invasion potential moderately increased in the presence of fibronectin as the chemoattractant^[15]. The cells with or without ectopic Id1 expression were equally seeded in the upper chamber, whereas, when the number of cells that invaded the Matrigel and attached to the lower filter was calculated, the invasion number of cells with ectopic Id1 expression was more than that of the control cells (Figure 3A). As shown in Figure 3B, an increase of about 2.3 fold in the invasion number of MCF-7 cells was repeatedly observed. Transfection of FHL2 in mock-transfected cells did not significantly alter the invasion number. However, ectopic FHL2 expression in Id1-overexpressed cells markedly attenuated Id1-enhanced cell invasion effect (Figure 3A and 3B). These results suggested that FHL2 can effectively antagonize Id1-enhanced cell invasion through associating with it.

DISCUSSION

The biological function of Id proteins is achieved through antagonizing the activity of their associated bHLH transcription factors^[1-4].

Although the molecular mechanism involved in this process has been well dissected, additional regulators should work in concert with Ids to modulate Id-controlled transcription programme and cellular behavior. FHL2 was previously identified as a novel interactor of the four Id protein members^[13]. In this study, we show that FHL2 can effectively repress the inhibitory effect of Id1 on E47-mediated transcription by cotransfection assays, demonstrating that FHL2 is a functional repressor of Id1. Furthermore, we show that FHL2 can also effectively antagonize Id1-promoted proliferation and invasion of human breast cancer MCF-7 cells. These findings underscore an important function of FHL2 in regulating Id1 signaling pathway and suggest that FHL2 may act as a tumor suppressor in human breast cancer cells.

Our previous data demonstrated that FHL2 can physically interact with all members of Id protein family including Id1^[13]. Ectopic FHL2 expression in neuroblastoma cells markedly reduces the transcriptional and cell cycle promoting functions of Id2. FHL2 was revealed to be an important repressor of the oncogenic activity in human neuroblastoma cells. Consistent with the observation that FHL2 can effectively repress Id2-mediated transcription activity in multiple cell types^[13], in this study, we demonstrated that FHL2 can also effectively repress Id1-mediated transcription programme in MCF-7 and 293T cells (Figure 1). This repression was blocked by Id1-del mutant, which can not associate with FHL2. These data further suggest that the FHL2 inhibition of Id1 transcription activity is caused by the formation of FHL2-Id1 complex.

Id proteins have differential function activities in different cell types^[5, 6]. Id1 was suggested to be the only member of the Id family the expression of which correlated with the aggressive phenotype in several human breast cancer cells including estrogen sensitive and insensitive cell lines^[7]. Consistent with the previous reports^[7], Id1 promoted proliferation and invasive growth of human breast cancer MCF-7 cells. As a non-selective functional repressor of Id protein family, in this study we demonstrated that FHL2 can effectively antagonize Id1-promoted cell proliferation and invasive phenotype. Our data suggest that FHL2 may act as an oncogenic repressor in Id1-positive human breast cancer cells.

Expression of Id1 in breast cancer cells has been increasingly recognized to be an important

marker to value tumor progression and disease prognosis^[6-8]. We first identified that FHL2 is a potent repressor of oncogenic activities of Id1 in breast cancer cells. These findings provide basis for further investigating the functional roles of FHL2-Id1 signaling in the carcinogenesis and development of human breast cancer. Testing FHL2 and Id1 expressions in human breast cancer cells might be beneficial to differentiate different clinopathological subtypes of human breast cancers.

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