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Original Research

Endophilin B1 regulates EGFR endocytic degradation in prostate cancer cell

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Abstract: Prostate cancer (Pca) is one of the most common types of cancer for elder men. Aberrant expression of epidermal growth factor receptor (EGFR) and EGFR downstream signaling have been known to contribute to disease progression in prostate cancer. EGF-stimulated EGFR is internalized and the process of endocytic degradation of EGFR mediates its signaling which is frequently dysregulated in many kinds of cancer. In the present study, we demonstrated that endophilin B1 expression was inhibited and EGFR expression was significantly increased in prostate cancer cell lines. We demonstrated that suppression of endophilin B1 increased EGFR levels via delaying EGFR internalization triggered by EGF and its intracellular degradation. Endophilin B1 decreased also sustained EGFR downstream signaling such as Erk1/2 phosphorylation in response to EGF stimulation and promoted prostate cancer cell proliferation which is EGF independent. Our data indicated that endophilin B1 mediated the biological function of EGFR in cancer cell proliferation through regulating the EGFR endocytic trafficking and downstream signaling.

Key words: Epidermal growth factor receptor (EGFR), endophilin B1, prostate cancer, intracellular trafficking.

Introduction

Prostate cancer (PCa) is one of the most common types of cancer in men above the age of 55 years, and unfortunately, only locally advanced and low-grade PCa could be treated. For the vast majority early stage patients respond to androgen deprivation, but most patients will develop androgen-independent prostate cancer (AIPC), and there is still no specific treatments for AIPC. Although the mechanism of the processes of transition to androgen-independent prostate cancer remains undetermined, remarkable advances have been made in the field of prostate cancer research in recent decades.

The epidermal growth factor receptor (EGFR) belongs to the family of ErbB receptor tyrosine kinase (RTK). EGFR signaling regulates key cellular programs, including proliferation, differentiation and motility in different cell types (1). EGFR is initially activated at the cell membrane surface, activated EGFR is internalized and rapidly transferred to early endosomes which are also called as sorting endosomes and eventual delivered to lysosomes (2). Since degradation in lysosomes will terminate receptor signaling, EGFR signaling will be enhanced when receptor internalization and degradation be inhibited.

Endophilin B1 was initially identified as a Bax-binding protein, therefore it is also called Bif-1 (Bax-interacting factor-1) or SH3GLB1 (3), which is mainly localized in cytoplasm and intracellular membranes, and little is found in the nucleus (4,5). Aberration of endophilin B1 function has been found in neurodegenerative diseases and cancers. In addition to its function in Bax related apoptosis, many studies also showed that endophilin B1 plays important roles in regulation of membrane dynamics and autophagy (6,7). It has been found that there is an amino-terminal N-BAR domain at the N-terminus of endophilin B1 and a carboxy-terminal SH3 domain at the C-terminus, which has the membrane binding activities (8,9). On the basis of intracellular localization and its lipid binding and bending activity, we could speculate that endophilin B1 may also plays an important role in receptor endocytic trafficking in cells.

In the current study, we here report a novel function of endophilin B1 in mediating EGF induced cell proliferation and EGFR endocytosis. We observed that suppression of endophilin B1 increased cell proliferation and inhibited cell response to EGF stimulation. Furthermore, we found that suppression of endophilin B1 delayed EGFR degradation and maintained EGFR downstream signal for a longer time. In addition, we also found that cell surface EGFR level will maintain a higher level when endophilin B1 was knockdown. Taken together, our results suggested that endophilin B1 played positive roles in EGFR trafficking and signaling pathway.

Materials and Methods

Materials

DMEM, RPMI 1640 culture medium,Fetal bovine serum (FBS), penicillin and trypsin-EDTA were pur-

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chased from GIBCO. EGF was from PeproTech. Antibodies recognizing EGFR, phospho-Erk1/2, Erk1/2, and β-actin were purchased from Cell Signaling Technology. Monoclonal mouse anti-human phospho-EGFR (Y1173) antibody was purchased from Upstate Biotechnology. Infrared-labeled secondary antibody (Goat anti-rabbit IR Dye@680CW, Goat anti-mouse IR Dye @800CW) were purchased from LI-COR. Small interfering RNAs (siRNAs) oligos were obtained from Invitrogen. Sequence of the siRNA target endophilin B1 is as follows: 5′- CACAGUGUUACCAGUAUAUTT-3′.

Cell culture and transfection

The human prostate cancer cell line (LNCaP and DU145) originally purchased from American Type Culture Collection (ATCC). Cells were routinely maintained in the regular culture medium as described by ATCC. Briefly, LNCaP cells and DU145 cells were grown in RPMI 1640 medium and DMEM medium respectively, all medium supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C in 5% CO_2 atmosphere. In gene knockdown experiments, siRNAs were transfected into the cells using the lipofectamine® RNAiMAX (Invitrogen). In experiments to assess protein phosphorylation, all treatments were performed in serum-free medium following a 16-hour period of serum starvation.

Cell Proliferation analysis

Cell proliferation was evaluated by MTT assay. Briefly, cells (2000 cells/well) with transfection of specific endophilin B1 siRNA were seeded in 96-well plates for 24 hours and subsequently subjected to EGF (10 ng/ml) stimulation. At the end of different time point, MTT solution was added to culture medium (20 μl/well) and incubate for 3 hours, subsequently solubilized in dimethyl sulfoxide (100 μl/well), cell proliferation was evaluated by measuring absorbance at 570 nm using 96-well plate reader.

Quantitative Real-Time PCR (qPCR)

RNA extraction, cDNA synthesis and qPCR were performed as described before (10). β-actin mRNA was used for normalization. Primer sequences are listed in Table 1.

Immunoblotting and Signaling analysis

Cells were grown to confluency followed by incubation with EGF (50 ng/ml) at 37°C for different time courses (5, 15, 30 and 60 min). And then cells were harvested by scraping and were subsequently lysed in cold RIPA buffer containing protease inhibitors. The phosphatase inhibitors (NaF and Na_3VO_4) were added to the RIPA buffer for detecting phosphorylation of protein. Protein sample was separated by 10% SDS-PAGE and was electro-transfered to a nitrocellulose membrane. After 2 hours blocking with 5% BSA at room temperature, the membrane was incubated with primary antibody overnight at 4ºC. After washing with TBST buffer, the membrane was incubated with the Infrared-labeled secondary antibody for 2 hours at room temperature, followed by washing with TBST buffer, the specific protein bands were visualized and scanned by LI-COR's Odyssey Infrared Image System.

Table 1. Primers sequences.

Primer	Nucleotide sequence
EGFR-F	CTTTCGATACCCAGGACCAA
EGFR-R	ACTTCCTGGCTAGTCGGTGT
EndoB1-F	GCCGTGCAGTTCACAGAAGA
EndoB1-R	TGGCATTTGGATTTGGCTGC
β-actin-F	CTGGAACGGTGAAGGTGACA
β-actin-R	AAGGGACTTCCTGTAACAATGCA

Cell surface biotinylation

Cell surface EGFR levels were determined by biotinylation assay. Briefly, Cells were grown to confluency followed by incubation with EGF (50 ng/ml) at 37°C for different times (5, 15, 30 and 60 min). Afterwards, receptor trafficking was arrested by cooling cells on ice. After washing with cold PBS, cells were treated with Sulfo-NHS-SS-biotin (500 μg/ml in PBS) which is reversible and membrane-impermeable cross-linker for 1 hour on ice. And then the quenching buffer (glycine, 100 mM in PBS) was added to stop the reaction. Cells were lysed with cold RIPA buffer, and immobilized Streptavidin-beads (Pierce) were used to precipitate the biotinylated proteins. The amount of biotinylated surface EGFR was quantified by immunoblotting as described above. The experiments were repeated for 3 times with the same results.

Biotinylation based internalization assay

We quantify EGFR internalization by cell surface biotinylation assay. Briefly, cells grown to confluency, pre-treated with lysosomal blocker NH_4CL (50 mM) for 2 hr. Subsequently, cells were surface-biotinylated with Sulfo-NHS-SS-biotin (500 μg/ml in PBS) for 1 hour on ice. Then the reaction was stopped on ice by quenching buffer (glycine, 100 mM in PBS), after washing with cold PBS, cells were incubated in pre-warmed medium supplemented with EGF (50 ng/ml) at 37°C for different period (5, 15, 30 and 60 min) to trigger the endocytosis of biotinylated surface EGFR. Cells were cooled on ice, the remaining biotinylated surface proteins were cleaved of biotin-tag by incubating with the reducing buffer (DTT, 50 mM in PBS). Meanwhile, internalized biotinylated proteins were protected from biotin-striping. Cells were lysed with cold RIPA buffer, these biotinylated proteins were precipitated with immobilized Streptavidin-beads (Pierce). The amount of internalized EGFR was quantified by immunoblotting. These experiments were repeated for 3 times with the same results.

Statistical analysis

All data were expressed as mean ± SD unless otherwise noted. Student *t-test* was used to evaluate the significance difference of control group and treatment group. A value of $P < 0.05$ was considered to be statistically significant.

Results

The abnormal expression of Endophilin B1 and EGFR in prostate cancer cell lines

EGFR has been found over-expressed in most kinds of tumors of epithelial origin (11) and decreased endophilin B1 expression was also found in many kinds of

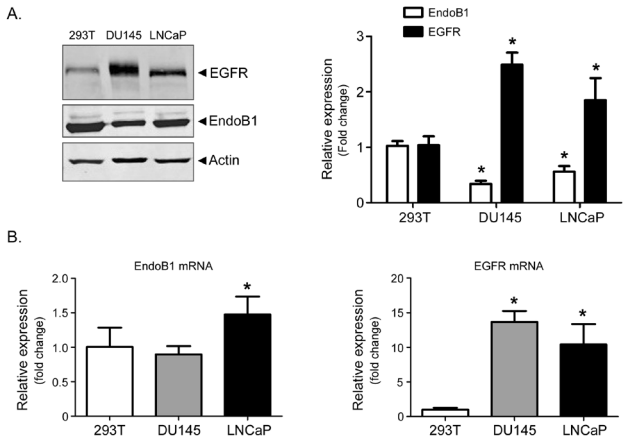

Figure 1. EGFR and endophilin B1 expression in prostate cancer cells. Differentially expressed genes in protein (**A**) and mRNA levels (**B**) in prostate cancer cell lines (DU145, LNCaP). Data are presented as means ± SD of 6 independent experiments. Asterisks indicate significant difference ($P < 0.05$) compared with the control cell line - 293T.

cancer (4). Firstly, we examined the expression levels of EGFR and endophilin B1 in prostate cancer cell lines (DU145 and LNCaP) compared with 293T cells which was set as normal cell control. EGFR levels on protein level in prostate cancer cells (DU145 and LNCaP) showed 1.7-2.3 fold increased levels compared with that in 293T cells, whereas endophilin B1 showed approximately significantly decreased protein levels (Fig. 1A). Meanwhile, the data from qRT-PCR also showed the same trend in both EGFR and endophilin B1 mRNA levels (Fig. 1B).

Silencing of endophilin B1 induces the increased expression of EGFR

To investigate the effects of endophilin B1 on EGFR expression and signaling, we knocked down endophilin B1 in LNCaP cells with specific siRNA. As shown by immunoblotting (Fig. 2), endophilin B1 in LNCaP cells was successfully declined by more than 70% in both protein and mRNA levels. Meanwhile, with the down-regulation of endophilin B1, we also found the slightly increase of EGFR (Fig. 2).

Suppression of endophilin B1 delays EGFR degradation and downstream signaling

As described above, knockdown of endophilin B1 enhance EGFR level in prostate cancer cells. To further explore how endophilin B1 plays its roles in LNCaP cells, we examined whether endophilin B1 affected

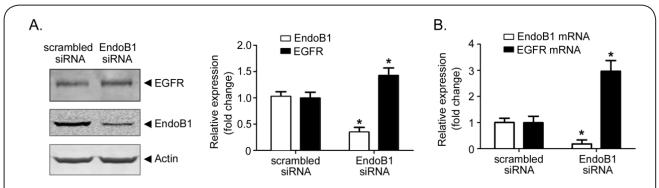

Figure 2. Knockdown of endophilin B1 induces EGFR expression increase. A. Immunoblot analysis shows knockdown of Endophilin B1 and induced EGFR expression in protein level. **B,** qRT-PCR analysis shows significant suppressed endophilin B1 and significant increases of EGFR transcript. Data are presented as means ± SD of 3 independent experiments. Asterisks indicate significant difference ($P < 0.05$) compared with the control group.

EGFR signaling. First, we investigated whether suppression of endophilin B1 affected EGF (50ng/ml) triggered EGFR degradation. We found that knockdown of endophilin B1 significantly delayed EGF-stimulated EGFR degradation, meanwhile, we also found knockdown of endophilin B1 sustained higher receptor activation as assessed by EGFR phosphorylation on Y1173. Meanwhile, an important downstream signaling of EGFR pathway, phosphorylated Erk1/2, was also found sustained at high level in endophilin B1 knockdown cells (Fig. 3).

Suppression of endophilin B1 delays EGFR endocytosis from cell surface

To figure out which step of EGFR trafficking was affected by endophilin B1, we examined the EGF-induced EGFR trafficking in endophilin B1 suppressed cells. First, we examined cell surface EGFR levels based on biotinylation assay. We found that the amount of the surface EGFR was higher in endophilin B1 knocked-down cells after EGF treatment (Fig. 4A). In control group, after EGF treatment, the surface EGFR levels dramatically decreased due to the EGF-triggered internalization. In endophilin B1 knocked-down group, EGFR internalization was slowed significantly, especially from 5 min to 30 min after EGF treatment, suggesting that endophilin B1 might plays important roles in EGFR internalization.

Suppression of endophilin B1 enhances intracellular accumulation of EGFR triggered by EGF

To further verify whether endophilin B1 is involved in EGFR internalization, we biotinylated membrane surface proteins using a non-permeable cleavable cross-linker (Sulfo-NHS-SS-biotin) in LNCaP cells and then stimulated cells with EGF for various intervals (5, 15, 30 and 60 min) to induce the endocytosis of biotinylated

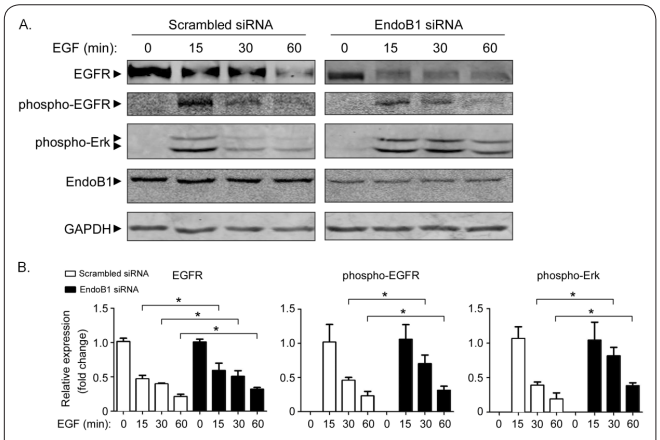

Figure 3. Suppression of endophilin B1 delays EGF-triggered EGFR degradation and sustains EGFR downstream signaling in LNCaP cells. A. knockdown of endophilin B1 expression resulted in enhanced level of total EGFR, phospho-EGFR and the downstream Erk signaling. Cells were transfected with scrambled (control group) or endophilin B1 (treatment group) siRNAs followed by EGF treatment for different periods. Then the amount of total EGFR, phospho-Tyr1173-EGFR and phospho-Erk1/2 were examined. **B.** Quantification of the gray degree values in A. Data are presented as means ± SD of 3 independent experiments. Asterisks indicate significant difference ($P < 0.05$) compared with the control group.

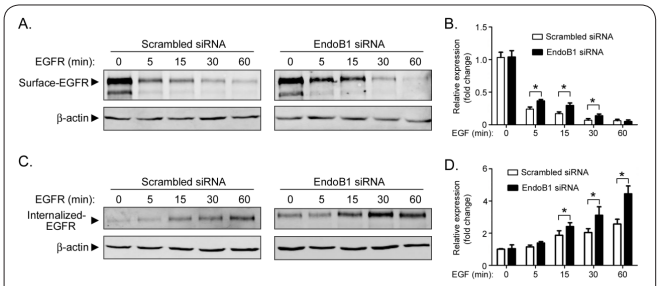

Figure 4. Knockdown of endophilin B1 delays EGF-triggered EGFR internalization and degradation. A-B. LNCaP cells were transfected with endophilin B1 siRNA for 48 hours. The cells were treated with EGF (50 ng/ml) for different time intervals as indicated. Then the cell surface proteins were labeled with biotin and biotinylated surface-EGFR was immunoprecipitated and detected by Western blot (A). Quantification of the gray degree values was shown in B. **C-D** LNCaP cells were transfected with endophilin B1 siRNA. Then the cell surface proteins were labeled with biotin followed by EGF treatment (50 ng/ml) for different time intervals. After EGF treatment, surface biotin-tag was cleaved by DTT. Then the internalized biotinylated EGFR was immunoprecipitated and detected by Western blot (C). Quantification of the gray degree values was shown in D. Data are presented as means ± SD of 3 independent experiments. Asterisks indicate significant difference ($P < 0.05$).

surface EGFR. Then the cells were incubated with DTT to strip biotin-tag. Therefore, only internalized biotinylated EGFR remained biotin-tag. Our results showed that the amount of internalized EGFR in endophilin B1 knocked-down cells was higher than that in control cells, especially from 15 min to 60 min after EGF treatment (Fig. 4B), indicating that the EGFR degradation was delayed when endophilin B1 expression was suppressed.

Silencing of endophilin B1 promotes cell proliferation but suppresses EGF reactivity

To explore the biological role of endophilin B1 on prostate cancer cells, we assessed the effect of suppression of endophilin B1 on proliferation of LNCaP cells by MTT assay. In control group, around 1.5-1.8 fold increase of LNCaP cell proliferation was observed from day 1 to day 3, and EGF (10 ng/ml) significantly increased the LNCaP cell proliferation (Fig. 5 left panel). In the endophilin B1 knocked-down group, we found that there was a nearly 2.4-2.8 fold increase in cell proliferation from day 2 to day 3, which is much higher than that in control group. However, EGF treatment could not further promote the LNCaP cell proliferation. These results strongly suggested that the endophilin B1 might affect cell proliferation through EGFR signaling pathway.

Discussion

It is well documented that EGFR is over-expressed in most cancer types in our body (11). In prostate cancer, many evidences proved that aberrant expression of EGFR is involved in androgen-independent growth (12,13). It has been demonstrated that EGFR expression is increased with the progression of prostate cancer from an androgen-dependent stage to an androgen-independent stage (14). For example, in prostate cancer

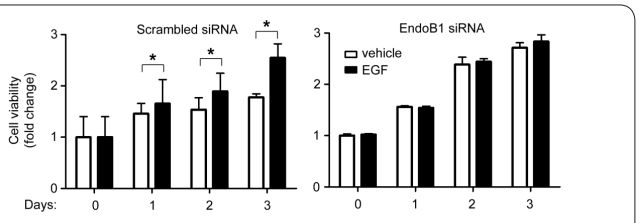

Figure 5. Suppression of endophilin B1 promotes LNCaP cell proliferation but losses EGF reactivity. LNCaP cells were transfected with scrambled or endophilin B1 siRNAs for 24 hours. Then the cells were seeded in 96-well plate for 24 hours followed by EGF treatment (10 ng/ml). MTT assay was performed to evaluate the cell viability. Data are presented as means ± SD of 3 independent experiments. Asterisks indicate significant difference ($P < 0.05$) compared with the EGF untreated group.

bone metastases, EGFR expressed much higher level than prostate tumor of the primary site (15), and higher expression of EGFR in androgen-independent prostate cell lines than in androgen-dependent cell lines(16). It is generally accepted that EGFR plays important roles in the progression of prostate cancer, the tyrosine kinase activity of EGFR is thus considered to be a target of prostate cancer therapy. Recently, there are some other studies reported that EGFR has a kinase independent pro-survival function in cancer cells (17,18), another study also found suppressing the EGFR expression instead of its tyrosine kinase activity might lead to higher efficiency of EGFR targeted therapy for prostate cancer (14). Because receptor sorting and signaling are closely linked, trafficking of the EGFR have been given more and more research concerns in recent years (19,20,21). The internalization process of EGFR on cell membrane can be divided into two classes: clathrin-mediated and clathrin-independent endocytosis. Numerous adaptor proteins interact with the EGFR in this process, thus further affect the endocytic machinery (22).

Previous studies indicated that endophilin B1 is a tumor suppressor by regulating mitochondrial morphology and mitochondrial apoptosis (23,24). Recent studies have reported low expression of endophilin B1 in many kinds of cancers including prostate cancer (4). Loss of endophilin B1 promotes tumor growth in nude mice (24), and endophilin B1 knockout mice have a higher incidence of tumor (6). Another study found that endophilin B1 was highly expressed in the early stages of the development of prostate adenocarcinoma, indicating it might play an important role in prostate tumor development (4). Endophilin B1 has proven to be an adaptor protein in clathrin-mediated endocytosis, and the role in this process is thought to promote the internalization of receptor (25). However, some studies also suggested a relatively minor role for endophilin protein in clathrin-mediated endocytosis (CME). A latest research presented a new clathrin-independent endocytic pathway called fast endophilin-mediated endocytosis (FEME), in which endophilin play an important role. Taken together, these studies demonstrated that endophilin B1 plays important roles on cancer, but the mechanism and the roles of endophilin B1 in tumorigenesis are not yet fully understood.

In this study, we found that both endophilin B1 and EGFR are abnormal expressed in prostate cancer cell lines - LNCaP, DU145,which representing different de-

velopment stages of prostate cancer. Endophilin B1 had lower expression in these cancer cells, whereas EGFR had relatively higher expressions, which is consistent with previous reports (4). We also found that knock-down of endophilin B1 promoted LNCaP cell proliferation which is EGF independent, indicating that loss of endophilin B1 might play important roles in cancer cell viability, further confirming the tumor suppressor gene function of endophilin B1.

There are many evidences indicating that endophilin B1 possibly controls the movement of receptor (20,21,26-28). In particular, it has now been demonstrated that endophilin protein can bind to EGFR through CIN85 and Cbl (28), and thereby plays important roles in endocytosis. In this study, we found knocked-down of endophilin B1 significantly delayed the internalization of membrane EGFR, indicating that endophilin B1 plays important roles in endocytosis. The transport of EGFR after internalization is finally going to lysosome for degradation or to recycling endosome for going back to membrane. In our study, we found the amount of intracellular EGFR is higher in endophilin B1 knocked-down cells than in control cells, indicating the EGFR degradation in lysosome was inhibited. Other studies have come to similar conclusion. It is reported that suppression of endophilin B1 expression delays EGFR degradation in breast cancer cell (20) and in PLC/PRF/5 cells (21). However, Wan et al. reported that knock-down of endophilin B1 expression accelerated degradation of TrkA, and demonstrated endophilin B1 also contributed to recycling of NGF/TrkA to the cell surface in PC12 cells (26,27). Consistent with these findings, another study also reported that loss of endophilin B1 promoted the co-localization of internalized EGFR with LAMP1, a marker for lysosomes, and the rate of EGF-stimulated EGFR degradation was significantly enhanced in HeLa cells (7). In summary, these studies all indicated that endophilin B1 played important roles in the trafficking of cell membrane receptors, despite it might be cell and receptor specific.

Acknowledgments

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