Oncogene (2005) 24, 7203–7212

www.nature.com/onc

Expression of p66^{Shc} protein correlates with proliferation of human prostate cancer cells

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p66^{Shc}, an isoform of Shc adaptor proteins, is shown to mediate various signals, including cellular stress. However, little is known about its involvement in carcinogenesis. We previously showed that p66^{Shc} protein level is upregulated by steroid hormones in human carcinoma cells and is higher in prostate cancer (PCa) specimens than adjacent noncancerous cells. In this study, we investigated the role of p66^{Shc} protein in PCa cell proliferation. Among different PCa cell lines tested, p66^{Shc} protein level showed positive correlation with cell proliferation, that is, rapid-growing cells expressed higher p66^{Shc} protein than slow-growing cells. Exposure of slow-growing LNCaP C-33 cells to epidermal growth factor (EGF) and 5α-dihydrotestosterone (DHT) led to upregulation of proliferation and p66^{Shc} protein level. Conversely, growth suppression of fast-growing cells by cellular form of prostatic acid phosphatase (cPAcP) expression, a negative growth regulator, downregulated their p66^{Shc} protein level. Additionally, increased expression of p66^{Shc} protein by cDNA transfection in LNCaP C-33 cells resulted in increased cell proliferation. Cell cycle analyses showed higher percentage of p66^{Shc}-overexpressing cells at S phase (24%) than control cells (17%), correlating with their growth rates. On the other hand, transient knock-down of p66^{Shc} expression by RNAi in rapidly growing cells decreased their proliferation as evidenced by the reduced cell growth as well as S phase in p66^{Shc}-knocked down cells. The p66^{Shc} signaling in cell growth regulation is apparently mediated by extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK). Thus, our results indicate a novel role for p66^{Shc} in prostate carcinogenesis, in part, promoting cell proliferation.
Oncogene (2005) **24,** 7203–7212. doi:10.1038/sj.onc.1208852; published online 19 September 2005

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Received 17 December 2004; revised 4 May 2005; accepted 4 May 2005; published online 19 September 2005

Keywords: p66^{Shc}; prostatic acid phosphatase; prostate cancer; cell proliferation

Introduction

Shc (Src homolog and collagen homolog) proteins are adaptor molecules that contain SH2 and PTB domains (Ravichandran, 2001). They exist in three different isoforms with molecular masses of 46, 52 and 66 kDa. Shc proteins are conventionally known to transduce the mitogenic signals from receptor tyrosine kinases to downstream targets, such as, extracellular signal-regulated kinases/mitogen-activated protein kinases (ERK/MAPK) (Ravichandran, 2001). All the isoforms contain three functional domains – an SH2 domain, a PTB domain and a CH1 domain with three conserved tyrosine residues that are phosphorylated with respect to various signals (Ravichandran, 2001). Additionally, p66^{Shc} has a unique CH2 domain at the N-terminus of the protein, which contains a serine residue that could be phosphorylated under stress signals (Migliaccio *et al*., 1999).

Different members of the Shc proteins exhibit distinct expression patterns and biological functions. For example, p52^{Shc} and p46^{Shc} are expressed in most of the cells, while p66^{Shc} protein is expressed predominantly in epithelial cells (Migliaccio *et al*., 1997). Both p52^{Shc} and p66^{Shc} are distributed throughout the cytosol, whereas p46^{Shc} localizes to mitochondria (Ventura *et al*., 2004). Nevertheless, recent studies indicate that in response to stress signals, such as, H_2O_2 treatment, a fraction of cytosolic p66^{Shc} is translocated to mitochondria, where it is associated with heat–shock protein to mediate apoptotic response (Orsini *et al*., 2004). Despite that p66^{Shc}, like p52^{Shc}/p46^{Shc}, is phosphorylated at its tyrosine residues during epidermal growth factor (EGF) treatment and forms complexes with Grb-2, there are certain functional differences between p66^{Shc} and the other two Shc members. First, p66^{Shc}, unlike p52^{Shc}, could not transform NIH3T3 mouse fibroblast cells *in vitro* (Migliaccio *et al*., 1997). Second,

overexpression of p66Shc protein in cell lines, such as, HeLa, CHO and COS-1 cells, does not increase EGF-induced ERK/MAPK activation (Migliaccio *et al*., 1997; Okada *et al*., 1997). Third, p66Shc is phosphorylated at Ser^{36} in its CH2 domain under various stress signals such as H_2O_2, UV radiation and exposure to chemicals, such as, Taxol, and thus could serve as an apoptotic sensitizer to stress signals (Migliaccio *et al*., 1999; Yang and Horwitz, 2000). Finally, p66Shc acts as a negative regulator of human and mouse T-cell survival and proliferation (Pacini *et al*., 2004) and has been implicated in the control of lifespan in mammals (Migliaccio *et al*., 1999). Thus, data from the above studies clearly indicate p66Shc as a transducer of signals in response to cellular stress. However, its role in human carcinogenesis is not understood.

In human carcinoma cells, p66Shc may be involved in regulating their proliferation. Among ErbB-2-positive ovarian cancer cell lines, a positive correlation between p66Shc and ErbB-2 is observed (Xie and Hung, 1996). In breast cancer cell lines, elevated expression of p66Shc protein is observed in those with highly metastatic ability and similar phenomenon is seen in lymph node-positive breast tumors (Jackson *et al*., 2000). However, another study shows that p66Shc protein level inversely correlates with the expression of ErbB-2, a prognostic marker for breast cancer cell lines (Xie and Hung, 1996). Additionally, studies on archival breast cancer specimens have showed that the expression of p66Shc protein inversely correlates with the relapse of breast cancer (Davol *et al*., 2003). Hence, the role of p66Shc in carcinogenesis requires further investigations.

We previously showed that sex steroid hormones including estrogens and androgens upregulate p66Shc protein level in human carcinoma cell lines (Lee *et al*., 2004a). These data suggest the role of p66Shc protein in sex hormone-regulated cancer cell proliferation. Furthermore, p66Shc level is significantly elevated in cancerous cells compared to the neighboring noncancerous cells in clinical archival prostate cancer (PCa) specimens (Lee *et al*., 2004a). Therefore, p66Shc might play a role in the development and/or progression of PCa. Hence, in this study, we explored the role of p66Shc in the regulation of PCa cell proliferation that could lead to cancer progression.

Results

Expression of p66Shc protein in different human PCa cell lines

Based on our previous observations on archival PCa specimens that p66Shc protein level is elevated in cancerous cells, we analysed the expression profile of p66Shc in different PCa cell lines, which exhibit different growth rates. As shown in Figure 1, Western blot analyses with an anti-Shc antibody (Ab) recognizing all three isoforms of Shc protein showed that rapid-growing PC-3, DU 145 and TSU-Pr1 PCa cells expressed higher levels of p66Shc protein as compared to slow-growing MDA PCa2b and LNCaP C-33 cells. The low level of p66Shc protein in MDA PCa2b cells was detected only after prolonged exposure (data not shown). The protein levels of p52Shc and p46Shc were similar in all the cell lines examined. The phenomenon that rapidly growing cells express high level of p66Shc protein was also observed with another Ab that specifically reacts with p66Shc protein alone (Figure 1). p66Shc protein levels in the rapid-growing cells were approximately 4–13-fold higher than that in slow-growing LNCaP C-33 cells and over 10-fold higher than in MDA PCa2b cells. Interestingly, among those PCa cells, the p66Shc protein level inversely correlated with the expression level of cellular form of prostatic acid phosphatase (cPAcP) (Figure 1), the major protein tyrosine phosphatase and a negative growth regulator in prostatic epithelia (Lin *et al*., 1998, 2001).

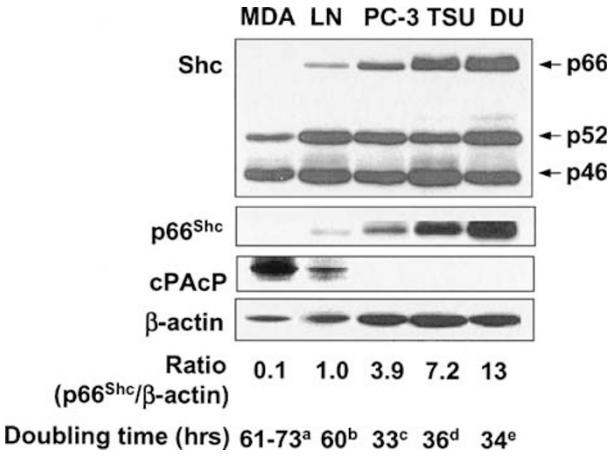

Figure 1 Expression of p66Shc protein in different PCa cells. LNCaP C-33, PC-3, TSU-Pr1 and DU 145 cells were plated in a medium supplemented with 5% fetal bovine serum (FBS), while MDA PCa2b cells were plated in a medium containing 20% FBS for the experiments. An aliquot of total cell lysates from MDA PCa2b (MDA), LNCaP C-33 (LN), PC-3 (PC), TSU-Pr1 (TSU), and DU145 (DU) cells was electrophoresed, transferred to nitrocellulose membranes, and incubated with Abs against Shc, p66Shc and cPAcP proteins, respectively. The level of β-actin protein was detected as an internal loading control. The intensity of hybridization band was semiquantified using a densitometer. The relative level of p66Shc protein to the corresponding β-actin protein was calculated and then normalized to that of LNCaP C-33 cells. The ratio was shown at the bottom of the figure. Similar results were observed in three sets of independent experiments. The doubling time of each cell line has been given as a measure of their growth rates ([a]Navone *et al*., 1997; [b]Horoszewicz *et al*., 1983; [c]Kaighn *et al*., 1979; [d]Iizumi *et al*., 1987; [e]Stone *et al*., 1978)

To examine further the correlation of p66Shc protein expression with cell growth, we analysed p66Shc protein level in different passages of LNCaP cells, utilizing Ab recognizing all the three isoforms of Shc protein. As reported earlier (Lin *et al*., 1998; Igawa *et al*., 2002), the growth ratio of C-81 cells was approximately three times higher than that of C-33 cells, while C-51 cells exhibit a growth rate faster than C-33 cells but slower than C-81 cells (Figure 2a). Western blot analyses revealed that p66Shc protein expression was higher in C-81 cells than in

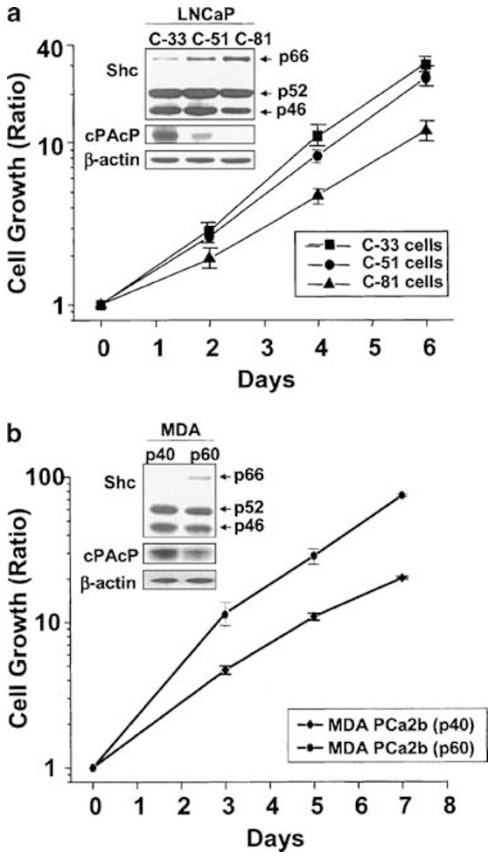

Figure 2 The growth rate and the expression of p66Shc protein in different passages of LNCaP and MDA PCa2b cells. (**a**) Different LNCaP cells were seeded and maintained as described in Materials and methods. The total cell number was counted on day 2, 4 and 6. Similar results were observed in three sets of independent experiments performed in duplicates (mean ± s.d.). Western blot analyses of Shc, cPAcP and β-actin proteins in LNCaP C-33, C-51 and C-81 cells were performed by incubating with the corresponding Abs (inner panel). (**b**) Different MDA PCa2b cells were seeded and maintained as described in Materials and methods. The total cell number was counted on day 3, 5 and 7. Similar results were observed in two sets of independent experiments done in duplicates (mean ± s.d.). Immunoblot analyses of Shc, cPAcP and β-actin proteins in both low (MDA p40) and high (MDA p60) passage MDA PCa2b cells were performed by incubating with the corresponding Abs (inner panel)

C-51 and C-33 cells, whereas p52Shc protein level was approximately similar in all cells (Figure 2a, inner panel). Since Shc family members are tyrosine phosphorylated in mediating the growth signals from tyrosine phosphorylated receptor tyrosine kinases (Ravichandran, 2001; Lee *et al*., 2004b), we analysed the tyrosine phosphorylation level of p66Shc protein by Western blotting, following immunoprecipitation with anti-Shc antibody, in different LNCaP cells. Unexpectedly, we could not detect any tyrosine phosphorylation of p66Shc protein in different passages of LNCaP cells by the enhanced chemiluminescence (ECL) method (data not shown, Lee *et al*., 2004b). Similar negative results were obtained by using the antibody against pY239 or pY317 of p52Shc that could also react with the corresponding sites in p66Shc protein with the total cell lysate proteins (Lee *et al*., 2004b). Nevertheless, cPAcP was highly expressed in C-33 cells, while it was only barely detected in C-81 cells, thus, inversely correlating with their relative p66Shc protein levels (Figure 2a, inner panel). Similarly, MDA PCa2b cells of passage number 60 (MDA p60) grew faster than those of passage number 40 (MDA p40) (Figure 2b). Concurrently, MDA p60 cells expressed a higher level of p66Shc protein than MDA p40 cells (Figure 2b, inner panel). The low level of p66Shc protein in MDA p40 cells could be detected only after prolonged exposure (data not shown). Furthermore, the elevated level of p66Shc protein in MDA p60 cells corresponded to the decreased cPAcP protein (Figure 2b, inner panel). Thus, the data collectively indicate that the expression level of p66Shc protein correlates, positively with PCa cell proliferation rate and, inversely with cPAcP expression.

Effects of growth manipulation on p66Shc protein level in different PCa cells

Since p66Shc protein level positively correlates with the PCa cell growth, we analysed whether manipulation of PCa cell growth would alter the expression level of p66Shc protein. We used EGF and 5α-dihydrotestosterone (DHT), which could upregulate the proliferation of LNCaP C-33 cells and downregulate the cPAcP (Lin *et al*., 1992, 1998; Meng and Lin, 1998; Lee *et al*., 2004b), and examined their effects on the protein levels of p66Shc in those cells. As shown in Figure 3a, p66Shc protein level was upregulated in LNCaP C-33 cells treated for 24 h with 10 ng/ml EGF or 10 nM DHT, approximately 2.5–3-fold higher than that in the corresponding control cells.

Owing to the inhibitory effect of cPAcP on PCa cell proliferation (Lin *et al*., 1998, 2001), we examined whether increased proliferation by inhibiting cPAcP activity would lead to the elevation of p66Shc protein in those cells. LNCaP C-33 cells that express endogenous cPAcP were exposed to different concentrations of L(+)-tartrate, a classical inhibitor of PAcP (Lin *et al*., 1992; Meng and Lin, 1998). In the presence of 2.5 mM L(+)-tartrate, the expression level of p66Shc protein was elevated by approximately 2.5-fold (Figure 3b), corresponding to the decrease in cPAcP activity (data not shown). 5 mM L(+)-tartrate had the similar effect as 2.5 mM concentration. Furthermore, the increase of p66Shc in L(+)-tartrate-treated cells correlated with increased ERK/MAPK activation (Figure 3b) and cell proliferation (data not shown). It should be noted that L(+)-tartrate had no effect on the levels of p52Shc and p46Shc proteins. Thus, elevated p66Shc protein level correlates with ERK/MAPK activation as well as cell proliferation in cPAcP-inhibited PCa cells.

As an additional approach, we examined the p66Shc protein expression in PAcP cDNA-transfected stable subclones of LNCaP C-81 cells that have reduced proliferation rates (Lin *et al*., 1998, 2001; Meng and Lin, 1998). Western blot analyses showed that LN-23 and LN-40 subclone cells, which express exogenous cPAcP, had lower levels of p66Shc protein when

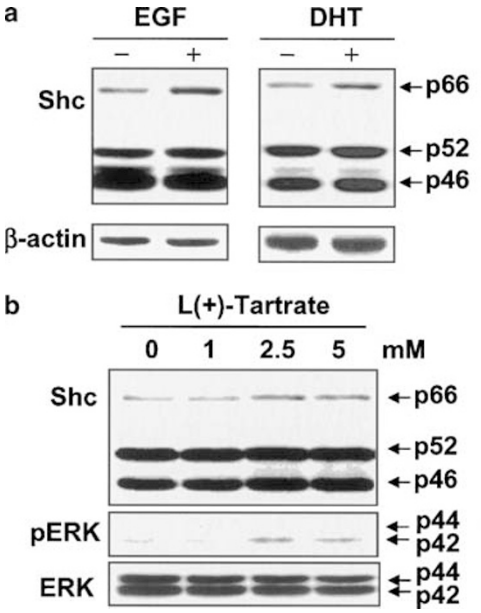

Figure 3 Effects of EGF, DHT and L(+)-tartrate on p66Shc protein expression in LNCaP C-33 cells. (**a**) LNCaP C-33 cells were preincubated in a steroid-reduced medium for 48 h and then treated with EGF (10 ng/ml) or DHT (10 nM) for 24 h. Control cells received the solvent alone. Total cell lysates were used for Western blot analyses of Shc protein level. The level of β-actin protein was detected as a loading control. Similar results were obtained from three sets of independent experiments. (**b**) LNCaP C-33 cells were treated with 0, 1, 2.5 and 5 mM of L(+)-tartrate for 16 h. Total cell lysates were used for analysing the protein levels of Shc and ERK/MAPK as well as the phosphorylation level of ERK/MAPK. Similar results were obtained from two sets of independent experiments

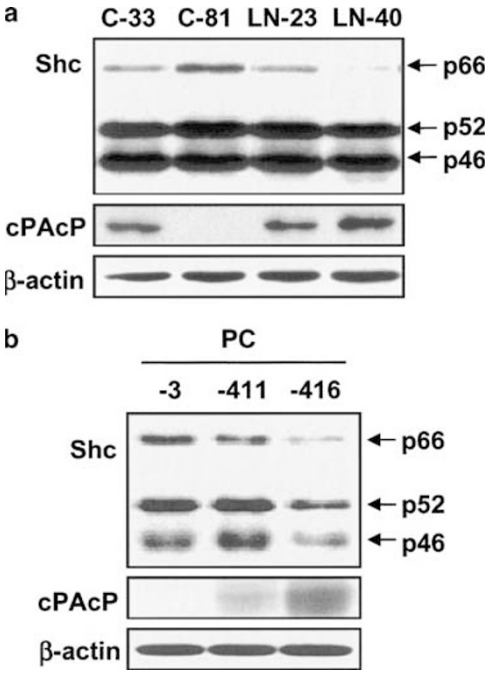

Figure 4 Expression of p66Shc protein in the PAcP cDNA-transfected LNCaP C-81 and PC-3 stable subclone cells. (**a**) The protein levels of Shc and cPAcP in LNCaP C-33 and C-81 cells as well as LN-23 and LN-40 cells were analyzed by Western blot analyses. The level of β-actin protein was analysed as a loading control. (**b**) Western blot analyses of Shc, cPAcP and β-actin proteins in PC-3, PC-411 and PC-416 cells. Similar results were obtained from two sets of independent experiments

compared to C-81 parental (Figure 4a). Similarly, PAcP cDNA-transfected PC-3 stable subclone cells that had slower growth rates compared to their parental cells (Lin *et al*., 1998; Meng and Lin, 1998) also expressed lower levels of p66Shc protein than their parental cells (Figure 4b). Taken together, the results indicated that manipulation of PCa cell growth leads to a corresponding change in p66Shc expression level.

Effect of p66Shc protein expression on the growth of PCa cells

We determined the effect of p66Shc protein on the growth rate of PCa cells. Initially, LNCaP C-33 cells were transiently transfected with a cDNA encoding the wild-type p66Shc protein and cell growth was analysed. Results indicated that transient elevation of p66Shc protein in LNCaP C-33 cells resulted in a reduction in the doubling time by approximately 26%, indicating an increased cell proliferation, which correlated with the increased activation of ERK/MAPK (data not shown). We subsequently established stable subclones of LNCaP C-33 cells, that is, S-32 and S-36 cells, which stably express the exogenous p66Shc protein. For experimental control, the vector alone-transfected stable subclone cells, that is, V-1 cells were established as well. Despite that different passages of LNCaP cells exhibit morphological changes, we did not observe any noticeable morphological difference between the subclone cells overexpressing p66Shc and V-1 and LNCaP C-33 cells (data not shown). As shown in Figure 5a (left panel), in S-32 and S-36 subclone cells, elevated levels of p66Shc protein correlated with the activation of ERK/MAPK, indicated by increased levels of phosphorylation. The cell growth was initially evaluated by analysing the doubling time of those cells. Results showed that elevated p66Shc protein levels correlated with rapid cell growth with a reduction in the doubling time of p66Shc-overexpressing stable subclones, that is, approximately 30 h for S-36 cells and 33 h for S-32 cells compared to the control cells, that is, 45 h for V-1 and C-33 parental cells (Figure 5a, right panel). We subsequently analysed the cell cycle distribution of those cells with flow cytometry. The S-32 and S-36 cells exhibited higher proliferation rates than the parental C-33 and V-1 cells, as indicated by the percentage of cell population in the S phase of cell cycle, that is, 17, 17.6, 22.3 and 24.1% for LNCaP C-33, V-1, S-32 and S-36 cells, respectively (Figure 5b). The data collectively suggest that elevated p66Shc protein in PCa cells, possibly through the activation of ERK/MAPK, plays a role in promoting their proliferation resulting in an increased percentage of cells in the S phase of the cell cycle.

To further confirm the role of p66Shc in PCa cell growth regulation, we determined whether downregulation of p66Shc protein, using a plasmid-based RNAi

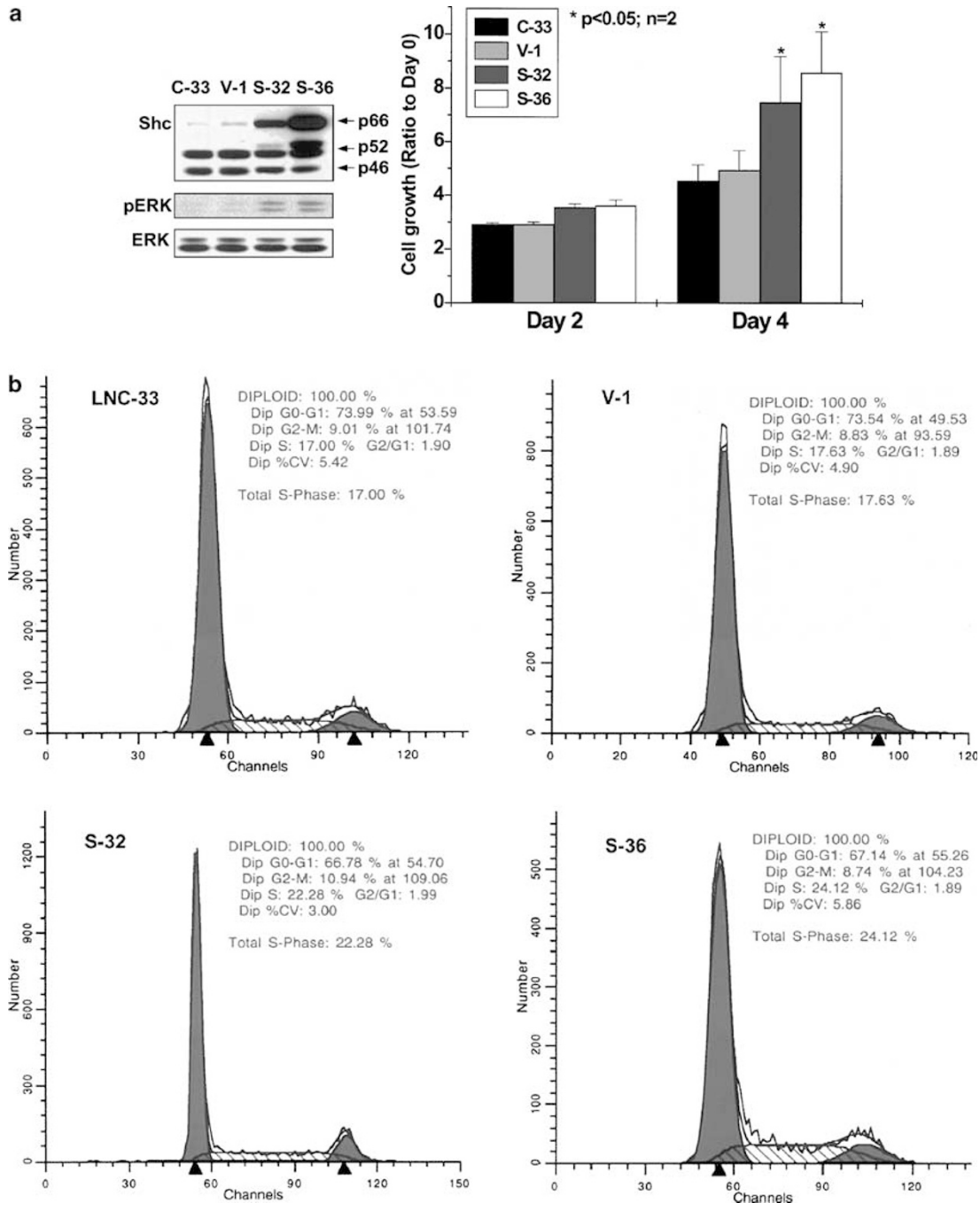

Figure 5 p66^{Shc} protein expression, phosphorylation of ERK/MAPK, and the growth of stable subclones of p66^{Shc} cDNA-transfected LNCaP C-33 cells. (**a**) Western blot analyses of Shc (left panel) and cell growth (right panel) in LNCaP C-33, vector alone-transfected (V-1), and two stable subclones of p66^{Shc} cDNA-transfected LNCaP C-33 cells (S-32 and S-36). Aliquots of the same set of whole-cell lysates were immunoblotted with an anti-phospho-ERK/MAPK Ab and, after stripping, followed by an anti-ERK/MAPK Ab. The cell growth data are the representative of two sets of independent experiments in duplicates (**b**) Histograms of cell cycle distributions of S-32 and S-36 cells by flow cytometric analyses. LNCaP C-33 parental and V-1 cells were used as controls. Similar results were obtained from two sets of independent experiments

approach, would lead to a decrease in their growth rate. We transfected LNCaP C-81 and PC-3 cells with pSUP-p66 to knock down their p66^{Shc} expression. In order to normalize the differences in the transfection efficiency of those cells, we cotransfected with pTRACER-CMV along with pSUP-p66. As shown in Figure 6a (left panel), transient knock-down of p66^{Shc} in C-81 cells correlated with the decrease in activation of ERK/MAPK, indicated by decreased levels of phosphorylation. Transient knock-down of p66^{Shc} protein by about 40% in rapid-growing LNCaP C-81 cells using pSUP-p66 resulted in an approximately 30% decrease in cell proliferation (Figure 6a, right panel). Cell cycle distribution of p66^{Shc}-knocked-down C-81 cells indicated

that they exhibited approximately 30% decrease in their proliferation rates than the vector-alone transfected cells, as indicated by the decrease in the percentage of cell population in the S phase of cell cycle, that is, 14.8% for p66Shc-knocked-down cells vs 22.6% for vector-alone-transfected cells (Figure 6b). Similarly, in PC-3 cells, an approximately 45% knock-down of p66Shc protein by RNAi led to the decrease in ERK/MAPK activation, as indicated by its decreased phosphorylation (Figure 6c, left panel) that correlated with approximately 35% reduction in their proliferation rate as determined by the cell counting (Figure 6c, right panel). Cell cycle analysis of those p66Shc-knocked-down PC-3 cells showed that there was a decrease in S phase from 18.3% in vector-alone-transfected cells to 13.6% for p66Shc-knocked-down cells (Figure 6d). Thus, the data collectively indicate that p66Shc protein plays a critical role in regulating the proliferation of PCa cells.

Discussion

Shc proteins play a critical role in regulating PCa cell proliferation, in part by mediating tyrosine phosphorylation

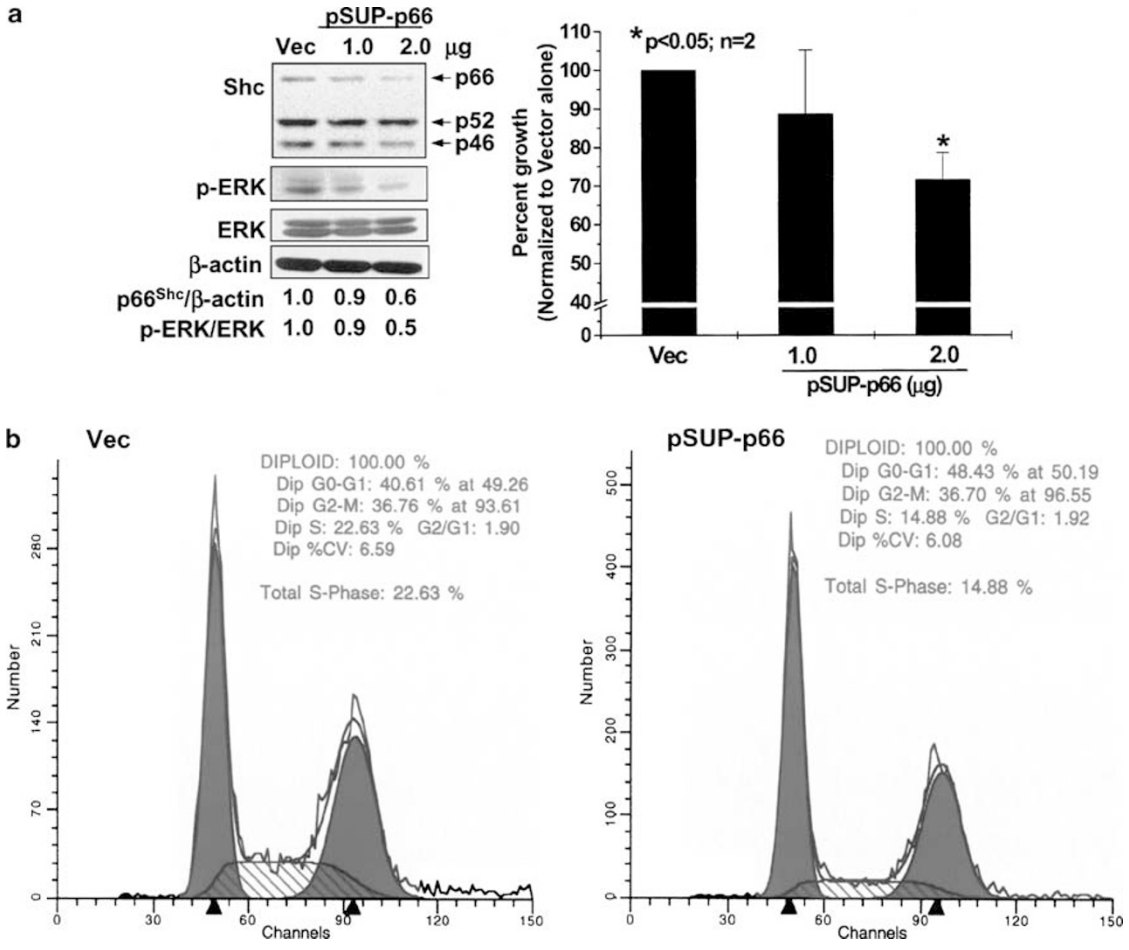

Figure 6 Effect of knock-down of p66Shc expression by siRNA on PCa cell growth. (**a**) Western blot and cell growth analyses of p66Shc–knocked-down LNCaP C-81 cells. LNCaP C-81 cells were plated at a density of 1×10^5 cells/well of a six-well plate for 48 h. The cells were transiently transfected with pSUP-p66. Control cells were transfected with pSUPER vector alone (Vec). After 3 days following transfection, cells were harvested by trypsinization and counted in a Coulter cell counter (right panel). Cell lysates were analysed for p66Shc protein level and ERK1/2 phosphorylation. To normalize the transfection efficiency, cells were cotransfected with pTRACER-CMV, a plasmid expressing green fluorescent protein (GFP). The figure is a representative of three sets of independent experiments in duplicates. (**b**) Cell cycle analysis of p66Shc-knocked-down LNCaP C-81 cells. 7.5×10^5 LNCaP C-81 cells were plated in regular medium on 100 mm dishes and transfected with pSUP-p66. Control cells were transfected with vector alone (Vec). After 3 days, cells were trypsinized, fixed with 70% ethanol and stained with propidium iodide. Cells were analysed with a flow cytometer for the distribution of cell cycle. Cells were cotransfected with pTRACER-CMV for estimating the transfection efficiency. The figure is a representative of three sets of independent experiments. (**c**) Western blot and cell growth analyses of p66Shc-knocked-down PC-3 cells. PC-3 cells were transfected with pSUP-p66, as described for LNCaP C-81 cells. Cell growth was analysed 3 days post-transfection. To normalize the transfection efficiency, cells were cotransfected with pTRACER-CMV. The figure is a representative of two sets of independent experiments performed in duplicates. (**d**) Cell cycle analysis of p66Shc-knocked-down PC-3 cells. 7.5×10^5 PC-3 cells were plated in regular medium on 100 mm dishes and transfected with pSUP-p66. Control cells were transfected with vector alone. To normalize the transfection efficiency, cells were cotransfected with pTRACER-CMV. After 3 days, cells were trypsinized, fixed with 70% ethanol and stained with propidium iodide. Cells were analysed with a flow cytometer for the distribution of cell cycle. The figure is a representative of two sets of independent experiments

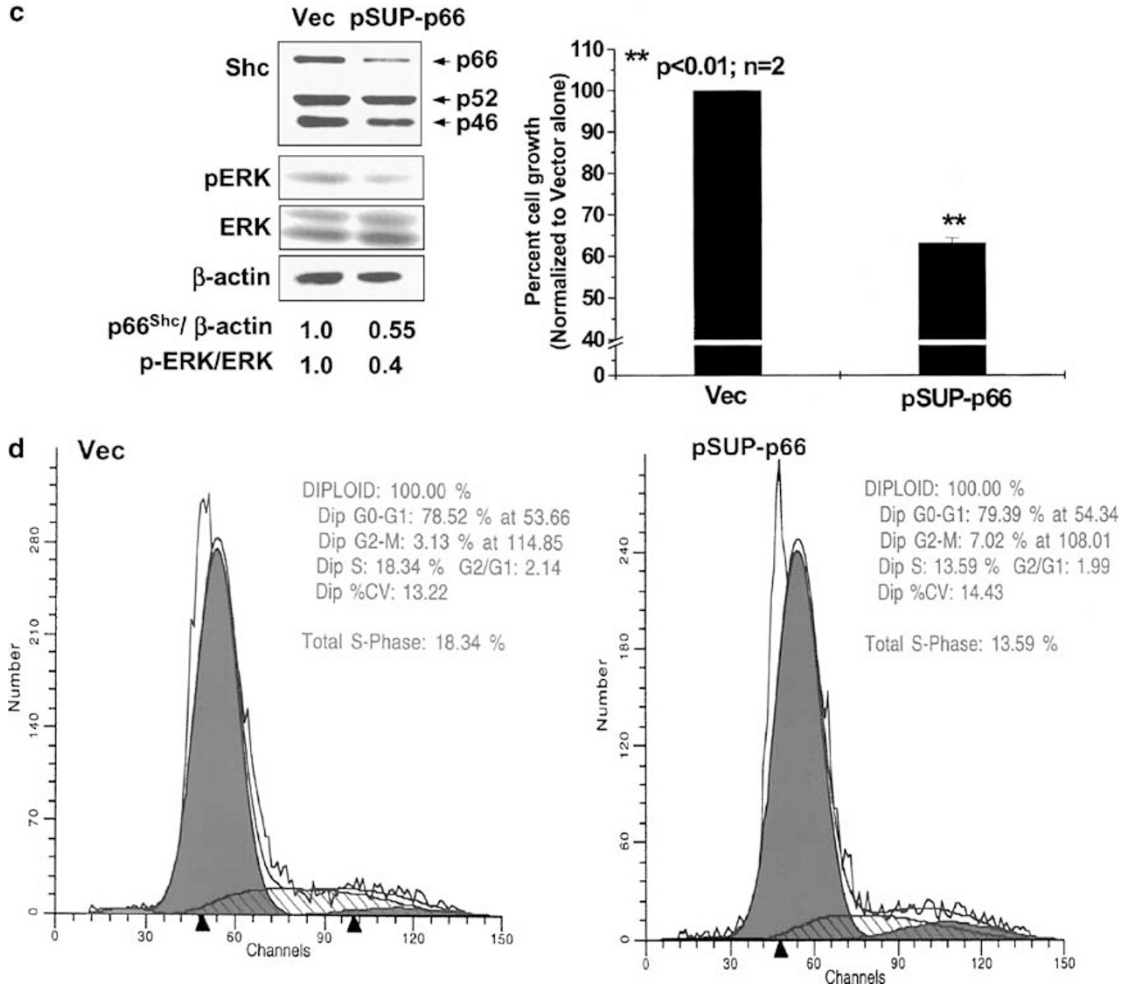

Figure 6 *Continued*

signals. For example, treatment of LNCaP C-33 cells with androgens leads to increased phosphorylation of p52^{Shc} at Y317 residue, which is required for DHT-induced proliferation, but not that of EGF (Lee *et al*., 2004b). Apart from p52^{Shc}, the role of other Shc members in the proliferation of PCa cells remains an enigma. Interestingly, androgen stimulation of PCa cells is accompanied with the upregulation of p66^{Shc} protein levels, but not its tyrosine phosphorylation levels (Lee *et al*., 2004a, b; Figure 3a). Furthermore, p66^{Shc} protein level is significantly elevated in archival human PCa specimens, higher in the cancerous cells compared to the adjacent noncancerous cells (Lee *et al*., 2004a). Hence, we hypothesize that p66^{Shc} protein is also involved directly in the regulation of PCa cell proliferation.

Our data in different PCa cell lines clearly show that p66^{Shc} protein level, but not p52^{Shc} or p46^{Shc} protein levels, consistently correlates with cell proliferation (Figures 1–4). Additionally, elevated expression of p66^{Shc} protein by cDNA transfection in LNCaP C-33 cells correlates with increased cell proliferation (Figure 5). Conversely, knock-down of p66^{Shc} protein level by its siRNA leads to decreased cell growth (Figure 6). Our cell line data corroborate our observations on the archival PCa tissue specimens that p66^{Shc} expression is lower in benign glandular cells, whereas in adjacent adenocarcinomatous cells, the expression of p66^{Shc} is higher (Lee *et al*., 2004a). Thus, we further propose that normal differentiated prostate epithelial cells, which have a slow growth rate, would express a low level of p66^{Shc} protein, whereas its elevated expression might contribute to prostate carcinogenesis. It should also be noted that the protein levels of other Shc isoforms, that is, p46^{Shc} and p52^{Shc}, do not consistently correlate with the PCa cell growth. Hence, we propose that p66^{Shc} protein level, but not p46^{Shc} or p52^{Shc} protein level, is directly involved in the regulation of cell growth. Nevertheless, a similar growth rate of the stable subclone cells that overexpress p66^{Shc} might be due to that the growth stimulation by high levels of p66^{Shc} reached the plateau (Figure 5a, right panel). This is comparable to the rapid-growing PCa cells, such as PC-3, TSU-Pr1 and DU 145 cells, which express increasingly higher levels of p66^{Shc} protein, but still have a similar doubling time (Figure 1). Collectively, the data support our hypothesis that elevated p66^{Shc} protein in prostate carcinomas plays a critical role in upregulating

the proliferation of those cancer cells and, thus, contributes to the tumorigenicity of human PCa.

Similarly, elevation of p66Shc protein is observed in breast cancer specimens, where p66Shc protein is elevated in the lymph node-positive breast carcinomas (Jackson *et al*., 2000). Nevertheless, other studies show that the level of p66Shc protein in relapsed breast cancers is decreased, significantly lower than that in primary, nonrelapsed tumors (Davol *et al*., 2003). The data collectively implicate that p66Shc protein plays a role in the tumorigenicity of those breast cancer cells, although the correlative relationship requires further clarification. It should be noted that the level of p66Shc protein in human carcinoma cells is altered by various treatments including steroids and growth factors (Figure 3a; Lee *et al*., 2004a). Hence, it is possible that the inconsistency in the correlativity in breast cancer studies may partly be related to the different treatment regimens that those patients were in, prior to obtaining the specimens. As described, some of those relapsed tumors had been obtained from patients who were under adjuvant hormonal therapy (Davol *et al*., 2003). It is thus expected that those hormone-ablated specimens would have a decreased level of p66Shc protein. In such case, a substantial number of cancer specimens that have low levels of p66Shc protein may tip the results of statistical analyses. Nevertheless, due to the small sample size in our study, we are unable to determine if the elevated p66Shc protein level could serve as a surrogate marker for PCa progression. The clinical application of p66Shc protein in PCa deserves further investigations.

The molecular mechanism by which p66Shc protein is involved in regulating cell proliferation deserves careful analyses. In PCa cells, p66Shc protein level inversely correlates with cPAcP and positively correlates with ERK/MAPK activation. In p66Shc cDNA-transfected stable subclone cells, ERK/MAPKs are activated, correlating with increased cell proliferation (Figure 5). Conversely, in p66Shc-knocked-down LNCaP C-81 and PC-3 cells, ERK/MAPKs are inhibited that correlate with decreased cell proliferation (Figure 6). This p66Shc-activated ERK/MAPK in p66Shc cDNA-transfected LNCaP cells is consistent with the observations that in cPAcP-inhibited LNCaP C-33 cells by L(+)-tartrate, p66Shc protein level is elevated and ERK/MAPK are activated (Figure 3b). We propose that the negative effect of cPAcP on p66Shc protein level is not through a direct mechanism, such as cPAcP-p66Shc protein interaction, although it requires further investigation. It should be noted that the inverse correlation of the level of cPAcP to p66Shc and ERK/MAPK phosphorylation is clinically significant, because in clinical PCa specimens, cPAcP is decreased (Loor *et al*., 1981; Pontes *et al*., 1981; Solin *et al*., 1990; Sakai *et al*., 1993; Lin *et al*., 2001) and p66Shc protein level and ERK/MAPK phosphorylation are elevated (Gioeli *et al*., 1999; Price *et al*., 1999; Lee *et al*., 2004a). This phenomenon is also similar to the observation in breast cancer cells that increased expression of p66Shc protein correlates with ERK/MAPK activation (Jackson *et al*., 2000). The observation that elevated p66Shc protein reduces the degree of EGF-induced ERK/MAPK activation in HeLa, CHO or COS-1 cells (Migliaccio *et al*., 1997; Okada *et al*., 1997) could be due in part to different cell types. Alternatively, it should be noted that in those p66Shc-elevated cells, the basal activity of ERK/MAPKs was upregulated. Thus, it could be expected that the degree of further stimulation of ERK/MAPK by EGF in those p66Shc-elevated cells is reduced. The mechanism of p66Shc activating ERK/MAPK requires further analysis.

Increased oxidative stress, through the generation of reactive oxygen species (ROS), in rapid-growing PCa cells might also account for the elevated expression of p66Shc protein in those cells. It is generally accepted that rapid-growing cells have activated metabolic reactions that lead to increased production of ROS (Klaunig and Kamendulis, 2004), which results in the elevation of p66Shc protein to mediate oxidative stress signals. This notion is supported by the observations that the expression of p66Shc protein is increased by raising intracellular ROS level, for example, by H_2O_2 treatment (Trinei *et al*., 2002; Pacini *et al*., 2004). Interestingly, treatment of cells with steroid hormones and growth factors, such as, DHT and EGF, results in the upregulation of ROS in those cells (Ripple *et al*., 1997, 1999; Meng *et al*., 2002) and the increased ROS could serve as a positive regulator of cell growth as observed in human hepatoma cells (Liu *et al*., 2002). Importantly, around 80% of the prostate tumor specimens showed an increase in NADPH oxidase 1 and intracellular ROS levels when compared to the adjacent noncancerous tissue (Lim *et al*., 2005). Thus, it could be speculated that the upregulation of p66Shc protein by DHT or EGF treatment in PCa cells might be through the upregulation of ROS in those stimulated cells. In summary, our data clearly support for a novel functional role of p66Shc protein in prostate carcinogenesis probably through promoting cell proliferation, although the detailed molecular mechanism(s) by which p66Shc protein upregulates PCa cell proliferation require(s) further studies. Further, the potential role for p66Shc protein in serving as a target for PCa therapy deserves further investigations.

Materials and methods

Materials

FBS, RPMI 1640 culture medium, glutamine and gentamicin were purchased from Invitrogen (Carlsbad, CA, USA). Charcoal/dextran-treated, certified FBS was obtained from HyClone (Logan, UT, USA). BRFF-HPC1 medium was obtained from Athena Environment Science, Inc. (Baltimore, MD, USA). Polyclonal Abs (#06–203) recognizing all three isoforms of Shc protein and the Ab (#07–150) specifically recognizing p66Shc protein were from Upstate Biotechnology Inc. (Lake Placid, NY, USA). Rabbit anti-PAcP antiserum was obtained as described previously (Lin *et al*., 1993). Polyclonal Abs against phospho-ERK/MAPK (Thr202/Tyr204) and ERK/MAPK were from Cell Signaling (Beverly, MA, USA). Anti-β-actin Ab, EGF and DHT were obtained

from Sigma (St Louis, MO, USA). Horseradish peroxidase-conjugated anti-rabbit and anti-mouse IgG were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). All other chemicals were as described previously (Lin *et al*., 1998; Meng and Lin, 1998; Lee *et al*., 2004a, b)

Cell culture

Human prostate carcinoma cell lines including LNCaP-FGC (doubling time 60 h) (Horoszewicz *et al*., 1983), MDA PCa2b (doubling time 61–73 h) (Navone *et al*., 1997), DU 145 (doubling time 34 h) (Stone *et al*., 1978) and PC-3 (doubling time 33 h) (Kaighn *et al*., 1979) were purchased from the American Type Culture Collection (Rockville, MD, USA). TSU-Pr1 cells (doubling time 36 h) (Iizumi *et al*., 1987) were obtained from Dr Jin-Tang Dong at Emory University School of Medicine (Atlanta, GA, USA). Human PCa cells except MDA PCa2b cells were routinely maintained in the regular culture medium, that is, phenol red-positive RPMI 1640 medium supplemented with 5% FBS, 1% glutamine and 0.5% gentamicin (Meng and Lin, 1998). Cells were split once per week, which was defined as one passage. LNCaP cells with passage numbers less than 33 were designated as C-33, those with numbers greater than 81 as C-81, and those with numbers between 34 and 80 as C-51 (Lin *et al*., 1998; Meng *et al*., 2000; Igawa *et al*., 2002). LNCaP C-33 cells express endogenous PAcP, while C-81 cells have lost PAcP expression (Lin *et al*., 1998). PAcP-cDNA-transfected C-81 stable subclone cells, that is, LN-23 and LN-40 cells, have slower growth rates (doubling time 72 and 90 h, respectively) than C-81 parental cells (doubling time 49 h) in a steroid-reduced condition (Meng and Lin, 1998). PC-3 cells do not express endogenous PAcP (Kaighn *et al*., 1979; Lin *et al*., 1998). PC-411 and PC-416 cells were PAcP cDNA-transfected PC-3 stable subclone cells with doubling time 40 and 54 h, respectively (Lin *et al*., 1998). MDA PCa2b cells were routinely maintained in the culture medium, that is, BRFF-HPC1 medium supplemented with 20% FBS, 1% glutamine and 0.5% gentamicin. For EGF or DHT treatment, LNCaP C-33 cells were steroid-starved for 48 h in a steroid-reduced medium, that is, phenol red-free RPMI 1640 medium containing 5% charcoal/dextran-treated, heat-inactivated certified FBS, 1% glutamine and 0.5% gentamicin. Cells were then exposed to EGF (10 ng/ml) or DHT (10 nM) for 24 h.

Cell growth determination

To determine the growth of different passages of LNCaP and MDA PCa2b cells, 3.2×10^4 of different LNCaP cells or 1×10^5 of different MDA PCa2b cells were seeded on six-well culture plates with duplicate wells and maintained in their respective culture medium. After 3 days, one set of attached cells was harvested and counted as day 0 with a Z1 model Coulter Counter (Coulter Corporation, Miami, FL, USA). The remaining attached cells were fed with fresh medium, and the total cell numbers were counted on days 2, 4 and 6 for LNCaP cells or day 3, 5 and 7 for MDA PCa2b cells. The fresh medium was added to the remaining LNCaP cells on days 2 and 4 or MDA PCa2b cells on days 3 and 5.

Immunoblotting

Subconfluent cells were harvested by scraping. After being spun at 4°C, 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 for immunoblotting were as described previously (Lin *et al*., 1998; Meng and Lin, 1998; Lee *et al*., 2004a, b). The p66Shc protein level was semiquantified by densitometric analyses of autoradiograms with different exposure time periods using Molecular Dynamics equipment and its software program (ImageQuant 3.0). The relative protein level was then normalized to the corresponding β-actin protein level.

Construction of p66Shc cDNA expression vector

The wild-type p66Shc cDNA constructed in the PINCO retroviral expression vector was from Dr A Raymond Frackelton Jr at the Brown University (Providence, RI, USA) with the approval from Dr Pier Giuseppe Pelicci at the European Institute of Oncology (Milan, Italy) (Migliaccio *et al*., 1999). The p66Shc coding region was amplified using this p66Shc PINCO plasmid as the template and two oligonucleotide primers: 5′-GAATTCAACT ATGGATCTCCTGCCCC-3′ and 5′-AAGCTTTAGGGCAGATCACAGTTT-CC-3′ (*Eco*RI and *Hind*III sites are, respectively, underlined). This PCR fragment was digested by restriction enzymes *Eco*RI and *Hind*III and then ligated with the *Eco*RI/*Hind*III-cut pcDNA 3.1A vector (designated as pcDNA 3.1A-p66Shc). The sequence of p66Shc cDNA coding region was confirmed by DNA sequencing at the UNMC Molecular Biology Core Facility.

Establishment of stable subclones of p66Shc cDNA-transfected LNCaP C-33 cells

LNCaP C-33 cells were transfected with pcDNA 3.1A-p66Shc plasmid encoding the wild-type p66Shc protein and subsequently selected by G418, as described previously (Lin *et al*., 1998). Two stable subclone cell lines, S32 and S36, were established and characterized in this study. V-1 cells were a subclone cell line of LNCaP C-33 cells transfected with the vector alone.

Flow cytometry

For experiments, cells were seeded at a density of 5×10^5 cells per T25 flask in the regular medium for 3 days. At 48 h after feeding with fresh medium, cells were trypsinized, harvested and washed twice by Hank's balanced salt solution. Cells were treated with 70% ethanol at 4°C for 1 h, washed with PBS, and spun down by centrifugation. The DNA of ethanol-fixed cells was stained by the PI staining reagent at 4°C for 30 min. The PI staining reagent was prepared in PBS, pH 7.4, containing 0.1% Triton X-100, 0.1 mM EDTA disodium salt, 0.05 mg/ml RNase A (50 U/mg), and 50 μg/ml propidium iodide (Telford *et al*., 1991). The determination of cell cycle distribution for single cell was carried out using a Becton-Dickinson fluorescence-activated cell sorter (FACSCalibur, Becton Dickinson, San Jose, CA, USA) at the UNMC Flow Cytometry Core Facility.

Construction of vector coding for p66Shc siRNA transcripts and transfection

To knock down p66Shc expression in PCa cells, pSUPER vector (OligoEngine Inc., USA) was used, which involves a plasmid-based RNAi approach. Sense and antisense strand oligonucleotides that code for p66Shc siRNA transcripts were designed, according to the instructions by OligoEngine Inc., USA, and were synthesized in the Molecular Biology core facility at UNMC. The target sequence was 5′-TGAGTCTCTGT CATCGCTG-3′ that lies in the CH_2 region of p66Shc (GenBank Acc. No. U73377). The oligos were annealed to each other and ligated with pSUPER vector predigested with *Bgl*II and *Hind*III. The plasmid was designated as pSUP-p66.

LNCaP C-81 cells and PC-3 cells were plated at a density of 1×10^5 cells/well of a six-well plate for 48 h. Cells were then transfected with different amounts of siRNA-encoding

pSUPER plasmid using LipofectAmine Plus reagent (Invitrogen, USA). After 4 h, cells were replenished with RPMI-1640 medium containing 10% FBS overnight and then fed with regular medium. The cells were harvested after 3 days by trypsinization and were counted using a Coulter counter. Owing to the differences in transfection efficiency of those cells, we cotransfected them with pSUP-p66 and pTRACER-CMV, a plasmid expressing full-length GFP (Invitrogen Inc., USA), in the ratio of 3 : 1. Cells were harvested after 3 days for analysis of cell growth and cell cycle.

Abbreviations

Ab, antibody; cPAcP, cellular form of prostatic acid phosphatase; DHT, 5α-dihydrotestosterone; ECL, enhanced chemiluminescence; EGF, epidermal growth factor; ERK/MAPK, extracellular signal-regulated kinases/mitogen-activated protein kinases; FBS, fetal bovine serum; GFP, green fluorescent protein; PCa, prostate cancer; ROS, reactive oxygen species.

Acknowledgements

We thank Dr Pier Giuseppe Pelicci at European Institute of Oncology (Milan, Italy) and Dr A Raymond Frackelton Jr at Brown University (Providence, RI) for the gift of the wild-type p66Shc cDNA; Dr Jin-Tang Dong at Emory University School of Medicine (Atlanta, GA) for TSU-Pr1 cell line. We thank Ms Jacquelynn Dahl's help for screening p66Shc stable subclone cells. We also thank the supports from the core facilities, including Molecular Biology, DNA sequencing, Tissue Procurement and Flow Cytometry at UNMC. This study was supported in part by NIH grant CA88184, P20RR017675, P20RR018759, NCI P30 CA36727, Department of Defense PC040587 and PC05096, and the Graduate Student Fellowship of UNMC.

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