Published in final edited form as:

Mol Carcinog. 2015 August ; 54(8): 618–631. doi:10.1002/mc.22129.

p66Shc longevity protein regulates the proliferation of human ovarian cancer cells

Sakthivel Muniyan^{1,#}, Yu-Wei Chou^{1,#}, Te-Jung Tsai^{1,2}, Paul Thomes¹, Suresh Veeramani¹, Benedict B. Benigno³, L. DeEtte Walker⁴, John F. McDonald⁴, Shafiq A. Khan⁵, Fen-Fen Lin¹, Subodh M Lele⁶, and Ming-Fong Lin^{1,7,8,9,*}

¹Department of Biochemistry and Molecular Biology, College of Medicine, University of Nebraska Medical Center, Omaha, NE 68198, USA

²College of Medicine, University of Nebraska Medical Center, Omaha, NE 68198, USA

³Ovarian Cancer Institute, 960 Johnson Ferry Road, Suite 130, Atlanta, GA 30342, USA

⁴Department of Biology, Georgia Institute of Technology, Atlanta, GA 30332, USA

⁵Center for Cancer Research and Therapeutic Development, Clark Atlanta University, Atlanta, GA 30314, USA

⁶Department of Pathology and Microbiology, University of Nebraska Medical Center, Omaha, NE 68198, USA

⁷Department of Surgery/Urology, College of Medicine, University of Nebraska Medical Center, Omaha, NE 68198, USA

⁸Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, Omaha, NE 68198, USA

⁹College of Pharmacy, Kaohsiung Medical University, Kaohsiung, Taiwan 807, ROC

Abstract

p66Shc functions as a longevity protein in murine and exhibits oxidase activity in regulating diverse biological activities. In this study, we investigated the role of p66Shc protein in regulating ovarian cancer (OCA) cell proliferation. Among three cell lines examined, the slowest growing OVCAR-3 cells have the lowest level of p66Shc protein. Transient transfection with p66Shc cDNA expression vector in OVCAR-3 cells increases cell proliferation. Conversely, knock-down of p66Shc by shRNA in rapidly growing SKOV-3 cells results in decreased cell growth. In estrogen (E2)-treated CaOV-3 cells, elevated p66Shc protein level correlates with ROS level, ErbB-2 and ERK/MAPK activation, and cell proliferation. Further, the E2-stimulated proliferation of CaOV-3 cells was blocked by antioxidants and ErbB-2 inhibitor. Additionally, in E2-stimulated cells, the tartrate-sensitive, but not the tartrate-resistant, phosphatase activity decreases; concurrently, the tyrosine phosphorylation of ErbB-2 increases. Conversely, inhibition of

*Corresponding Author: Ming-Fong Lin, Ph.D., Department of Biochemistry and Molecular Biology, College of Medicine, University of Nebraska Medical Center, 985870 Nebraska Medical Center, Omaha, NE 68198-5870, USA, TEL: (402) 559-6658, FAX: (402) 559-6650, mlin@unmc.edu.

#These authors contributed equally to this work

phosphatase activity by L(+)-tartrate treatment increases p66Shc protein level, ErbB-2 tyrosine phosphorylation, ERK/MAPK activation and cell growth. Further, inhibition of the ERK/MAPK pathway by PD98059 blocks E2-induced ERK/MAPK activation and cell proliferation in CaOV-3 cells. Moreover, immunohistochemical analyses showed that the p66Shc protein level was significantly higher in cancerous cells than in non-cancerous cells in archival OCa tissues (n=76; $p=0.00037$). These data collectively indicate that p66Shc protein plays a critical role in up-regulating OCa progression.

Keywords

p66Shc; estrogen; ROS; ErbB-2 signaling; tyrosine phosphatase

INTRODUCTION

Ovarian cancer (OCa) is one of the most common gynecological malignancies in the United States and is the fifth leading cause of cancer deaths in women with the five-year survival rate of 46% [1]. Although significant advances have been made in OCa research [2]; survival to incidence ratio is still poor and the overall cure rate is only 30% [3]. The high mortality rate is at least in part because 70% of the OCa cases are diagnosed at advanced stages [4]. Further, most of cancers develop resistance after initial treatments [5]. Therefore, further studies are needed to delineate the underlying molecular mechanism in the progression of OCa.

In mammalian cells, the Shc A family includes 3 members, 46-, 52-, and 66-kDa isoforms [6,7]. They are derived from the same gene by alternative splicing, and all three Shc isoforms share a Src-homology 2 (SH2) domain, a collagen-homology (CH1) region and a phosphotyrosine-binding (PTB) domain. In addition, p66Shc isoform has a unique N-terminal (CH2) domain. It was shown that Shc proteins transmit receptor protein tyrosine kinase signals to downstream targets such as extracellular signal-regulated kinases/mitogen-activated protein kinases (ERK/MAPK) [8]. Further studies reveal that Shc proteins can exert mitogenic [6,9,10] and anti-apoptotic [11] effects, independent of Ras, in addition to its role in mediating apoptotic signal [12,13]. Therefore, Shc proteins may serve as a common convergent point of signaling pathways in regulating apoptosis and cell proliferation.

Recent advances indicate that these three proteins exhibit diverse biological activities [14]. For example, p66Shc, but not p52 or p46, functions as the longevity gene in mammals [7,15]. The effects of p66Shc protein on apoptosis and mitogenesis are mediated in part through its authentic oxidase activity in regulating mitochondrial metabolism. Following its translocation to the mitochondrial inner-membrane, which can be mediated by S36 phosphorylation-dependent and -independent processes, p66Shc protein interacts with cytochrome *c* to generate reactive oxygen species (ROS) [14,16,17]. p66Shc can also produce ROS via the Rac1-SOS signaling pathway at the plasma membrane [18]. It is thus hypothesized that in contrast to p52Shc that serves as a receptor tyrosine kinase (RTK)

adaptor protein [19,20], p66Shc plays a predominant role in mitochondrial ROS metabolism and oxidative stress [7,14].

p66Shc protein is predominantly expressed in epithelial cells and its aberrant expression is shown to be associated with several types of human cancer [20–23]. p66Shc protein can also mediate thyroid cell proliferation in a TSH-dependent manner [24]. Further, steroid and growth factor stimulation of prostate, testis and breast cancer cells are accompanied with an increase of p66Shc protein level [20]. Thus, due to the potential importance of p66Shc in steroid-related carcinogenesis [14], the molecular mechanism of p66Shc in mediating steroid-stimulated ovarian cell proliferation deserves further investigation.

In two OCa cell lines, p66Shc protein level was shown to be correlated with ErbB-2 expression, a prognostic marker of the cancer [25]. Nevertheless, the biological significance of this correlative relationship and the role of p66Shc in clinical ovarian carcinomas require further investigation. In parallel, estrogens are known to play a regulatory role in ovarian cell growth and involved in ovarian carcinogenesis [26,27]. In this report, our data show the association of p66Shc and ErbB-2 protein via ERK/MAPK with estrogens in promoting OCa cell proliferation. Furthermore, p66Shc protein is elevated in clinical ovarian carcinomas, higher than in non-cancerous ovarian cells. Thus, p66Shc protein can serve as a useful target for OCa therapy.

MATERIALS AND METHODS

Reagents, cDNA and Antibodies

RPMI 1640 medium, glutamine, gentamicin and 2',7'-dichlorodihydrofluorescein diacetate (H₂DCF-DA) were purchased from Invitrogen (Carlsbad, CA, USA). Fetal bovine serum (FBS) and Charcoal/dextran-treated, certified FBS were obtained from Atlanta Biologicals (Lawrenceville, GA, USA). Protein molecular weight standard markers, acrylamide, and the protein assay kit were obtained from Bio-Rad (Hercules, CA). Myc-tagged wild-type p66Shc cDNA was constructed in pcDNA3.1 vector [10]. Polyclonal Abs recognizing all three isoforms of Shc protein was purchased from Upstate Biotechnology Inc. (Lake Placid, NY, USA). Polyclonal antiphospho-ErbB-2 (pY1221/2) and anti-phospho-ERK/MAPK (Thr202/Tyr204) were purchased from Cell Signaling Technology (Danvers, MA, USA). Mouse monoclonal anti-phosphotyrosine (4G10), PD98059 and AG879 were from Millipore Corporation (Temecula, CA, USA). Polyclonal anti-ErbB-2 (C-18), anti-cyclin D1, anti-cyclin B1, anti-PCNA, anti-ERK/MAPK, horseradish peroxidase-conjugated anti-rabbit and anti-mouse IgG were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-β-actin, β-estradiol (E2), N-Acetyl cysteine (NAC), vitamin E succinate (VES), *p*-nitrophenyl phosphate and L-(+)-tartaric acid were obtained from Sigma (St Louis, MO, USA). An enhanced ECL detection system was purchased from Pierce (Rockford, IL).

Cell Culture

OCa cell lines, OVCAR-3, CaOV-3 and SKOV-3 cells, were purchased from the American Type Culture Collection (Manassas, VA). These cells were maintained per ATCC instructions: OVCAR-3 cells express functional estrogen receptors and are estrogen-

sensitive cells. They are routinely maintained in phenol red-positive RPMI 1640 medium supplemented with 20% FBS, 0.01 mg/ml bovine insulin, 2 mM glutamine and 50 µg/ml gentamicin. CaOV-3 cells are also positive for estrogen receptor and estrogen-sensitive and are routinely maintained in DMEM medium supplemented with 10% FBS, 2 mM glutamine and 50 µg/ml gentamicin. SKOV-3 cells express an inactive mutant of estrogen receptor and are maintained in McCoy's 5a medium supplemented with 10% FBS, 2 mM glutamine and 50 µg/ml gentamicin.

For E2 treatment, 1×10^4 cells/cm² of CaOV-3 cells were seeded in 6-well plates. The cells were allowed to attach for 2 days and the medium was replaced with a steroid-reduced medium (phenol red-free DMEM containing 5% charcoal/dextran-treated, heat-inactivated certified FBS, 2 mM glutamine and 50 µg/ml gentamicin) for 48 hours and the cells were then exposed to 10 nM E2. After a specified time period, cells were harvested by trypsinization and the cell number was counted by Cellometer™ Auto T₄. Briefly, cell number counting was conducted by Trypan blue assay based on the principle that live cells with intact cell membrane do not take up the dye, whereas dead cells stains with. The ratio of live cell number in the experimental group to that of the control group was calculated for indicating cell proliferation.

Immunoblotting

Subconfluent cells were harvested by scraping. The pelleted cells were rinsed with ice-cold 20 mM HEPES-buffered saline, pH 7.0, and then lysed in ice-cold cell lysis buffer containing protease and phosphatase inhibitors. The detailed protocols of immunoblotting were described previously [20,28,29]. The p66Shc protein level was analyzed by western blotting and then semiquantified by densitometric analyses of autoradiograms with different exposure time periods using the ImageJ software program (<http://rsb.info.nih.gov/>). The relative protein level was calculated by normalizing to the corresponding β-actin protein level.

Transfection

For cDNA transient transfection experiments, OVCAR-3 cells were plated at a density of 2×10^4 cells/cm² and transfected with pcDNA-p66 cDNA expression vector encoding the full-length wild-type p66Shc protein, using Lipofectamine and Plus reagents. Control cells were transfected with pcDNA vector alone [10]. Four hours after transfection, the cells were fed with regular medium and all cells were then harvested after 48 hours by trypsinization, and cell growth determination and immunoblotting were carried out. To knockdown endogenous p66Shc expression in SKOV-3 cells, pSUP-p66, a plasmid-based small interfering RNA system targeted against the CH2 region of p66Shc, was used [10]. Transfection of pSUP-p66 was performed as described above for cDNA transfection.

ROS Determination

Intracellular ROS level was measured by using the H₂DCF-DA as a fluorescent probe as described previously [17]. Briefly, the cells were rinsed with Krebs-Ringer buffer and exposed to 10 µM H₂DCF-DA in the dark for 30 min at 37°C. After the incubation, the cells were subsequently trypsinized and resuspended in Krebs-Ringer buffer. Intracellular

fluorescence was quantified by a flow cytometer (Becton-Dickinson's, Franklin Lakes, NJ, USA) by measuring the fluorescence intensity at excitation and emission wavelengths of 488 nm and 525 nm, respectively. The ROS levels were calculated as the relative ratio by normalizing to that of the control cells.

Acid Phosphatase Activity Determination

p-Nitrophenyl phosphate was used as the substrate to quantify the phosphatase activity by measuring the absorbance of released *p*-nitrophenol in KOH solution at 410 nm [29]. For acid phosphatase activity, pH 5.5 was used; for the neutral phosphatase activity, pH 7.0 was used [30]. L-(+)-tartrate is a classical inhibitor of phosphatases [29] and thus was used as an inhibitor in the assay to distinguish the phosphatase subfamily [28].

Generation of Anti-p66Shc Protein Antibody (Ab)

A peptide of eighteen amino acids corresponding to the CH2 domain of p66Shc was selected [22], synthesized and used for immunization of rabbits for the generation of polyclonal antibodies (GeneScript, NJ, USA). The rabbit anti-p66Shc antisera were stored in aliquots at -20°C until use. To validate the specificity of Ab to p66Shc protein, western blot analyses were performed with total cell lysate proteins from p66Shc-overexpressed and -knocked down prostate cancer cells, respectively. Immunohistochemistry staining was subsequently performed on human archival OCa tissues with Abs which were pre- and post-absorbed by p66Shc over-expressed human prostate cancer cells (details were described and shown in Figure 7) or the immunogen peptide as described previously [31].

Immunohistochemistry

The protocol for the usage of human ovarian archival specimens was approved by the Institutional Review Board at UNMC. To analyze p66Shc expression, we purchased two commercially available tissue arrays (OV1004 and OVC961; Biomax, Rockville, MD, USA) containing 76 human OCa archival specimens (40 serous adenocarcinoma, 18 endometrial adenocarcinoma, 10 mucinous adenocarcinoma and 8 other subtypes of carcinoma) and 22 non-cancerous tissues (12 normal tissue and 10 benign tumors) with duplicate cores per case. Immunohistochemical staining was carried out as described previously [20,30,32]. Due to the heterogeneity of ovarian carcinomas, each sample was given a composite score based on the intensity (1 to 3) and the extent of tissue staining (%). The intensity as well as the extent of staining was evaluated by a clinical pathologist (S.M.L.) in the double blind condition as described previously [30].

Statistical Analysis

All experiments in cell proliferation analyses were performed by at least two sets of independent experiments in duplicates or triplicates and the mean or the most representative results are presented. Statistical procedures for *p*-value analysis were performed by Student's *t*-test with 2 populations to determine the significance of comparison. To compare the difference in the expression level of p66Shc protein in non-cancerous vs. malignant tissue on the array, independent sample test was carried out using SPSS software program (Version 16.0; SPSS Inc., IL, USA). *p*<0.05 was considered statistically significant.

RESULTS

Cell Proliferation and p66Shc Protein Levels in Human Ovarian Cancer Cell Lines

We investigated if p66Shc protein plays a role in regulating the proliferation of OCa cells by analyzing cell proliferation and p66Shc protein levels in three human OCa cell lines. As shown in Figure 1A, among three OCa cell lines examined, OVCAR-3 cells had the slowest growth rate indicated by cell number counting. Western blot analyses showed that OVCAR-3 cells expressed a greatly lower level of p66Shc protein than rapidly growing SKOV-3 and CaOV-3 cells (Figure 1B). In this set of experiments, the p66Shc protein in OVCAR-3 cells was not clearly detected in the same amount of total cell lysate proteins as CaOV-3 and SKOV-3 cells by an ECL reagent; while it could be seen with a greater amount of cell lysate protein with prolonged exposure (data not shown). Furthermore, the level of cyclin D1 protein, which is a positive cell cycle regulator and conventionally used as a parameter for indicating cell proliferation, validated cell proliferation by cell number counting (Figure 1B). These data showed that the proliferation of these three OCa cells correlate with p66Shc protein levels.

Effect of p66Shc Protein Expression on the Proliferation of Ovarian Cancer Cells

To determine the effect of p66Shc protein expression on the growth rate of OCa cells, we transfected OVCAR-3 cells with a p66Shc cDNA expression vector encoding the wild type p66Shc protein [10]. Elevated expression of the wild-type p66Shc protein in OVCAR-3 cells resulted in the significant increase of cell proliferation ($p<0.05$) as indicated by cell number counting, which was further validated by elevated cyclin D1 protein levels (Figures 2A & 2B).

Conversely, we knocked down the expression of p66Shc protein in rapidly growing SKOV-3 cells by transfecting these cells with a pSUP-p66 vector encoding shRNA to p66Shc mRNA [10]. Reduced p66Shc protein expression by shRNA concurred with decreased cell proliferation as shown by cell number counting ($p<0.01$) as well as cyclin D1 protein levels (Figures 2C & 2D). In summary, the data taken together indicate a functional role of p66Shc protein in regulating the proliferation of OCa cells.

Estrogen Effect on Cell Proliferation, p66Shc Protein Level and ROS Production in CaOV-3 Cells

Since estrogens are known as a positive growth regulator of OCa cells [26,27], we investigated estrogen effects on p66Shc protein levels and cell proliferation in OCa CaOV-3 cells. While CaOV-3 cells are considered to be estrogen-sensitive cells and have a slower growth rate in a steroid-reduced medium than in regular medium, these cells grew very well in both steroid-reduced and regular culture conditions (data not shown). In the presence of 10 nM E2, the growth rate of CaOV-3 cells was increased significantly ($p<0.05$, Figure 3A) and the p66Shc protein level was also elevated (Figure 3B), higher than that in the corresponding control cells despite that those cells expressed a high basal level of p66Shc (See Figures 1B and 3B for comparison). Since p66Shc is an authentic oxidase involved in ROS production [16,17], we determined the intracellular ROS level in estrogen-treated CaOV-3 cells. As shown in Figure 3A, in E2-treated cells, ROS production was increased

with concurrent elevation of p66Shc protein level (Figure 3B). We further assessed the role of estrogen receptor (ER) in this mode of regulation utilizing tamoxifen, an ER antagonist in clinical usage. The E2-increased CaOV-3 cell proliferation and ROS production was competitively blocked by tamoxifen while no significant effect on the basal growth rate or ROS level (Figures 3A and 3B; $p < 0.05$). Similar results of ROS production by E2 treatment and competitive inhibition by tamoxifen were observed in OVCAR-3 cells (data not shown). Thus, the data together showed a positive correlation between p66Shc protein level, ROS production and estrogen stimulation on OCa cell proliferation.

To examine whether ROS is mediating estrogen-stimulated OCa cell proliferation, estrogen-sensitive CaOV-3 cells were treated with 10 nM E2 in the presence or absence of antioxidants, vitamin E succinate (VES) and N-acetyl cysteine (NAC), respectively. In the absence of E2, the treatment of VES and NAC could reduce the basal cell growth, following the dose of VES (Figure 3C) and NAC (Figure 3D), apparently due to the high basal level of p66Shc protein (Figures 1B and 3B). In the absence of antioxidants, E2 significantly stimulated cell proliferation ($p < 0.01$; Figure 3C, column # 1 vs. 4 & Figure 3D, column # 1 vs. 5; also Figure 3A); while, both VES and NAC respectively abolished estrogen-induced cell proliferation following a dose-dependent manner ($p < 0.01$, Figures 3C & 3D). The elevated cyclin D1 level by E2 was reduced by NAC and VES, respectively (Figure 3E). Thus, E2-stimulated OCa cell proliferation is at least in part via ROS production.

Estrogen Effects on Phosphatase Activity and ErbB-2 Tyrosine Phosphorylation in CaOV-3 and OVCAR-3 Cells

It has been shown that ROS stimulates cell proliferation in part by inhibiting the phosphatase activity for activating the corresponding tyrosine kinase specific activity [33,34]. We analyzed the effect of E2 on phosphatase activity. We initially quantified the tartrate-sensitive and the tartrate-insensitive acid phosphatase activity by the classical phosphatase activity assay in E2-treated CaOV-3 cells. In E2-treated cells, the tartrate-sensitive acid phosphatase activity was decreased, significantly lower than in the corresponding control cells ($p < 0.05$), while the tartrate-insensitive acid phosphatase activity was increased in E2-treated CaOV-3 cells (Figure 4A). We analyzed protein tyrosine phosphorylation profiles in estrogen-treated CaOV-3 cells. As shown in Figure 4B, in the absence of E2, the basal tyrosine phosphorylation level of a 185 kDa protein was very high in CaOV-3 cells, which was further elevated by E2 treatment. Since ErbB-2 exhibits a molecular weight of 185 kDa and is involved in regulating androgen-stimulated prostate cancer cell proliferation [35], we analyzed the phosphorylation status of ErbB-2 protein with a site-specific Ab. The phosphorylation level of Y1221/2 of ErbB-2 protein was greatly increased (lower panel, Figure 4B), indicating the activation of ErbB-2 protein in E2-treated CaOV-3 cells in which the tartrate-sensitive phosphatase activity is inhibited. These data showed that despite a high basal tyrosine phosphorylation level of a 185 kDa protein in CaOV-3 cells, in the presence of E2, the tyrosine phosphorylation level of ErbB-2 protein was further elevated.

We further determined the tartrate-sensitive and the tartrate-insensitive acid phosphatase and neutral phosphatase activities in E2- vs. H_2O_2 -treated OVCAR-3 cells. In E2-treated and

H₂O₂-treated cells, both the tartrate-sensitive neutral phosphatase activity (Figure 4C) and tartrate-sensitive acid phosphatase activity (data not shown) were decreased, significantly lower than in the corresponding control cells ($p < 0.05$). Nevertheless, the tartrate-insensitive neutral phosphatase activity (Figure 4C) and the tartrate-insensitive acid phosphatase activity (data not shown) were slightly increased in both E2-treated and H₂O₂-treated OVCAR-3 cells (Figure 4C, $p < 0.05$). These data indicate that the tartrate-sensitive phosphatase may function as a negative growth regulator, which is decreased in growth-stimulated OCa cells. We analyzed protein tyrosine phosphorylation profiles in estrogen-treated and H₂O₂-treated OVCAR-3 cells in which the phosphatase activity is decreased. As shown in Figure 4D, the Tyr-P level of a molecule with 185 kDa was increased, correlating with the increased phosphorylation level of Y1221/2 of ErbB-2 protein (lower panel, Figure 4D). Thus, in both E2-treated OCa cells, the TSP activity is inhibited and ErbB-2 is activated by tyrosine phosphorylation.

To determine the relationship between estrogens, p66Shc protein and ErbB-2 tyrosine phosphorylation in OCa cell growth, OCa cells were cultured in the presence of E2 and/or L(+)-tartrate. As shown in Figure 5A (Lane 1 vs. Lane #2 and #3), similar to E2 treatment, tartrate treatment increased p66Shc protein level and tyrosine phosphorylation of ErbB-2 (pY1221/2) in CaOV-3 cells. In addition, tartrate treatment significantly increased ERK/MAPK activation. Further, tartrate induced cell growth is clearly evidenced by the increase in cyclin B1 level (Figure 5A). Similarly, in OVCAR-3 cells, the inhibition of phosphatase by L(+)-tartrate was associated with elevation of p66Shc protein level, ErbB-2 tyrosine phosphorylation (pY1221/2) and ERK/MAPK activation (Figure 5B). We semiquantified the ErbB-2 tyrosine phosphorylation level in both OCa cells by densitometric analyses using Image J program followed by calculating the ratio with reference to control and E2-treated group in CaOV-3 and OVCAR-3 cells, respectively. The ratio of ErbB-2 tyrosine phosphorylation level in both E2-induced and L(+)-tartrate treated OCa cells was significantly higher than the control cells (Figures 5A and 5B). Interestingly, in both E2-induced and L(+)-tartrate treated OCa cells, the activation level of ErbB-2 were similar (Figures 5A and 5B, lane #2 vs. lane #3). The combination of E2 with L(+)-tartrate treatment had only a partial, added effect on p66Shc protein elevation, ErbB-2 activation, and cell proliferation in both OCa cells (Figures 5A and 5B, lanes #2 & #3 vs. lane #4). The data indicates that the two pathways at least are partially overlapping.

Further, to determine whether inhibition of ErbB-2 blocks the estrogen-induced cell growth, CaOV-3 cells were treated with different concentrations of ErbB-2 inhibitor AG879 in the presence or absence of E2. As shown in Figure 5C, AG879 inhibits the basal growth of CaOV-3 cells in the absence of estrogen, indicating a role of ErbB-2 in regulating ovarian cancer cell growth. Estrogens significantly stimulated cell proliferation ($p < 0.05$, column #5 vs #1, Figure 5C); while AG879 completely abolished E2 effect on cell growth (Figure 5C). Western blot analyses showed that E2 treatment increased ErbB-2 tyrosine phosphorylation (primarily at Y1221/2 and to a lesser degree at Y1248) and p66Shc protein level (Figure 5D), which were decreased by AG879 treatment following a dosage response (Figure 5D). Interestingly, in the absence of E2, AG879 inhibited pY1221/2 but not pY1248 of ErbB-2. In those cells, estrogen-elevated PCNA were abolished by AG879, validating growth

inhibition as shown in Figure 5C. Thus, the results clearly indicate that estrogens promote CaOV-3 cell proliferation via p66Shc protein and ErbB-2 phosphorylation (pY1221/2).

Estrogen effects on ERK/MAPK activation and cell proliferation in CaOV-3 Cells

To determine the downstream signaling molecule involved in estrogen-induced CaOV-3 cell proliferation, immunoblot analyses were performed on analyzing pERK/MAPK activation. In CaOV-3 cells, E2-treatment significantly increased cell proliferation (Figure 6A) and ERK activation shown by elevated pERK/MAPK levels (Figure 6B). The increase in cell proliferation was further supported by increased p66Shc and cyclin D1 protein levels (Figure 6B). Further, to examine the role of ERK/MAPK in E2-induced growth signal in CaOV-3 cells, those cells were treated with either PD98059 alone or PD98059 plus E2. As shown in Figure 6A, inhibition of ERK/MAPK significantly blocked E2-induced cell proliferation. Western blot analyses showed that PD98059 treatment inhibits both basal and estrogen-induced ERK/MAPK activation in CaOV-3 cells (Figure 6B). Nevertheless, PD98059 treatment only had partial effect on estrogen-induced p66Shc level, indicating that ERK/MAPK transduces the signals from p66Shc induced by estrogens. The PD98059 inhibitory effect on E2-induced CaOV-3 cell proliferation was further confirmed by cyclin D1 protein level (Figure 6B).

Characterization of Anti-p66Shc Protein Antibody (Ab)

To investigate the clinical significance of p66Shc protein expression in ovarian carcinomas, we generated the custom-made anti-p66Shc Abs and analyzed the specificity of these Abs. The commercial anti-Shc Ab that recognizes all three Shc family members was used as a reference. Among 12 antisera generated, only #18A Ab showed the specific reaction to p66Shc protein by western blot analyses. To test the specificity of this Ab, we took the advantage of our p66Shc-overexpressed S32 and S36 prostate cancer subclone cells which express high levels of p66Shc protein [10]. As shown in Figure 7A, immunoblotting results showed that #18A Ab only reacts with p66Shc protein, but not p52 or p46 Shc proteins, which co-migrated with p66Shc protein that reacted with the commercial anti-Shc isoform Ab (Figure 7A, Lanes #1&2 vs. #3&4). The specificity of Ab was further shown by shRNA experiments that knockdown p66Shc in S32 subclone cells correlates with decreased reactivity of #18A Ab (Figure 7A, Lanes #5–8). Further, after absorption with the total cell lysates from p66Shc-overexpressed S36 subclone cells, immunoblotting analyses revealed that the reactivity of the Ab to p66Shc is greatly decreased (Figure 7A, Lane #9 vs.10). Similarly, after absorption with the immunogen peptide, the reactivity of #18A Ab to p66Shc was decreased (data not shown). The #18A Ab (Figure 7B, Left panel) could readily react with p66Shc protein in OCa specimens; while the reactivity of post-absorbed Ab was greatly reduced (Figure 7B, Right panel). The data collectively indicate that #18A Ab specifically recognizes the 66kDa isoform of Shc proteins.

p66Shc Protein Expression in Archival Ovarian Cancer Tissues

To corroborate clinical relevance, we conducted immunohistochemistry staining of p66Shc protein in two tissue arrays containing 76 OCa archival specimens and 22 non-cancerous ovarian specimens. Figure 7C shows a representative staining pattern of p66Shc protein in an OCa tissue specimen vs. non-cancerous ovarian tissue. The immunohistochemistry

staining shows the predominant epithelial expression of p66Shc protein. Due to the heterogeneity of p66Shc protein staining in OCa tissues, we calculated the staining index by multiplying the staining intensity with the percentage of stained cells. We performed statistical analyses on the staining index of cancerous vs. non-cancerous tissues. The mean value of the composite score for p66Shc staining in non-cancerous epithelial samples was 0.95 ± 0.07 ($n=22$, mean $\pm$ SE), while the staining index in the cancerous tissue had a composite score of 1.36 ± 0.05 ($n=76$, mean $\pm$ SE). Statistical analyses showed that p66Shc staining index is significantly higher in carcinoma tissue than in non-cancerous epithelial cells ($p=0.00037$). The elevated level of p66Shc protein in cancerous tissue suggests its positive role involving in OCa progression.

DISCUSSION

p66Shc protein is proposed to play a critical role in regulating the growth and tumorigenicity of steroid hormone-sensitive human cancer cells [14]. We analyzed if p66Shc also plays a role in regulating the proliferation of human OCa cells. To investigate the involvement of p66Shc protein in regulating the proliferation of OCa cells, we used three OCa cell lines as the model system. Our results clearly show that the p66Shc protein level, but not p52 or p46Shc protein, correlates with the cell proliferation rate. Among them, OVCAR-3 cells grow very slow and show a very low level of p66Shc protein that could be detected only after loading a large amount of cellular protein with a prolonged exposure period with ECL reagents (data not shown). In slow-growing OVCAR-3 cells, elevated expression of p66Shc protein by cDNA transfection results in an increase of cell proliferation rates (Figures 2A and 2B). The only approximately 25% increase in cell number is in part due to the poor transfection efficiency of these slow-growing cells (data not shown). Supportively, knock-down of p66Shc protein level by its shRNA in rapid-growing SKOV-3 cells is associated with decreased cell proliferation by over 50% (Figures 2C and 2D). In parallel, p66Shc protein levels are higher in ovarian carcinoma cells than in non-cancerous cells (Figure 7C). Our data clearly support the notion that p66Shc protein plays a critical role in regulating the proliferation of OCa cells.

Our results further demonstrate that E2 increases p66Shc protein level and the growth rate of CaOV-3 cells (Figure 3). Similarly, treatments of steroid hormones androgens and estrogens increase p66Shc protein levels as well as cell proliferation rates in hormone-sensitive human prostate LNCaP C-33 and MDA PCa2b, testicular Tera-1 and Tera-2, and breast MCF-7 cancer cells, respectively [20]. In parallel, in thyroid cancer cells, p66Shc protein as well as cell growth is up-regulated in a TSH-dependent manner [24]. Further, in OCa cells, the functional estrogen receptors are required because tamoxifen, an antagonist in clinical usage, can effectively block estrogen action on ovarian cell proliferation and p66Shc level (Figures 3A & 3B), as observed in breast cancer [20]. Thus our results clearly demonstrate that estrogen-induced OCa cell proliferation is associated with p66Shc protein elevation.

One of the hallmarks of rapidly dividing cells is their ability to produce high amounts of intracellular ROS that in turn can promote cell cycle activity [36]. Increased production of ROS can promote cell proliferation and tumor progression [37,38]. In parallel, estrogens have been shown to increase the risk of OCa progression in part through promoting cell

proliferation [39–42]. The accelerated cell proliferation correlates with the increased level of intracellular ROS [43]. In estrogen-treated CaOV-3 cells, the cell proliferation increases and intracellular ROS level and p66Shc protein level are also elevated (Figures 3A and 3B). Conversely, antioxidants VES and NAC effectively block the estrogen-induced growth of CaOV-3 cells in a dose-dependent manner (Figures 3C and 3D). Thus, estrogens stimulate CaOV-3 cell proliferation apparently in part through ROS production. This ROS involvement in growth regulation is also seen in the basal growth of CaOV-3 cells (Figure 3C, Column #1–3; Figure 3D, Column #1–4) that express a high basal level of p66Shc protein and exhibit rapid cell growth (Figure 1). In ovarian cancers, higher levels of ROS-generating enzymes, such as p66Shc and superoxide dismutase (SOD), are found in cancerous cells than in non-cancerous cells in clinical archival carcinomas [Figure 7C; 43]. Further, p66Shc may play a role in regulating mitochondrial ROS metabolism and oxidative stress response [7,17,30]. This suggests that OCa cells expressing an elevated level of p66Shc protein have a higher proliferation potential than those cells expressing a low level of p66Shc protein (Figures 1 & 2). The importance of p66Shc protein in promoting OCa cell proliferation is further supported by immunohistochemical analyses that p66Shc protein level is significantly higher in cancerous cells than in non-cancerous cells in archival OCa specimens (Figure 7C, $p=0.00037$). Hence, the results of the present study reveal a non-genomic estrogen signal pathway in regulating hormone-sensitive OCa cell proliferation. Aberrant elevation of p66Shc can promote OCa cell progression.

In the tyrosine phosphorylation signal pathway, it is evidenced that ROS stimulates cell proliferation in part by inhibiting the phosphatase activity, leading to the activation of corresponding tyrosine kinases [30,33,34]. Several phosphatases, including acid, neutral and alkaline phosphatases, have been shown to dephosphorylate phosphotyrosine residues in various protein substrates [30,44–48]. This activity of phosphotyrosine dephosphorylation may allow them to function as negative cell growth regulators. As shown in Figure 4, estrogen treatments significantly reduce the tartrate-sensitive phosphatase activity and increase the tartrate-insensitive phosphatase activity in both CaOV-3 and OVCAR-3 cells, similar to the androgen effect on prostate cancer cells [49]. The elevated tartrate-resistant phosphatase activity in rapidly growing OCa cells is in consistent with observations in clinical OCa tissue in which TRAP is increased [50]. In the present study, we further observed the decreased tartrate-sensitive phosphatase activity and, concurrently, the increased tyrosine phosphorylation of ErbB2 at Y1221/2, correlating with estrogen-promoted cell proliferation.

Further, treatment of both CaOV-3 and OVCAR3 OCa cells with either estrogens or L(+)-tartrate shows elevated levels of p66Shc protein, ErbB-2 tyrosine phosphorylation (pY1221/2) and ERK/MAPK activation, correlating with cell growth indicated by cyclin B1 elevation (Figures 5A and 5B). L(+)-tartrate shows only a partial added effects to estrogens on ErbB-2 tyrosine phosphorylation. In parallel, estrogen-stimulated cell proliferation in CaOV-3 was effectively blocked by ErbB-2 inhibitor AG879, following a dose-dependent manner (Figure 5C). ErbB-2 inhibitor AG879 effectively decreased Y1221/2 phosphorylation of ErbB-2 protein in those cells, independent of E2 status. Interestingly, in those same cells, AG879 only abolished Y1248 phosphorylation in the presence of E2

(Figure 5D). The data together indicate that ErbB-2 signaling via pY1221/2 plays a critical role in regulating the proliferation of CaOV-3 cells, independent of estrogens.

We also observed that estrogen exerts its stimulatory effects on OCa cells via the ERK/MAPK signaling pathway (Figure 6). Inhibition of ERK/MAPK with PD98059 abolishes E2-induced CaOV-3 cell proliferation (Figures 6A and 6B). In parallel, the same ERK/MAPK signaling pathway is activated in steroid-stimulated breast and prostate cancer cells [10,14]. Therefore, our results together with previous observations suggest that steroids up-regulate cell proliferation at least in part via activating the ERK/MAPK signaling pathway in hormone-sensitive cells. Thus, we propose that E2 and ROS inhibit the tartrate-sensitive phosphatase and thus promote OCa cell proliferation by increasing p66Shc protein level and ErbB-2 tyrosine phosphorylation, through ERK/MAPK activation.

In summary, our results reveal a functional role of p66Shc protein in regulating the proliferation of OCa cells. The elevated level of p66Shc protein in ovarian carcinomas may serve as a viable target for its therapy. Further studies should also clarify the identity of the tartrate-sensitive phosphatase that is involved in the regulation of ErbB-2 signaling by estrogen-induced ROS in OCa cells. This phosphatase may also serve as a novel target for the development of human OCa therapy.

Acknowledgments

This work was supported in part by the National Cancer Institute, National Institutes of Health [R01 CA88184]; Department of Defense [W81XWH-06-1-0070, W81XWH-08-1-0459]; Nebraska DHHS [LB 506 #2010-18]; the Nebraska Research Initiative; and the University of Nebraska Medical Center Bridge Fund.

ABBREVIATIONS

Ab	antibody
E2	β -estradiol
ECL	enhanced chemiluminescence
ER	estrogen receptor
ERK/MAPK	extracellular signal-regulated kinases/mitogen-activated protein kinases
FBS	fetal bovine serum
cFBS	charcoal/dextran-treated FBS
H₂DCF-DA	2',7'-dichlorodihydrofluorescein diacetate
OCa	Ovarian cancer
PTB	protein tyrosine binding
PTK	protein tyrosine kinase
RTK	receptor tyrosine kinase
Shc	Src homologue and collagen homologue
Src	Rous Sarcoma virus protein

ROS	reactive oxygen species
TSP	tartrate-sensitive phosphatase

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Figure 1.

Cell proliferation and p66Shc protein levels in different human ovarian cancer cell lines. (A) OVCAR-3 (OV), SKOV-3 (SK) and CaOV-3 (Ca) cells were plated in 6-well plates in duplicates with a density of 1×10^4 cells/cm² in their respective regular medium. Cells were allowed to attach for 48 hours and then refreshed with their respective regular medium. Cells were harvested 48 hours later by trypsinization and cell proliferation was analyzed by cell counting. (B) Immunoblotting was performed for analyzing Shc proteins and Cyclin D1 proteins in the total cell lysate protein with 40 µg per lane. The level of β-actin protein was detected as an internal loading control. The ratio of p66Shc protein to the corresponding β-actin protein is shown at the bottom of the figure. Similar results were observed in 3 sets of independent experiments (n=2×3, *p<0.05, compared with OVCAR-3 cells).

Figure 2.

Effect of altered p66Shc expression on ovarian cancer cell proliferation. (A) OVCAR-3 cells were plated in 6-well plates in duplicates at 2×10^4 cells/cm² for 48 hours. Cells were then transfected with pcDNA-p66 cDNA encoding the full-length wild-type p66Shc protein for 4 hours. Control cells were transfected with pcDNA vector alone. Cells were then refreshed with their regular media, and then harvested after 48 hours for cell counting. The data shown are representative of two sets of independent experiments in duplicates ($n=2 \times 2$, $*p < 0.05$). (B) Immunoblotting was performed for analyzing p66Shc and Cyclin D1 protein levels in OVCAR-3 cells transfected with p66Shc cDNA or the vector alone. The level of β-actin protein was detected as an internal loading control for calculating the relative ratio of p66Shc to β-actin. (C) SKOV-3 cells were plated in 6-well plates in duplicates at 1×10^4 cells/cm² for 48 hours. Cells were then transfected with pSUP-p66 encoding shRNA against the CH2 region of p66Shc mRNA. Control cells were transfected with pSUP vector alone.

Cells were refreshed with their regular medium and harvested after 48 hours by trypsinization and cell proliferation was analyzed by cell counting. Similar results were observed in 2 sets of independent experiments in duplicates ($n=2 \times 2$, $**p < 0.01$). (D) Immunoblotting was performed for analyzing p66Shc and Cyclin D1 protein levels in p66Shc shRNA transfected SKOV-3 cells. The level of β -actin protein was detected as an internal loading control. The p66Shc protein level was normalized to β -actin for calculating the ratio.

Figure 3.

Estrogen effects on p66Shc protein and reactive oxygen species (ROS) levels in CaOV-3 cells. (A) CaOV-3 cells were plated in duplicates at a density of 8×10^3 cells/cm² for 48 hours and then steroid starved for another 48 hours. Cells were then treated with β -estradiol (10 nM) alone, β -estradiol in combination with tamoxifen (1 μ M) or tamoxifen alone in steroid-reduced condition. Cells were then harvested after 24 hours for ROS determination using DCF-DA as a fluorescent probe and cell growth determination by cell number counting. (B) Immunoblotting was performed for analyzing p66Shc protein levels in CaOV-3 cells. The level of β -actin protein was detected as an internal loading control and

for calculating the relative ratio. (C and D) CaOV-3 cells were plated in duplicates at 1×10^4 cells/cm² in regular medium and allowed to attach for 48 hours. Cells were steroid starved in a steroid-reduced medium for 48 hours, and then treated with 10 nM β -estradiol (E2) in the presence or absence of different doses of vitamin E succinate (VES) (C) or N-acetyl cysteine (NAC) (D). Cells were harvested after 48 hours of treatment by trypsinization and cell proliferation was analyzed by cell number counting. (E) Immunoblotting was performed in E2 and antioxidants treated CaOV-3 cell lysates for Cyclin D1 protein levels. β -actin protein levels were detected as an internal loading control. Similar results were obtained from 3 sets of independent experiments in duplicates ($n=2 \times 3$, $*p < 0.05$, $**p < 0.01$; correlation coefficient: $r_1 = -0.969$ for NAC in the absence of E2, $r_2 = -0.952$ for NAC in the presence of E2).

Figure 4.

Estrogen effects on phosphatase activity and tyrosine phosphorylation profiles in ovarian cancer cells. (A) CaOV-3 cells were plated at 1×10^4 cells/cm² in regular media and allowed to attach for 48 hours. Cells were steroid-starved in a steroid-reduced medium for 48 hours and then treated with 10 nM β -estradiol (E2) for 24 hours. Cells were harvested by trypsinization and the tartrate-sensitive and the tartrate-insensitive acid phosphatase activity in the total cell lysate protein was determined. Similar results were obtained from 5 sets of independent experiments ($n=5$, $*p<0.05$). (B) Immunoblotting analyses were performed on CaOV-3 cell lysates with anti-Tyr(P) 4G10 Ab and antiphospho-Y1221/2 ErbB-2 Ab, respectively. After stripping, membranes were rehybridized with anti-ErbB-2 protein Ab (C-18) for the protein level. The arrow indicates the position of molecular mass of 185 kDa. Similar results were obtained from 3 sets of independent experiments. (C) OVCAR-3 were plated at 1×10^4 cells/cm² in regular media and allowed to attach for 48 hours. Cells were

steroid starved in a steroid-reduced medium for 48 hours and then treated with 10 nM β -estradiol (E2) and H_2O_2 for 24 hours. Cells were harvested by trypsinization and the tartrate-sensitive and the tartrate-insensitive neutral phosphatase activity in the total cell lysate protein was determined. Similar results were obtained from 5 sets of independent experiments ($n=5$, $*p<0.05$). (D) Immunoblot analyses were performed on OVCAR-3 cell lysates with anti-Tyr(P) 4G10 Ab and antiphospho-Y1221/2 ErbB-2 Ab, respectively. After stripping, membranes were rehybridized with anti-ErbB-2 protein Ab (C-18) for the protein level. The arrow points out the molecular mass of 185 kDa position. Similar results were obtained from 3 sets of independent experiments. The ratio of pY1221/2 to ErbB-2 protein was calculated after densitometric analyses.

Figure 5.

Effects by estrogens and phosphatase inhibitor L(+)-tartrate on Shc and ErbB-2 phosphorylation in OCa cells. (A) CaOV-3 cells were plated at 3×10^4 cells/cm² in duplicates in regular medium. After steroid starvation, cells were treated with 10 nM β -estradiol (E2) and/or L(+)-tartrate for 16 hours. Cells were harvested and immunoblotting analyses were performed to analyze pErbB-2 (phospho-Y1221/2), p66Shc, pERK/MAPK and Cyclin B1 protein levels. The membranes were then stripped and re-hybridized with anti-ErbB-2 protein and anti-ERK/MAPK protein Ab. β -actin protein level was detected as an internal loading control. (B) OVCAR-3 cells were plated at 6×10^4 cells/cm² in duplicates in regular medium.

After steroid starvation, cells were treated with 10 nM β -estradiol (E2) and/or L(+)-tartrate for 16 hours. Cells were harvested and immunoblotting was performed to analyze pErbB-2 (phospho-Y1221/2), p66Shc, pERK/MAPK and Cyclin B1 protein levels. The membranes were then stripped and re-hybridized with anti-ErbB-2 and anti-ERK/MAPK Ab. β -actin protein level was detected as an internal loading control. (C) CaOV-3 cells were plated at 1×10^4 cells/cm² in duplicates in regular medium. After steroid starvation, the cells were treated with different doses of ErbB-2 inhibitor AG879 in the presence or absence of 10 nM β -estradiol (E2) in steroid-reduced medium for 24 hours. Cell growth was analyzed by cell counting. (D) Cell lysates from E2 and ErbB-2 inhibitor treated samples were subjected to immunoblotting analyses with antiphospho-Y1221/2 and antiphospho-Y1248 ErbB-2 Ab, respectively. After stripping, membranes were hybridized with anti-ErbB-2 Ab (C-18) for the protein levels. The level of Shc and PCNA proteins were also analyzed. β -actin protein was detected as an internal loading control. Similar results were obtained from 4 sets of independent experiments ($n=2 \times 4$, $*p<0.05$, $**p<0.01$; correlation coefficient: $r_1 = -0.888$ (AG879 in the absence of E2), $r_2 = -0.942$ (AG879 in the presence of E2)).

Figure 6.

Effects of ERK/MAPK inhibitor PD98059 on estrogen-induced ERK/MAPK activation and cell proliferation in CaOV-3 cells. (A) CaOV-3 cells were plated in 6-well plates in duplicates at a density of 2×10^4 cells/cm² and allowed to attach for 48 hours. Cells were steroid starved for 48 hours and then treated with solvent ethanol, 10 nM β-estradiol (E2) in ethanol, PD98059 alone or the combination of E2 with PD98059. Cells were harvested after 48 hours of treatment by trypsinization and cell proliferation was analyzed by cell counting. (B) Immunoblotting was performed for analyzing p66Shc and Cyclin D1 protein levels and the phosphorylation level of ERK/MAPK. The membrane for pERK was stripped and re-hybridized with anti-ERK1/2 protein Ab. β-actin protein level was detected as an internal loading control. The ratio of p66Shc protein to the corresponding β-actin protein is shown at

the bottom of the figure. Similar results were observed in 2 sets of independent experiments ($n=2 \times 2$, $**p < 0.01$).

Figure 7.

Characterization of anti-p66Shc #18A Ab and immunohistochemistry staining of p66Shc protein in archival ovarian carcinomas. A. The total cellular lysate proteins from LNCaP C-33 prostate cancer and its p66Shc-overexpressed subclone cells were used for analyzing the specificity of anti-p66Shc protein #18A Ab by immunoblotting. The total cell lysate proteins of S36 subclone cells and LNCaP C-33 parental cells were resolved in SDS-PAGE and probed with a commercially available anti-Shc Ab (Lanes 1 & 2) or #18A antisera (Lanes 3 & 4). Conversely, the lysate proteins of S32 subclone cells transfected with shRNA or control vector were probed with commercially available anti-Shc Ab (Lanes 5 & 6) or

#18A antisera (Lanes 7 & 8). Further, pre- and post-absorbed #18A Ab were used to detect p66Shc in S36 cell lysate proteins (Lane #9 vs. #10). B. Specificity of #18A anti-p66Shc antisera in immunostaining of human ovarian tissue archival specimen (Serous papillary ovarian cancer stage IIIc, grade 3). Subsequent sections of paraffin-embedded, formalin-fixed ovarian tissue were reacted with pre- and post-absorbed #18A antisera. C. Representative immunohistochemical staining of p66Shc protein in human ovarian cancer tissue array. Intense staining was observed in serous adenocarcinoma cancer tissues (left panel) when compared to non-cancerous ovarian epithelial region (right panel). Bars, 100 μ m. Contrast and color balance were uniformly adjusted for comparison.