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Ell3 stimulates proliferation, drug resistance, and cancer stem cell properties of breast cancer cells via a MEK/ERK-dependent signaling pathway

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ABSTRACT

Ell3 is a RNA polymerase II transcription elongation factor that is enriched in testis. The C-terminal domain of Ell3 shows strong similarities to that of Ell (eleven–nineteen lysine-rich leukemia gene), which acts as a negative regulator of p53 and regulates cell proliferation and survival. Recent studies in our laboratory showed that Ell3 induces the differentiation of mouse embryonic stem cells by protecting differentiating cells from apoptosis via the promotion of p53 degradation. In this study, we evaluated the function of Ell3 in breast cancer cell lines. MCF-7 cell lines overexpressing Ell3 were used to examine cell proliferation and cancer stem cell properties. Ectopic expression of Ell3 in breast cancer cell lines induces proliferation and 5-FU resistance. In addition, Ell3 expression increases the cancer stem cell population, which is characterized by CD44 (+) or ALDH1 (+) cells. Mammosphere-forming potential and migration ability were also increased upon Ell3 expression in breast cancer cell lines. Through biochemical and molecular biological analyses, we showed that Ell3 regulates proliferation, cancer stem cell properties and drug resistance in breast cancer cell lines partly through the MEK–extracellular signal-regulated kinase signaling pathway. Murine xenograft experiments showed that Ell3 expression promotes tumorigenesis *in vivo*. These results suggest that Ell3 may play a critical role in promoting oncogenesis in breast cancer by regulating cell proliferation and cancer stem cell properties via the ERK1/2 signaling pathway.

1. Introduction

Breast cancer is the most common malignancy in women and a significant cause of morbidity and mortality. Similar to other tumors, breast cancer shows intratumoral heterogeneity. Although the mechanism(s) underlying this heterogeneity are not understood, a growing body of evidence suggests that a small population of cells, called cancer stem cells (CSCs), which show some phenotypic similarities with adult tissue stem cells, contribute to tumor heterogeneity [1]. CSCs are operationally defined by self-renewal, as well as their ability to differentiate into the non-self-renewing multiple lineages of the bulk tumor [2,3]. Human breast CSCs are characterized according to their expression of specific cell surface markers (e.g., $CD44^{+high}CD24^{-low}$), aldehyde dehydrogenase (ALDH) enzyme activity (ALDH-positive cells), and mammosphere formation in suspension culture [2,3]. CSCs mediate tumor metastasis and drug resistance, which contributes to relapse of cancer [4,5]. Thus, the successful targeting of this cell population is critical to successful cancer treatment.

The RAS/RAF/MEK signaling transduction pathway critically regulates proliferation and apoptosis in various cell types. These proteins represent a group of serine/threonine kinases that mediate signal transduction from the cell surface towards both nuclear and cytosolic targets in response to a variety of extracellular stimuli. Deregulation of the RAS/RAF/MEK pathway is detected in more than 30% of human tumors, mainly resulting from mutations in RAS and/or B-RAF [6]. In addition, recent studies reveal that the RAS/RAF/MEK pathway is associated with CSCs and sensitivity to targeted therapy [7,8]. Drug resistant breast cancer cells showing increased activation of the RAS/RAF/MEK pathway also show properties consistent with CSCs [9].

Ell3 is a Pol II transcription elongation factor enriched in testis. The C-terminal domain of Ell3 shows strong similarities with that of the Ell (eleven–nineteen lysine-rich leukemia) gene, which acts as a negative regulator of p53 and regulates cell proliferation and survival [10,11].

Ell3 primes gene activation in embryonic stem cells (ESCs) by marking the enhancers of developmentally-regulated genes [12].

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The binding of Ell3 to inactive or poised enhancers is essential for the recruitment of the super elongation complex (SEC) upon differentiation signaling. Recent studies in this laboratory demonstrated that Ell3 enhances the differentiation of mouse ESCs by protecting differentiating cells from apoptosis through the promotion of p53 degradation [13]. Given the central function of Ell3 in p53 stability, we investigated the role of Ell3 in breast cancer. We found that ectopic expression of Ell3 promotes cell proliferation and induces 5-FU drug resistance in both breast cancer cell lines and non-tumorigenic MCF-10A cells. Moreover, the cancer stem cell properties of breast cancer were increased upon Ell3 expression. ERK1/2 was phosphorylated upon Ell3 expression, and chemical inhibition of ERK activity compromised the effect of Ell3 on the breast cancer cell lines. Our data suggest that Ell3 functions to promote oncogenesis in breast cancer cell lines by regulating the ERK signaling pathway.

2. Materials and methods

2.1. Cell culture and establishment of Ell3 overexpressing breast cancer cell lines

MCF-7, BT-20 and MCF-10A cell lines were purchased from American Type Culture Collection (ATCC, Teddington, UK). MCF-7 and BT-20 cells were cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Ell3-overexpressing (Ell3 OE) breast cancer cell lines were generated by the chromosomal integration of an Ell3 expression plasmid, which was constructed by cloning PCR-amplified Ell3 cDNA into pcDNA3.1 vectors (Invitrogen, Carlsbad, CA). Three independent Ell3 OE cell lines were established and all experiments were repeated in each cell line to confirm the results.

2.2. Western blot analysis

Protein expression was analyzed as previously described [13]. Briefly, cells were washed twice with cold phosphate buffered saline (PBS) and lysed with tissue lysis buffer (20 mM Tris-base, pH 7.4, 137 mM NaCl, 2 mM EDTA, 1% Triton X-100, 25 mM β-glycerophosphate, 2 mM sodium pyrophosphate, 10% glycerol, 1 mM sodium orthovanadate, 1 mM phenylmethysulfonyl fluoride, and 1 mM benzamidine). Total cell extracts (50 μg) were resolved by SDS–PAGE, transferred to Immobilon-P membranes (Millipore, Bedford, MA; http://www.Millipore.com), and blotted with antibodies to Ell3 (ab67415, Abcam), p53 (#2524, Cell Signaling, Denver, MA; http://www.cellsignal.com), Caspase-3 (#9665, Cell Signaling), Caspase-9 (#9504, Cell Signaling), and β-actin (sc-47778, Santa Cruz, USA). Immunoreactivity was detected by enhanced chemiluminescence (ECL; Amersham, Piscataway, NJ, USA; http://www.amershambiosciences.com).

2.3. Real-time reverse transcriptase-PCR

Total RNA was prepared from cells using TRIzol (Invitrogen) and 2–5 μg of total RNA was reverse-transcribed into cDNA using the SuperScriptII™ First-Strand Synthesis System (Invitrogen) according to the manufacturer's instructions. Real-time PCR was performed in triplicate with the Quantitect SYBR Green PCR kit (Qiagen, Valencia, CA, USA, http://www.qiagen.com) and the CFX96 Real-time System (Bio-Rad Laboratories, Richmond, CA, USA, http://www.bio-rad.com). For quantification, target gene expression was normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

2.4. Apoptosis analysis using Annexin-V and Propidium iodide (PI) with flow cytometry

An Annexin-V fluorescein-isothiocyanate (FITC)–conjugated apoptosis detection kit incorporating PI was used as described in the manufacturer's protocol (LS-02–100, BioBud, Korea). Samples were analyzed by flow cytometry using FL1 (FITC) and FL2 (PI) laser lines.

2.5. Analysis of expression of ALDH$^+$ cells

ALDH activity was detected using the ALDEFLUOR assay kit (StemCell Technologies) as described by the manufacturer. Briefly, detached single cells were suspended in ALDEFLUOR assay buffer containing an ALDH substrate for 45 min at 37 °C. A specific inhibitor of ALDH, diethylaminobenzaldehyde (DEAB), was used as a negative control. Samples were analyzed by flow cytometry using the FL1 laser line.

2.6. Wound healing assay

Breast cancer cells were seeded in 60 mm tissue culture dishes at a density of 5×10^5. The next day, the cells were treated with 100 μg/ml of Mitomycin C (M-0503, Sigma–Aldrich) to induce cell growth arrest. After 2 h, cells were washed three times using dPBS and a wound was incised in the central region of the confluent culture followed by washing to remove detached cells and the addition of fresh culture medium. Phase contrast images of the wounded region were recorded under an inverted microscope 24 h after the wound was made.

2.7. Mammosphere formation

To induce sphere formation, cells were detached from the culture dishes using 0.05% Trypsin–EDTA solution and then were suspended in non-coated petri dishes. Cells were grown in serum-free DMEM/F12 medium containing B27 (10889–088, Invitrogen), 20 ng/ml epidermal growth factor, 20 ng/ml basic fibroblast growth factor (13256–029, Invitrogen), and 20 ng/ml insulin growth factor 1 (291-G1, R&D systems). Mammospheres were cultured for 5–7 days.

2.8. Soft Agar colony formation assay

A CytoSelect™ 96-well *invitro* Tumor Sensitivity Assay (CBA-150, Cell Biolabs, San Diego, USA, http://www.cellbiolabs.com) was used to analyze colony formation in soft agar. In brief, cells seeded in soft agar were incubated for 6–8 days at 37 °C and 3-(4,5-dimethylthiazol-2-yl)-2,5,-diphenyltetrazolium (MTT) solution was added to each well. The plates were incubated at 37 °C for 4 h and cells were detached to measure the absorbance at 570 nm in a 96-well microtiter plate reader.

2.9. Transfection with Ell3 small interfering RNA (siRNA)

Breast cancer cells were transfected with siRNA using SiGENOME (M-014601–01–0005), which was provided by Dharmacon (distributed by ThermoScientific/AbGene Ltd. Epsom, UK). Cells were transfected with either siRNA or plasmids using Lipofectamine 2000 (Invitrogen) in OPTIMEM medium (Invitrogen) according to the manufacturer's instructions. Nonspecific control siRNAs were purchased from Bioneer (Daejoen, Korea).

2.10. Statistical analysis

Graphical data are presented as the mean ± SD. Each experiment was performed at least three times and subjected to statistical analysis. Statistically significant differences between two groups were determined using the Student's *t*-test, and a p value < 0.05 was considered significant. All statistical analyses were performed using the SAS statistical package, v.9.13 (SAS Inc., Cary, NC, USA; http://www.sas.com/).

3. Results

3.1. Ell3. promotes cell proliferation in breast cancer cells

To understand the role of Ell3 in breast cancer cells, we first examined the expression of Ell3 in various breast cancer cell lines and in MCF-10A cells, a normal mammary epithelial cell line. Ell3 expression in the breast cancer cell lines was higher than that in MCF-10A cells (Fig. 1A). Thus, we first sought to determine whether stable Ell3 overexpression regulates breast cancer cell line characteristics. MCF-7 cells overexpressing Ell3 were generated by retroviral infection (Fig. 1B). As expected, Ell3 was mainly localized in the nucleus, regardless of its expression level (Fig. 1C). This observation is consistent with the known function of Ell3 as an RNA polymerase II elongation factor. We first examined the effect of Ell3 overexpression on MCF-7 cell proliferation by counting cells after seeding into culture dishes. Over a period of 4 days, Ell3 OE cells showed higher proliferation (as early as Day 1) and continued to grow faster than empty vector control cells (C) (Fig. 1D). To examine the role of Ell3 in the cell cycle, we analyzed the expression of cell cycle-related genes. As shown in Fig. 1E, the expression of cyclin A, which is a rate-limiting component required for the initiation of

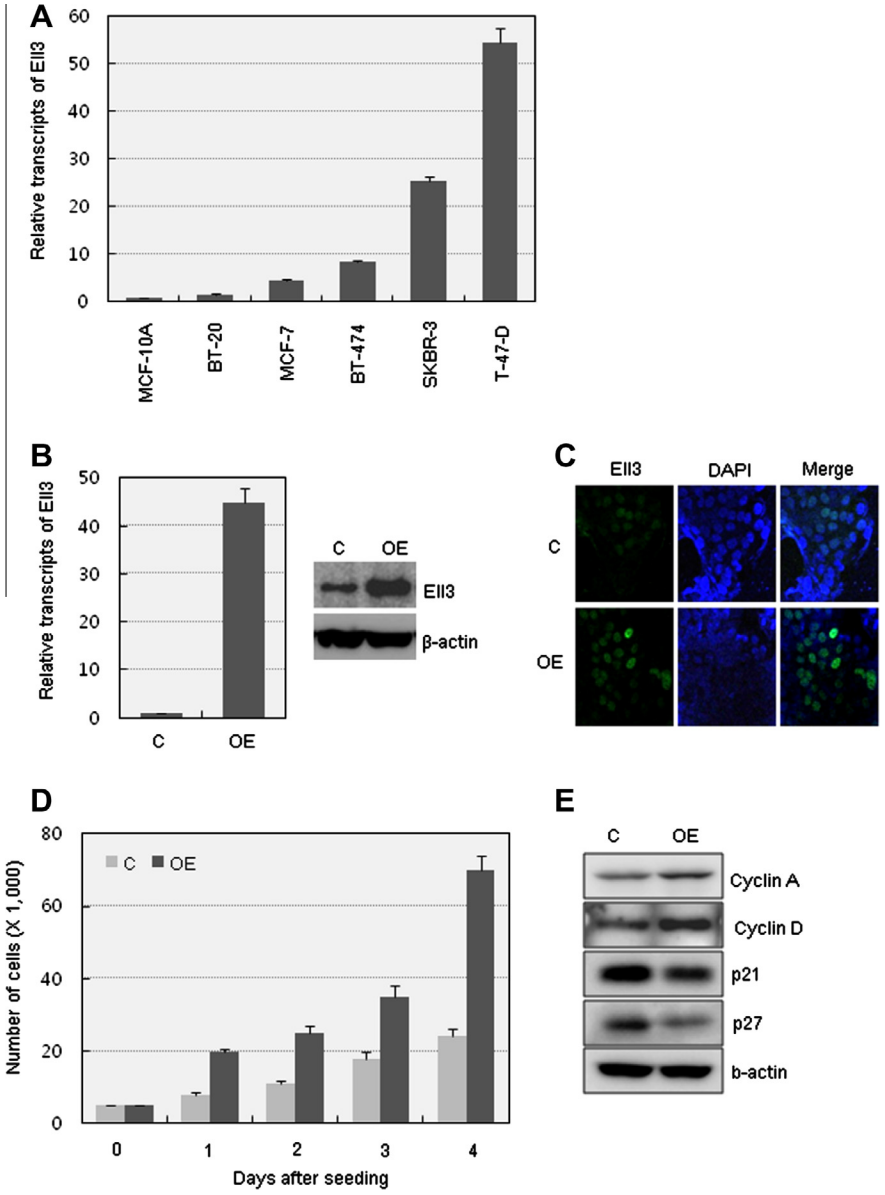

Fig. 1. Ell3 regulates the proliferation of MCF-7 cells. (A) Ell3 expression by the indicated breast cancer cell lines was analyzed by real-time RT-PCR. (B) Ell3-overexpressing MCF-7 cells were constructed and Ell3 expression was analyzed by RT-PCR and immunoblot analysis. (C) The subcellular localization of Ell3 was analyzed by immunocytochemical staining of Ell3 in Ell3-overexpressing MCF-7 cell lines. (D) The proliferation of Ell3-overexpressing MCF-7 cells was analyzed by counting cell numbers every 24 h for 4 days. (E) Expression of cell cycle regulatory proteins was analyzed by immunoblot analysis. Abbreviations: C, control parental cells; OE, Ell3-overexpressing MCF-7 cells.

DNA synthesis and entry into mitosis [14], was increased in Ell3 OE MCF-7 cells. In addition, another cell cycle regulator, cyclin D, which serves as a cell cycle switch by promoting entry into G2 phase in actively proliferating cells [15], was also highly expressed in Ell3 OE cells. By contrast, expression of cell cycle inhibitors such as p21 and p27 Cip/Kip was suppressed in Ell3 OE cells. Moreover, ectopic expression of Ell3 in MCF10A cells also promoted cell proliferation (Fig. S1A and B). These results suggest that Ell3 facilitates breast cancer cell proliferation by promoting cell cycle progression.

3.2. Ell3 overexpression reinforces cancer stem cell characteristics and 5-FU resistance in MCF-7 cells

Since CSCs have a greater ability to maintain tumorigenesis and mammosphere formation they can, in part, recapitulate breast tumorigenesis [16,17]; therefore, we next examined the mammosphere-forming ability of Ell3 OE cells. As shown in Fig. 2A, Ell3 OE cells formed more (and larger) mammospheres than the controls. Interestingly, Ell3 overexpression also enabled MCF-10A cells to form mammospheres in serum-free media (Fig. S1C). Cell migration is another prominent characteristic of CSCs [18] and, as expected, Ell3 OE cells showed increased migration ability compared with the control (Fig. 2B). Since the growth of Ell3 OE was faster than that of the control, we cannot rule out the possibility that the difference in migration ability was due to a difference in the cell proliferation. The self-renewing capacity of Ell OE cells was also examined in a colony forming assay. An MTT assay, performed on colonies formed in soft agar, revealed that Ell3 overexpression significantly increased the colony forming ability of MCF-7 cells (Fig. 2C). In accordance with the finding that the expression of CD44, the type I transmembrane glycoprotein receptor, is highly up-regulated in breast CSCs [2], the percentage

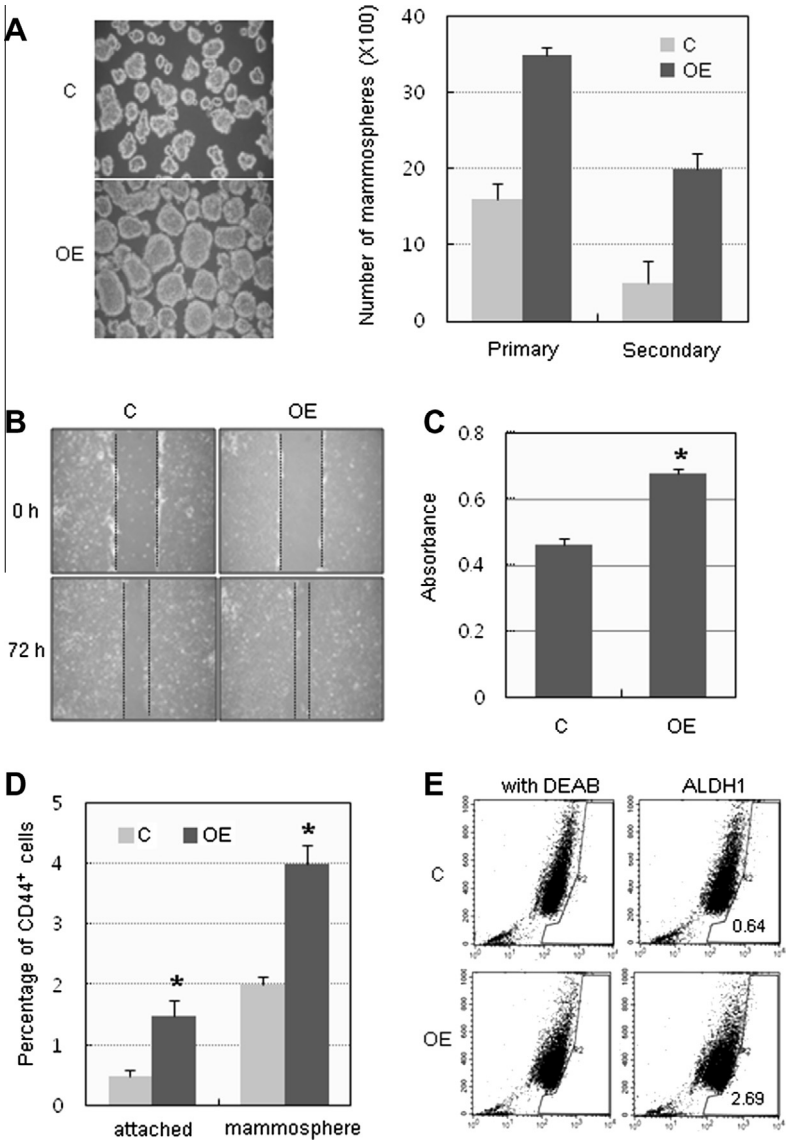

Fig. 2. Effect of Ell3 overexpression on the cancer stem cell characteristics of MCF-7 cells. (A) Ell3-overexpressing MCF-7 cells formed a greater number of mammospheres. The graph shows the number of primary and secondary mammospheres generated after 10 days in suspension culture. (B) The migration of Ell3-overexpressing MCF-7 cells was compared with that of control cells in an *in vitro* scratch wound healing assay. Photographs were taken 24 h after the wound was made. (C) The number of viable cells in colonies incubated for 7 days in soft agar was estimated using an MTT assay. (D) CD44-expressing cells within the Ell3-overexpressing MCF-7 cell and control cell populations were counted by flow cytometry. (E) ALDH1 enzymatic activity of Ell3-overexpressing MCF-7 and control cells as detected using the ALDEFLUOR assay. DEAB was used to inhibit the reaction of ALDH with the ALDEFLUOR reagent, providing a negative control. (F) Growth and mammosphere formation by Ell3-overexpressing MCF-7 cells and control cells in the presence of 5-FU were examined under a light microscope. Attached cells were incubated for 2 days and mammospheres were formed for 14 days. (G) Apoptosis of Ell3-overexpressing MCF-7 and control cells was examined by Annexin-V staining and FACS analysis of samples treated with 5-FU for 48 h. Data represent means of three independent experiments. $^{*}p < 0.05$. Abbreviations: C, control parental cells; OE, Ell3-overexpressing MCF-7 cells.

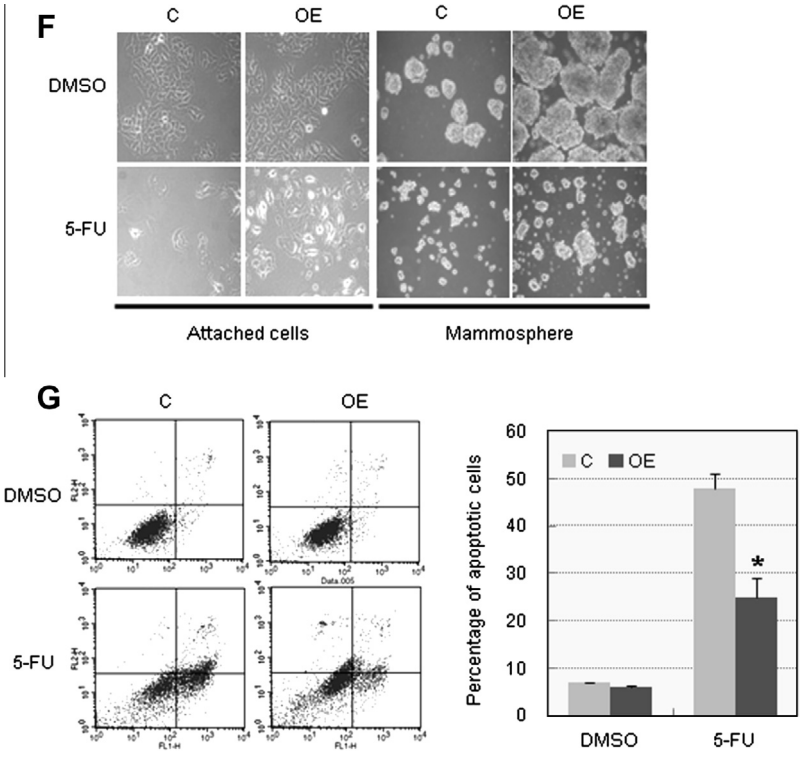

Fig. 2 (*continued*)

of CD44$^+$ cells was higher in Ell3 OE than in the controls (Fig. 2D). Finally the proportion of ALDH1(+) cells, which is indicative of CSCs, in the Ell3 OE cell population increased 3-fold compared with that in the control population (Fig. 2E). These results suggest that Ell3 expression reflects the CSC properties of breast cancer cells. 5-flourouracil (5-FU) is a pyrimidine analog used to treat several types of cancer, including breast cancer. The *invitro* antitumor efficacy of 5-FU in Ell3 OE was evaluated by examining the survival of attached cells and mammosphere formation by cells in suspension. As shown in Fig. 2F, Ell3 overexpression conferred 5-FU resistance upon attached and suspended MCF-7 cells. Especially, Ell3 OE formed larger mammospheres than the control. Quantitative analysis of 5-FU drug resistance in attached cells revealed that Ell3 OE was twice as resistant to 5-FU as the control cells (Fig. 2G). Ell3 overexpression in MCF-10A cells also induced drug resistance and increased cell survival in the presence of 5-FU (Fig. S1D). These results indicate that Ell3 expression might be involved in the mechanism underlying 5-FU resistance in breast cancer.

3.3. siRNA mediated suppression of Ell3 reverts the phenotype of Ell3 OE

Studies of the ectopic expression of Ell3 support a novel function for Ell3 as a regulator of cell proliferation, cancer stem cell properties, and drug resistance. To confirm that these phenotypes are indeed induced by Ell3 overexpression, we transiently knocked down Ell3 using siRNA and examined whether the phenotype of Ell3 OE reverted. First of all, we confirmed that 100 nM of siRNA was the optimal concentration required to reduced Ell3 expression to control levels (Fig. 3A). As expected, the proliferation, mammosphere formation and 5-FU drug resistance of Ell3 OE were reverted to levels similar to those observed in control cells (Fig. 3B–D). These results suggest that increased cell proliferation, cancer stem cell properties, and 5-FU resistance in Ell3 OE are induced by Ell3.

3.4. Ell3 increases oncogenesis in breast cancer cells by activating ERK1/2 signaling

To understand the mechanism(s) underlying the Ell3-mediated induction of cell proliferation in breast cancer, we examined whether Ell3 expression affects the phosphorylation of ERK1/2, which are both members of the mitogen-activated protein kinase superfamily that mediates cell proliferation and apoptosis [19]. Fig. 4A shows that ERK1/2 phosphorylation in Ell3 OE cells was significantly higher than that in the control. To confirm that the increased proliferation of Ell3 OE cells was induced by ERK1/2 activation, we inhibited ERK1/2 signaling using PD98059, a chemical inhibitor of the ERK pathway, and then examined cell growth. Under the conditions required for ERK1/2 to be dephosphorylated to control levels, the growth of Ell3 OE cells was similar to that of control cells (Fig. 4B). In addition, mammosphere formation by Ell3 OE cells was reduced in the presence of PD98059 (Fig. 4C). Resistance to 5-FU was also decreased to control levels by inhibiting ERK1/2 signaling (Fig. 4D). Taken together, our data suggest that Ell3-mediated stimulation of cell growth, increased cancer stem cell properties, and 5-FU drug resistance requires the activation of ERK1/2 signaling.

4. Discussion

Here, we examined the oncogenic activity of Ell3 in breast cancer cells. Ell3 is an RNA polymerase II transcription elongation factor, which shows approximately 50% sequence identity to both Ell and Ell2 throughout its ORF proteins [10]. The Ell C-terminal domain of a MLL-ELL fusion protein found in patients with acute myeloid leukemia represses p53 transcriptional activity by binding to it, which is sufficient to immortalize myeloid progenitors [10]. Since Ell3 shows a great degree of homology to the C-terminal domain of Ell, and induces the differentiation of mouse ESCs by regulating epithelial-mesenchymal transition and apoptosis [13], we

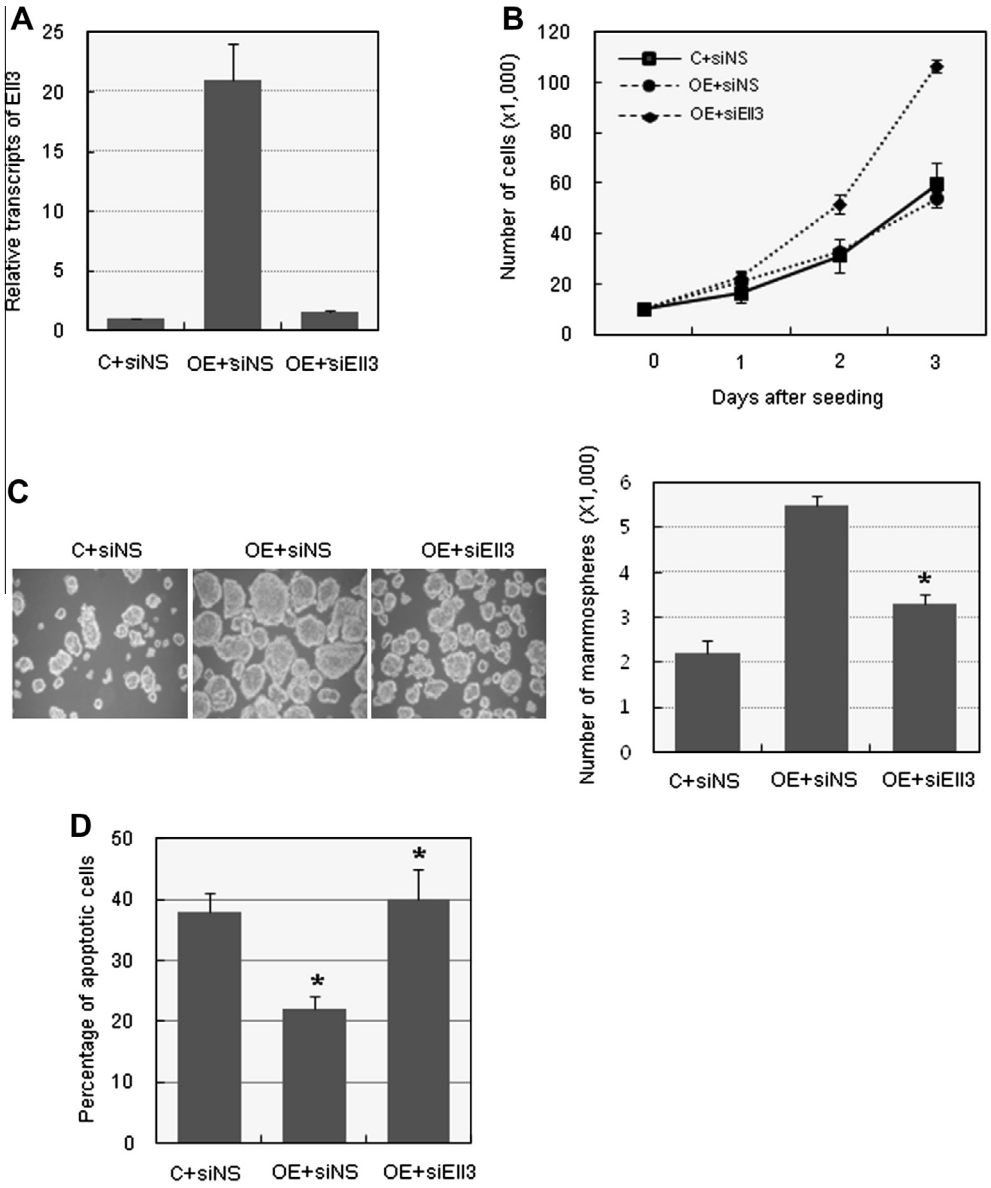

Fig. 3. siRNA mediated suppression of Ell3 inhibits proliferation, mammosphere formation, and drug resistance in Ell3 OE MCF-7 cells. (A) siEll3 or siNS was transfected into Ell3-overexpressing MCF-7 cells and Ell3 expression was analyzed by real-time RT-PCR. (B) Proliferation of control and Ell3-overexpressing MCF-7 cells transfected with siEll3 or siNS was analyzed by counting cell numbers every 24 h for 3 days. (C) Mammosphere formation by control and Ell3- overexpressing MCF-7 cells transfected with siEll3 or siNS was examined 7 days after incubating the cells in suspension culture. (D) Apoptosis induced by 5-FU was analyzed by FACS analysis of Annexin-V-stained Ell3-overexpressing MCF-7 cells transfected with siEll3 or siNS. Data represent the means of three independent experiments. $^{*}p < 0.05$. Abbreviations: C, control parental cells; OE, Ell3-overexpressing MCF-7 cells; siNS, nonspecific siRNA; siEll3, siRNA targeting Ell3.

reasoned that Ell3 might have similar oncogenic properties to those apparently mediated by Ell.

As expected, our results showed that Ell3 overexpression increased the proliferation of breast cancer cell lines. The oncogenic activity of Ell3 was further confirmed by increases in cell migration, the number of CSCs, drug resistance, and *in vivo* tumor formation by Ell3-overexpressing breast cancer cell lines. An association between cell proliferation and the ERK pathway is already well established. The ERK signaling pathway controls cyclin D1 expression and S phase progression in mouse ESCs [20]. In addition, the cyclin-dependent kinase inhibitor p21 (Waf1), is suppressed by ERK, which inhibits nuclear Sp1 binding to the promoter of p21 [21]. Ell3 overexpression-mediated cell proliferation was totally impaired by inhibiting the MEK/ERK kinase cascade. Thus, MEK/ERK signaling appears to determine the effect of Ell3 on the proliferation of MCF-7 cells.

Activation of the ERK signaling pathway promotes the expansion of breast cancer-initiating cells [22,23]. In line with these reports, our results show that Ell3 regulates cancer stem cell populations primarily via ERK signaling, as evidenced by the suppression of mammosphere formation by Ell3-overexpressing MCF-7 cells after the inhibition of ERK signaling. However, our results indicate that additional mechanisms involved in maintaining mammospheres are activated by Ell3, because ERK inhibition did not completely abolish the mammosphere-forming ability of Ell3-overexpressing MCF-7 cells.

In oral cancer, ERK1/2 activation in aggressively growing cell lines stimulates the expression of CD44 whereas inhibiting ERK1/

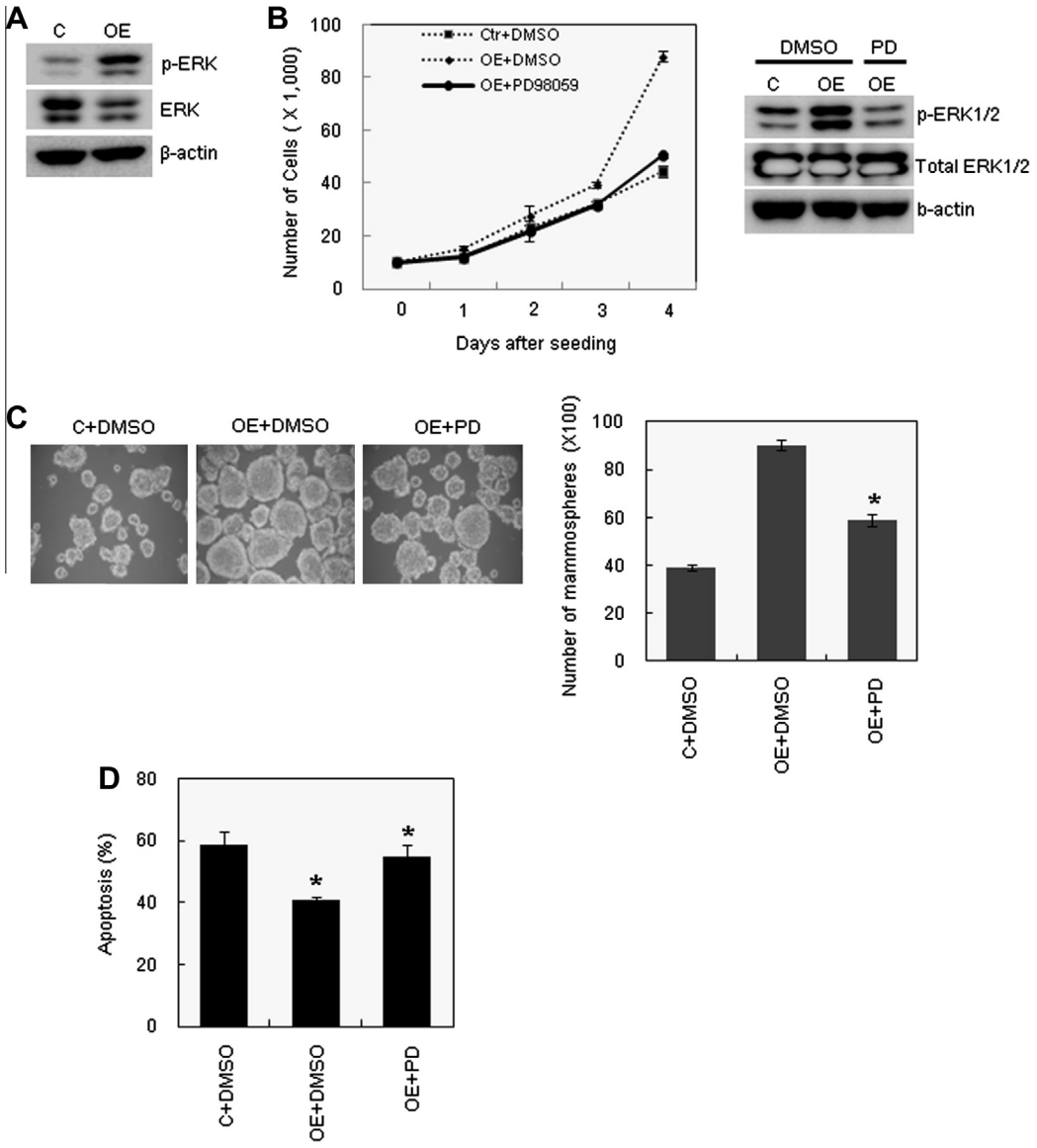

Fig. 4. Ell3 mediates proliferation, mammosphere formation, and drug resistance in Ell3 OE MCF-7 cells by activating ERK1/2 signaling. (A) Phosphorylation of ERK1/2 in control and Ell3-overexpressing MCF-7 cells as analyzed by immunoblotting. (B) Proliferation of Ell3-overexpressing MCF-7 cells in the presence of DMSO or PD98059 was compared with that of control cells by counting cell numbers every 24 h for 3 days. (C) Mammosphere formation by Ell3-overexpressing MCF-7 cells in the presence of DMSO or PD98059 was compared with that by control cells. (D) 5-FU resistance of Ell3-overexpressing MCF-7 cells in the presence of DMSO or PD98059 was compared with that of control cells. Data represent means of three independent experiments. $^{*}p < 0.05$. Abbreviations: C, control parental cells; OE, Ell3-overexpressing MCF-7 cells; PD, PD98059.

2 reduces CD44 expression by suppressing promoter activity, which suggests that CD44 is a critical target for ERK1/2 in aggressive tumors [23]. These data also suggest that increases in the number of $CD44^{+}$ cells within the Ell3-overexpressing MCF-7 cell population is mainly due to the increased activation of ERK1/2.

Similar to Ell3-overexpressing MCF-7 cells, the proliferation of BT-20 cell lines that were engineered to overexpress Ell3 was greater than that of the parental cell line (data not shown). More importantly, Ell3-overexpressing BT-20 cells gave rise to significantly larger tumors than control cells in the xenograft experiment (Fig. S2A). We also found that cyclin A and cyclin D expression was increased whereas p21 and p27 expression was decreased in Ell3-overexpressing tumors (Fig. S2B). These results strongly support that Ell3 promotes breast cancer growth by modulating the expression of cell cycle regulators. We speculate that the Ell3 increases oncogenecity of breast cancer cell lines by regulating expression of cell cycle regulators through the ERK signaling pathway. It would be interesting to identify the mechanism(s) underlying Ell3-induced activation of ERK signaling in breast cancer cell lines. It is also important to study whether Ell3 functions as an oncogenic factor in other cancers.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.06.114.

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