Down-regulation of BiP/GRP78 sensitizes resistant prostate cancer cells to gene-therapeutic overexpression of REIC/Dkk3

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Key words: REIC, Dkk, apoptosis, GRP78, ER stress

Abbreviations: Ad-LacZ, An adenovirus vetor carrying LacZ gene; Ad-REIC, An adenovirus carrying REIC/Dkk-3 gene.

Appropriate journal category: Cancer cell biology

Novelty and impact of the paper:

1. This paper describes that overexpression of BiP is a major factor for resistance of cancer cells to a promising gene-therapeutic adenenovirus vector carrying REIC/Dkk-3 (Ad-REIC).
2. Down-regulation of Bip by siRNA sensitizes resistant cancer cells to Ad-REIC.

(Summary)

We have recently shown that an adenovirus carrying REIC/Dkk-3 (Ad-REIC) exhibits a potent tumor-specific cell-killing function for various human cancers. It has also become evident that some human cancers are resistant to Ad-REIC-induced apoptosis. The aim of the present study was to determine the molecular mechanisms of resistance to Ad-REIC. First, we isolated resistant clones from a human prostate cancer cell line, PC3, after repeated exposure to Ad-REIC. Infection efficiency of the adenovirus vector and expression level of REIC/Dkk-3 in the resistant clones were similar to those in the parental PC3 cells. By screening for alteration in levels and functional status of proteins involved in Ad-REIC-induced apoptosis, we found that BiP/GRP78, an ER-residing chaperone protein, was expressed at higher levels consistently among resistant cells. Expression levels of BiP and rates of apoptosis induced by Ad-REIC were inversely correlated. Down-regulation of BiP with siRNA sensitized the resistant cells to Ad-REIC in vivo as well as in culture. These results indicate that BiP is a major determinant of resistance to Ad-REIC-induced apoptosis. Thus BiP is useful for diagnosis of inherent and acquired resistance of cancers and also as a target molecule to overcome resistance to the gene therapeutic Ad-REIC.

(Introduction)

REIC/Dkk-3 is a tumor suppressor gene that was first identified as a gene that is down-regulated in association with immortalization of normal human fibroblasts[1]. Subsequently, we found that overexpression of REIC/Dkk-3 using an adenovirus vector (Ad-REIC) has a dramatic therapeutic effect on various types of human and cancer, including those derived from the prostate, testis, pleura, and breast[2-5]. Infection with Ad-REIC selectively induced apoptotic cell death in cancer cells in a cell-autonomous manner, whereas normal cells did not undergo apoptosis upon infection with Ad-REIC. Furthermore, intratumoral injection of Ad-REIC was shown to inhibit metastasis of mouse prostate cancer in an orthotopic model[6]. These selective multi-targeting anti-cancer functions of Ad-REIC show great promise for clinical application, which will be shortly initiated.

One of the major challenges in cancer therapy is overcoming inherent and acquired resistance of cancer cells. Re-growth of such resistant cell populations annuls initially effective therapeutic measures and often leads to fatal outcomes. Cancer gene therapy may restore apoptosis in cancer cells that are resistant to conventional chemotherapeutic agents and/or radiation[7], but gene therapeutic modalities, including those using p53 and mda-7/IL-24, cannot be free from such a challenge[8,9]. Pataer et al. [10] isolated Ad-mda7/IL-24-resistant lung cancer cell lines and showed that the resistance was overcome by an inhibitor for HSP90. Clarification of molecular mechanisms underlying resistance to a therapeutic measure would contribute not only to overcoming the acquired resistance of cancer cells but also to diagnosis of inherently resistant cancer cases to avoid application of ineffective therapeutic modalities.

Although REIC/Dkk-3 is a secretory protein and assumed to

exert its physiological function through a yet unidentified cell surface receptor, the induction of apoptosis in cancer cells by Ad-REIC is mainly due to ER stress induced by overproduction of REIC/Dkk-3 in the cells[2,3]. Activation of c-Jun N-terminal kinase (JNK) triggered by ER stress is a critical event for apoptosis induced by Ad-REIC[2,3]. In the present study, we isolated resistant clones from a human prostate cancer cell line, PC3, that is sensitive to Ad-REIC and found that overexpression of BiP/GRP78, an ER-residing chaperone protein, is a major determinant of the acquired and inherent resistance to Ad-REIC. Down-regulation of BiP using siRNA restored sensitivity of tumors formed by transplantation of a resistant clone to therapeutic application of Ad-REIC *in vivo*.

Materials and methods

Cells culture

The human prostate cancer cell lines PC3 and LNCaP were cultivated in HAM'S F-12 K medium and RPMI 1640 (Nissui, Tokyo, Japan), respectively, with a supplement of 10% FBS. OUMS-24 cells (kindly provided by Dr. Masayoshi Namba) are normal human fibroblasts cultured in Dulbecco's modified MEM (Nissui, Tokyo, Japan) supplemented with 10% FBS. Human bladder cancer cell lines T24 and J82 were cultivated in McCoy's 5A medium and MEM, respectively, with 10% FBS. For isolation of clones resistant to Ad-REIC, PC3 cells were subjected to 3 cycles of massive killing by Ad-REIC and recovery over 3 months and then cloned by the limited dilution method. Apoptotic cells were identified as those with shrunken and fragmented nuclei stained with Hoechst33342 72 h after infection with Ad-REIC.

Western blot analysis

Western blot analysis was performed under conventional conditions using the following antibodies: Rabbit anti-human REIC/Dkk-3 antibody raised in our laboratory; rabbit anti-human GRP78 (BiP), rabbit anti-human GADD153 (CHOP), mouse anti-human mitochondrial HSP70 (GRP75), and rabbit anti-human ATF6 (N-terminus) antibodies (Abcam, Cambridge, MA); mouse anti-human HSP70, mouse anti-human HSP90 and mouse anti-phospho-serine/threonin (clone 22a) antibodies (BD Biosciences, San Jose, CA); mouse anti-human HSC70, mouse anti-human HSP27, goat anti-human GRP78, and rabbit anti-GRP94 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA); mouse anti-β-galactosidase antibody (Calbiochem); rabbit anti-human SAPK/JNK, mouse anti-human phospho-SAPK/JNK (Thr183/Tyr185), rabbit anti-human p38, rabbit anti-human phospho-p38

(Thr180/Tyr182), rabbit anti-human ASK1, rabbit anti-human phospho-ASK1 (Thr845) and rabbit anti-human IRE1α antibodies (Cell Signaling Technology, Beverly, MA); rabbit anti-human phospho-IRE1α (Ser724) antibody (ABR, Golden, CO); mouse anti-human Bcl-2, mouse anti-human Bcl-xL, mouse anti-human Bax and mouse anti-human Bad antibodies (BD Biosciences); rabbit anti-human calreticulin antibody (Novus Biologicals, Littleton, CO); rabbit anti-human DNAJA2 antibody (Proteintech Group, Chicago, IL); and mouse anti-human β-actin and mouse anti-human tubulin antibodies (Sigma, St Louis, MO).

Isolation of ERSE-binding proteins in vitro

A biotinylated 39-bp DNA fragment containing ER stress response element (ERSE) of the human BiP promoter was prepared using a forward primer (5'-biotin-agggccttcaccaatcggcggcctccacgacggggctgg-3') and a reverse primer (3'-ccagccccgtcgtggaggccgccgattggtgaaggccct-biotin-5'). Proteins that bind to the biotinylated DNA fragment were identified in nuclear extracts under conditions described previously[11].

Transfection of siRNA and decoy oligonucleotides in culture

Small interfering RNAs (siRNAs) against human BiP (Stealth™; siRNAs duplex oligonucleotide for BiP; CGAGUGACAGCUGAAGACAAGGGUA) and a control siRNA (Silencer negative control 2 siRNA) were purchased from Invitrogen and Ambion (Austin, Texas), respectively. Transfection of siRNAs (final concentration of 0.2 μM) was performed using FuGENE-HD (Roche). Decoy oligonucleotides for transcription factors were prepared by annealing complementary oligonucleotides having the following sequences: 5'-ccaatcggcggcctccacg-3' for ATF-6, 5'-atgaatcag-3' for AP-1, 5'-ttcctggaa-3' for STAT,

5'-cataactggc-3' and 5'-CCCAACTGGC-3' for c-Myb, and 5'-agcagccaatga-3' and 5'-ctgggccaatga-3' for C/EBP. Transfection of the oligonucleotides (final concentration of 10 μM) was performed using lipofectamine™ 2000 (Invitrogen) with NupherinTM reagent (Biomol).

Tumor growth assay in vivo

PC3-derived Cl-1 cells ($5x10^6$ in 100 μl PBS) mixed with 100 μl Matrigel (BD Biosciences) were subcutaneously injected into the right flank of adult male BALB/c *nu/nu* mice (SLC, Hamamatsu, Japan). One week after transplantation (~5 mm in diameter), 4 μg of siRNAs in 200 μl of TransIT-QR Hydrodynamic Delivery Solution (Mirus, Madison, WI) was injected intratumorally into 4 different sites. Twenty-four hours later, Ad-REIC or Ad-LacZ ($2x10^8$ pfu) in 200 μl of a buffer was injected intratumorally. Tumor volume was calculated using an empirical formula, V = 1/2 x [(shortest diameter)^2 x (longest diameter)]. PC-3M-luc-C6 Bioware cells (PC3-Luc; Caliper Life Sciences, Hopkinton, MA) transplanted under the same conditions as those for PC3 cells were used for monitoring the effect of siRNA *in vivo* using IVIS 2000 (Xenogen, Alameda, CA). The experiments were performed according to the ethical code for experimental animal handling of our university.

Statistical analysis

Results were expressed as means +/- SD unless otherwise indicated. Statistical analysis was performed by Mann-Whitney's U-test. P values equal to or less than 0.05 were considered statistically significant.

Results

Isolation and characterization of resistant clones from PC3 cells to Ad-REIC

To isolate resistant variants, PC3 cells were exposed to Ad-REIC at 20 MOI, which induced massive cell death. A small number of surviving cells were further propagated and re-infected with Ad-REIC under the same conditions. After three rounds of infection with Ad-REIC and recovery, the cells were seeded into a 96-well plate for single cell cloning. The resulting clones showed varying sensitivity to Ad-REIC (Fig. 1a), i.e., resistant (Cl-1~Cl-5), intermediate (Cl-6~Cl-9), and sensitive (Cl-10, Cl-11) clones were obtained. Infection efficiency of Ad-LacZ among the clones was not remarkably altered (Fig. 1b), excluding the possibility that the resistance to Ad-REIC was due to failure in infection. Expression level of REIC/Dkk-3 protein in Cl-1 cells was similar to that in PC3 cells after infection with Ad-REIC (Fig. 1c).

Since our previous studies showed that up-regulation of HSP70 and Bcl-2 may be responsible for conferring upon cells resistance to Ad-REIC[12,13], we first examined expression levels of heat shock proteins (HSPs)/chaperones and Bcl-2-related proteins of a representative resistant clone, Cl-1 (Fig. 1c). No significant change in expression of HSP90, HSP70, and HSC70 was observed in Cl-1 cells compared with that in PC3 cells. On the other hand, Bcl-2 and Bcl-xL were down- and up-regulated, respectively, in Cl-1 cells. Overall effect of the alteration, however, is probably not very significant, because both Bcl-2 and Bcl-xL have anti-apoptotic function and thus are mostly complementary[14]. We particularly noted that BiP, an ER-specific chaperone protein, was up-regulated in Cl-1 cells, since Ad-REIC induces apoptosis by triggering ER stress. BiP, but not HSPs, was up-regulated in all of the resistant clones isolated (Fig. 1d). The lower major band of BiP appeared genuine

since a single band corresponding to its size was detected with a different antibody against BiP (Fig. 4a).

Signal transduction leading to overexpression of BiP in Cl-1 cells

Various potential *cis*-acting regulatory elements were revealed by a computer search to be present in the promoter of BiP (Fig. 2a, upper panel). Among the corresponding decoy oligonucleotides, only that for ERSE suppressed expression of BiP upon transfection to Cl-1 cells (Fig. 2a, lower panel). The ERSE is known to be activated by binding with ATF6, a sensor protein truncated and phosphorylated by p38 under the ER stress conditions[15,16]. Truncated ATF6 was constitutively detected in Cl-1 cells, while the truncated form was observed in PC3 cells only upon infection with Ad-REIC, which evoked ER stress (Fig. 2b). *In vitro* fishing of transacting factors using the biotinylated ERSE resulted in identification of the activated ATF6 (Fig. 2c). Binding of constitutively active ATF6 together with YY1 in the Cl-1 cell extract to the ERSE was observed (Data not shown). p38 inhibitors abrogated the binding of ATF6 to the ERSE (Fig. 2d) and overexpression of BiP (Fig. 2e). p38 and its upstream kinase ASK1, but not IRE1α, were constitutively active in Cl-1 cells as indicated by phosphorylation (Fig. 2f). Inactive IRE1α in Cl-1 cells was confirmed by the lack of splicing of XBP1 mRNA (Fig. 2g). In Cl-1 cells, activation of IRE1α and JNK was not observed even upon exposure to Ad-REIC (Fig. 2f). These results indicate that Cl-1 cells were refractory to the induction of ER stress by Ad-REIC and that overexpression of BiP in Cl-1 cells was due to constitutive cleavage of ATF6 and activation of ASK1 for a yet unidentified reason.

Causative link between overexpression of BiP and resistance to

Ad-REIC

Next we investigated whether overexpression of BiP in the resistant clones is causatively linked to their resistance to Ad-REIC. Expression level of BiP was high in highly resistant clones but not in intermediate and sensitive clones (Fig. 3a). A reverse correlation (γ=-0.75) was found between the rate of cell apoptosis induced by Ad-REIC and BiP protein level among different clones. The correlation between Ad-REIC-induced apoptosis and protein level of either HSP70 (γ=0.003) or Bcl-2 (γ=-0.32) was not remarkable. A similar reverse correlation was observed among PC3 cells exposed for different times (1~4) to Ad-REIC and some other cancer cell lines (γ=-0.93; Fig. 3b). It should be noted that repeated exposure of PC3 cells to Ad-REIC progressively promoted expression of BiP and resistance to Ad-REIC.

Transfection of BiP siRNA efficiently down-regulated BiP expression in Cl-1 cells in culture (Fig. 4a). The effect of BiP siRNA was specific since levels of chaperone proteins such as HSP70, HSC70, HSP90, HSP27, GRP94, GRP75, calreticulin, and DNAJA2 were not altered by the siRNA. This treatment sensitized Cl-1 cells to induction of apoptosis by Ad-REIC, i.e., from 4% in the cells transfected with control siRNA to 38% in those with BiP siRNA (Fig. 4b). Similar sensitization of another resistant clone, Cl-3, and resistant bladder cancer cell lines, T24 and J82, was observed (Fig. 4c). It is notable that down-regulation of BiP in normal human fibroblasts (OUMS-24) did not result in sensitization, though the cells expressed BiP to a level similar to the levels in Cl-1 and T24 cells (Fig. 4b). Normal cells probably have additional protective mechanisms against Ad-REIC. Down regulation of BiP in Cl-1 cells resulted in alteration in the signal transduction from ER stress to activation of JNK, a critical event leading to apoptosis[2]. On infection of BiP siRNA-treated Cl-1 cells with

Ad-REIC resulted in phosphorylation of IRE1α and activation of the down stream JNK (Fig. 4d). Similar response was observed in PC3 cells but not in Cl-1 cells (Fig. 2d). These results indicate that expression level of BiP is the critical determinant for resistance of cancer cells to Ad-REIC.

Sensitization of Cl-1 cells to therapeutic Ad-REIC by BiP siRNA in vivo

Transfection of siRNA and successful down-regulation of the target gene *in vivo* were verified using luciferase siRNA in tumors formed by transplantation of PC3-Luc cells (Fig. 5a). Transfection of BiP siRNA under similar conditions resulted in marked down-regulation of BiP in tumors formed by Cl-1 cells (Fig. 5b). The down-regulation of BiP significantly suppressed the growth of tumors formed by transplantation of Cl-1 cells *in vivo* (Fig. 5c). Histological examination of the tumors revealed heterogeneous appearance of tissues, but in general the progressively growing tumors were mainly occupied by viable tumor cells, while those treated with BiP siRNA and Ad-REIC were composed of many degenerative cells (data not shown).

Discussion

In the present study, we showed that: 1) BiP was overexpressed in resistant clones derived from PC3 cells and intrinsically resistant cancer cells to Ad-REIC (Fig. 1), 2) rates of apoptosis induced by Ad-REIC and expression levels of BiP among different cell types were inversely correlated (Fig. 3), and 3) down-regulation of BiP resulted in sensitization of the resistant cells to Ad-REIC (Figs. 4 and 5). These results indicate that BiP is a critical determinant of sensitivity/resistance to Ad-REIC. This is conceivable since BiP is an ER-residing chaperone protein negatively regulating ER stress and Ad-REIC exerts its apoptosis-inducing activity through ER stress[2,3,12]. Application of purified REIC/Dkk-3 protein failed to induce apoptosis in cancer cells. Ad-mda-7/IL-24, a promising gene therapeutic agent, was also shown to exhibit its effect partly through ER stress[10,17]. Pataer et al.[10] isolated clones resistant to overexpression of mda-7/IL-24 from a lung cancer cell line and showed that the clones were partially sensitized by an HSP90 inhibitor, 17AAG. However, functional status of BiP was not described.

BiP has been attracting much interest for its role in resistance of cancer cells to a broad variety of therapeutic measures, many of which are not directly linked to ER stress[18]. Although BiP is primarily located in the ER, it has also been shown to interact with cytosolic executors of apoptosis, such as caspase 7 and BIK[19,20], and even to be present even on the cell surface[21]. The particularly good inverse correlation between rates of apoptosis induced by Ad-REIC and expression levels of BiP (Fig. 3) was probably due to the fact that Ad-REIC induces apoptosis via ER stress. This indicates that determination of BiP level in a given cancer is a valid test for the effectiveness of gene therapy using Ad-REIC, although BiP is considered to play a critical role in a broad variety

of cancer therapies[18].

Down-regulation of BiP sensitized resistant cancer cells not only to Ad-REIC as shown in the present study but also to many chemotherapeutic agents[18]. Transfection with siRNA *in vivo* was effective in reducing target proteins in the present experimental settings (Fig. 5). However, it is unlikely that a similar method can be applied to human cancer, and safer and more effective methods for gene delivery are needed. Substances to inhibit BiP have been intensively screened and some candidate molecules have been reported[22,23]. It should be noted that normal human fibroblasts, OUMS-24, were not sensitized to Ad-REIC by down-regulation of BiP (Fig. 4B). Combination of a BiP inhibitor with Ad-REIC should therefore lead to a potent and specific therapeutic approach against inherent and acquired resistant cancers in the near future.

Acknowledgements

This work was supported in part by grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (to N. Huh, 20015031) and from Special Coordination Funds for Promoting Science and Technology (H. Kumon). All the authors declare to have no conflict of interests.

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Figure Legends

FIGURE 1 - Isolation of PC3 cell clones resistant to Ad-REIC and overexpression of BiP in the resistant clones. (**a**) Rates of apoptosis induced by Ad-REIC (20 MOI) of PC3 cells and PC3-derived clones (Cl-1~Cl-11) that were obtained after exposure to Ad-REIC 3 times. (**b**) Infection efficiency of Ad-LacZ to PC3 cells and PC3-derived clones determined by staining with X-gal after infection with Ad-LacZ at 20 MOI. (**c**) Western blot analysis for proteins belonging to the family of heat shock/chaperone proteins and Bcl family proteins. Cell extracts were prepared 24 h after infection with Ad-LacZ or Ad-REIC at 20 MOI. Tubulin was used as a control. (**d**) Western blot analysis for heat shock/chaperone proteins of different cell lines and PC3-derived resistant clones (Cl-1~Cl-5). LNCaP and DU145 cells (prostate cancer) and HeLa cells (cervical cancer) are sensitive to Ad-REIC (data not shown).

FIGURE 2 - Signal transduction leading to overexpression of BiP in a resistant clone, Cl-1. (**a**) Identification of a critical *cis*-acting regulatory element(s) in the BiP promoter of Cl-1 cells. Upper panel: Candidate *cis*-elements in the BiP promoter identified by MOTIF Search provided by Kyoto University Bioinformatics Center. Lower panel: Down-regulation of BiP by transfection of synthetic decoy oligonucleotides corresponding the candidate *cis*-elements. ERSE, ER stress-responsive element. (**b**) Western blot analysis for ATF6 of PC3 and Cl-1 cells 24 h after infection with Ad-LacZ or Ad-REIC at 20 MOI. (**c**) Identification of ATF6 as a protein binding to ERSE *in vitro* (see Materials and Methods). P-ATF6, phosphorylated ATF6, was detected using a mouse anti-phospho-serine/threonine antibody. (**d**) *In vitro* fishing for ATF6 using the ERSE in cells exposed to inhibitors for p38 (SB203580 1 μM), JNK (SP600125 100

nM), and Akt (Akt I 10 μM) for 24 h. (**e**) Inhibition of BiP expression in Cl-1 cells by p38 inhibitors. The cells were exposed to inhibitors for p38 (SB203580, 1 μM; SC68376 10 μM) and for JNK (SP600125 100 nM) for 24 h. (**f**) Splicing of XBP1 mRNA examined by RT-PCR. Closed arrowhead, unspliced; open arrowhead, spliced. (**g**) Western blot analysis for proteins potentially involved in the signal transduction triggered by ER stress. P-, phosphorylated. Cell extracts in (**c**) and (**f**) were the same as those used for the experiment shown in (**b**).

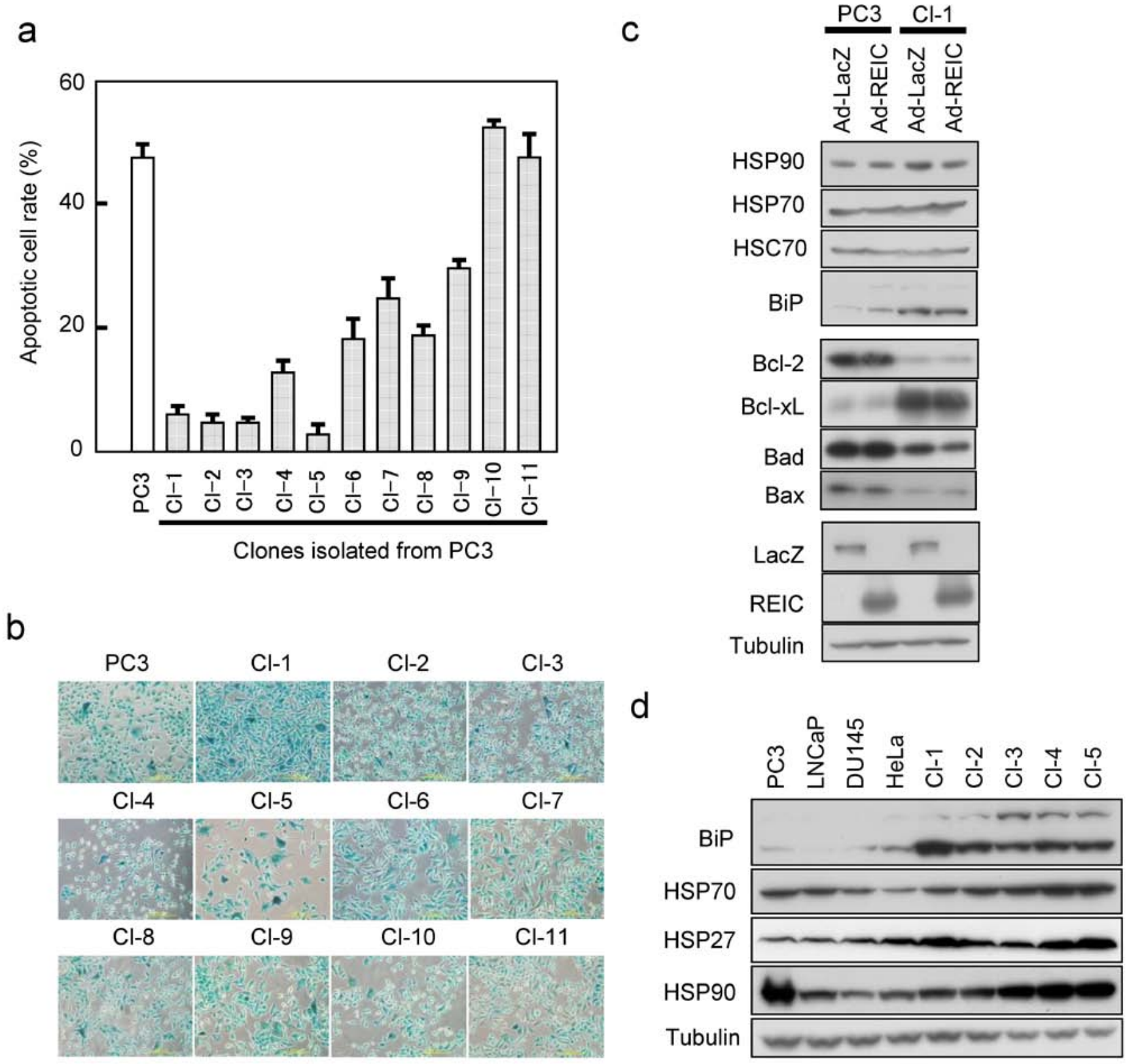
FIGURE 3 - Inverse correlation between expression level of BiP and sensitivity to killing effect of Ad-REIC. (**a**) Upper panel: Western blot analysis for BiP, HSP70 and Bcl-2 in PC3 cells and PC3-derived clones with varying resistance to Ad-REIC. Lower panel: Correlation between rates of apoptosis induced by Ad-REIC and expression levels of BiP among different clones. γ, correlation coefficient. (**b**) Analysis similar to that described in (**a**) in PC3 cells and PC3-derived populations that survived different cycles of Ad-REIC infection and recovery (C0~C4; 0 to 4 times). LNCaP is a sensitive prostate cancer cell line, and T24 and J82 are resistant bladder cancer cell lines.

FIGURE 4 - Sensitization of cancer cells to Ad-REIC by down regulation of BiP using siRNA. (**a**) down-regulation of BiP (detected with an antibody from Santa Cruz Biotechnology) by siRNA in Cl-1 cells shown by Western blot analysis. Cells were analyzed 48 h after transfection with siRNAs (0.2 μM). (**b**) sensitization of Cl-1 cells but not OUMS-24 cells to Ad-REIC by down-regulation of BiP using siRNA. (**c**) sensitization of different cell types to Ad-REIC (20 MOI) by transfection of BiP siRNA (0.2 μM) 24 h prior to the Ad-REIC challenge. (**d**) alteration in levels of proteins potentially

involved in the signal transduction triggered by ER stress in Cl-1 cells. P-, phosphorylated.

FIGURE 5 - Down-regulation of BiP resulted in sensitization of Cl-1 cells to Ad-REIC *in vivo*. (**a**) Down-regulation of luciferase activity of PC3-Luc cells by transfecting siRNA for luciferase *in vivo*. Open and shadowed columns are before and 3 days after application of siRNA normalized to the values of Day 0. Vertical bars, standard error among 5 mice. *, p=0.05. (**b**) Down-regulation of BiP in tumors formed by Cl-1 cells determined 3 days after transfection with the siRNA. Three tumors were examined. (**c**) Sensitization of Cl-1 cells to Ad-REIC by transfecting BiP siRNA *in vivo*. Vertical bars, standard error among 5 mice. *, $p<0.05$.

Figure 1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Apoptotic cell rate (%)
60
40
20
0
PC3
Cl−1
Cl−2
Cl−3
Cl−4
Cl−5
Cl−6
Cl−7
Cl−8
Cl−9
Cl−10
Cl−11
Clones isolated from PC3
b
PC3
Cl-1
Cl-2
Cl-3
Cl-4
Cl-5
Cl-6
Cl-7
Cl-8
Cl-9
Cl-10
Cl-11
c
PC3
Cl-1
Ad-LacZ
Ad-REIC
Ad-LacZ
Ad-REIC
HSP90
HSP70
HSC70
BiP
Bcl-2
Bcl-xL
Bad
Bax
LacZ
REIC
Tubulin
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PC3
LNCaP
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HSP27
HSP90
Tubulin

Figure 2

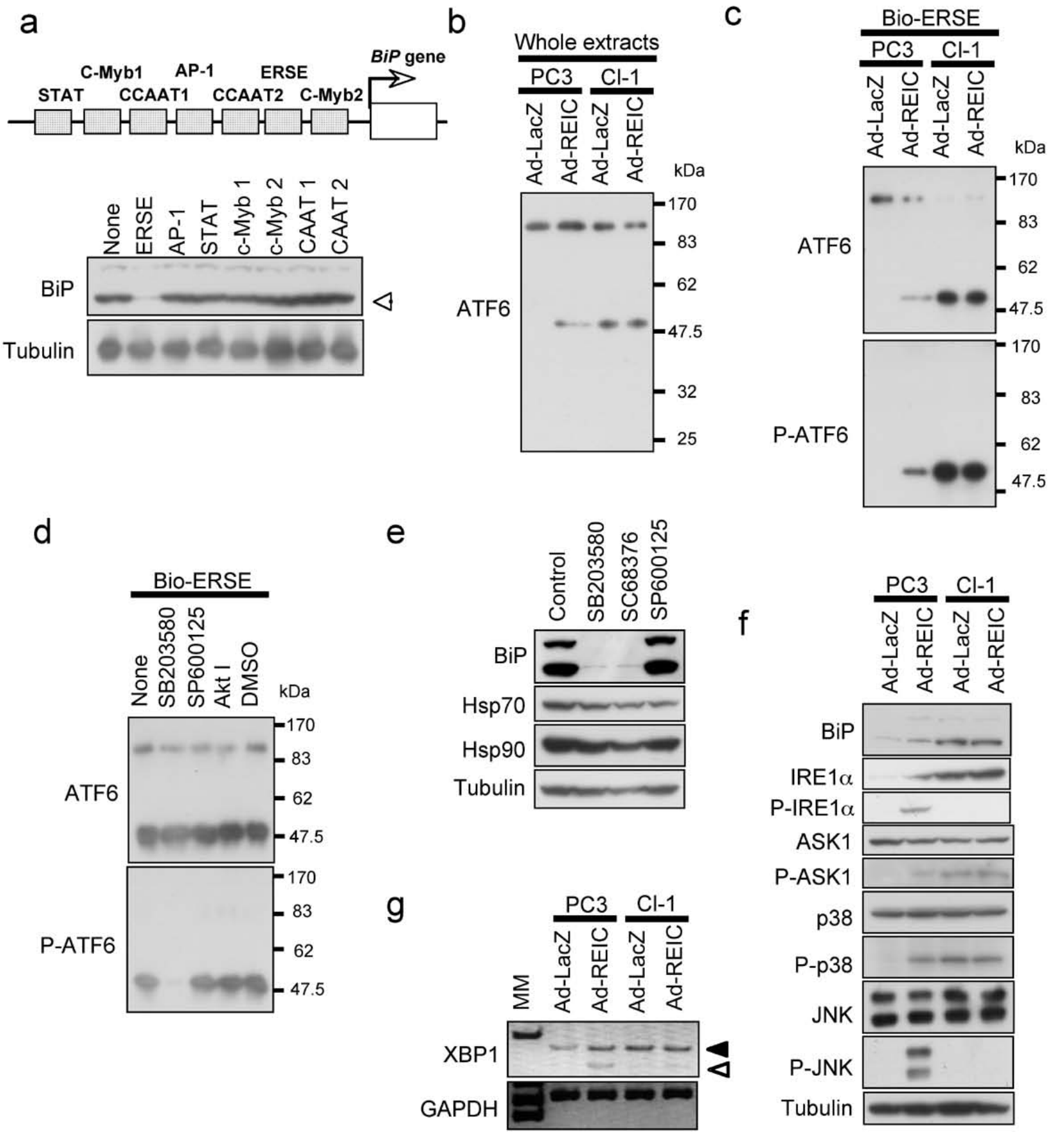

a
BiP gene
C-Myb1
AP-1
ERSE
STAT
CCAAT1
CCAAT2
C-Myb2
None
ERSE
AP-1
STAT
c-Myb 1
c-Myb 2
CAAT 1
CAAT 2
BiP
Tubulin
b
Whole extracts
PC3
Cl-1
Ad-LacZ
Ad-REIC
kDa
ATF6
170
83
62
47.5
32
25
c
Bio-ERSE
PC3
Cl-1
Ad-LacZ
Ad-REIC
kDa
ATF6
P-ATF6
170
83
62
47.5
d
Bio-ERSE
None
SB203580
SP600125
Akt I
DMSO
kDa
ATF6
P-ATF6
170
83
62
47.5
e
Control
SB203580
SC68376
SP600125
BiP
Hsp70
Hsp90
Tubulin
f
PC3
Cl-1
Ad-LacZ
Ad-REIC
BiP
IRE1α
P-IRE1α
ASK1
P-ASK1
p38
P-p38
JNK
P-JNK
Tubulin
g
PC3
Cl-1
MM
Ad-LacZ
Ad-REIC
XBP1
GAPDH

Figure 3

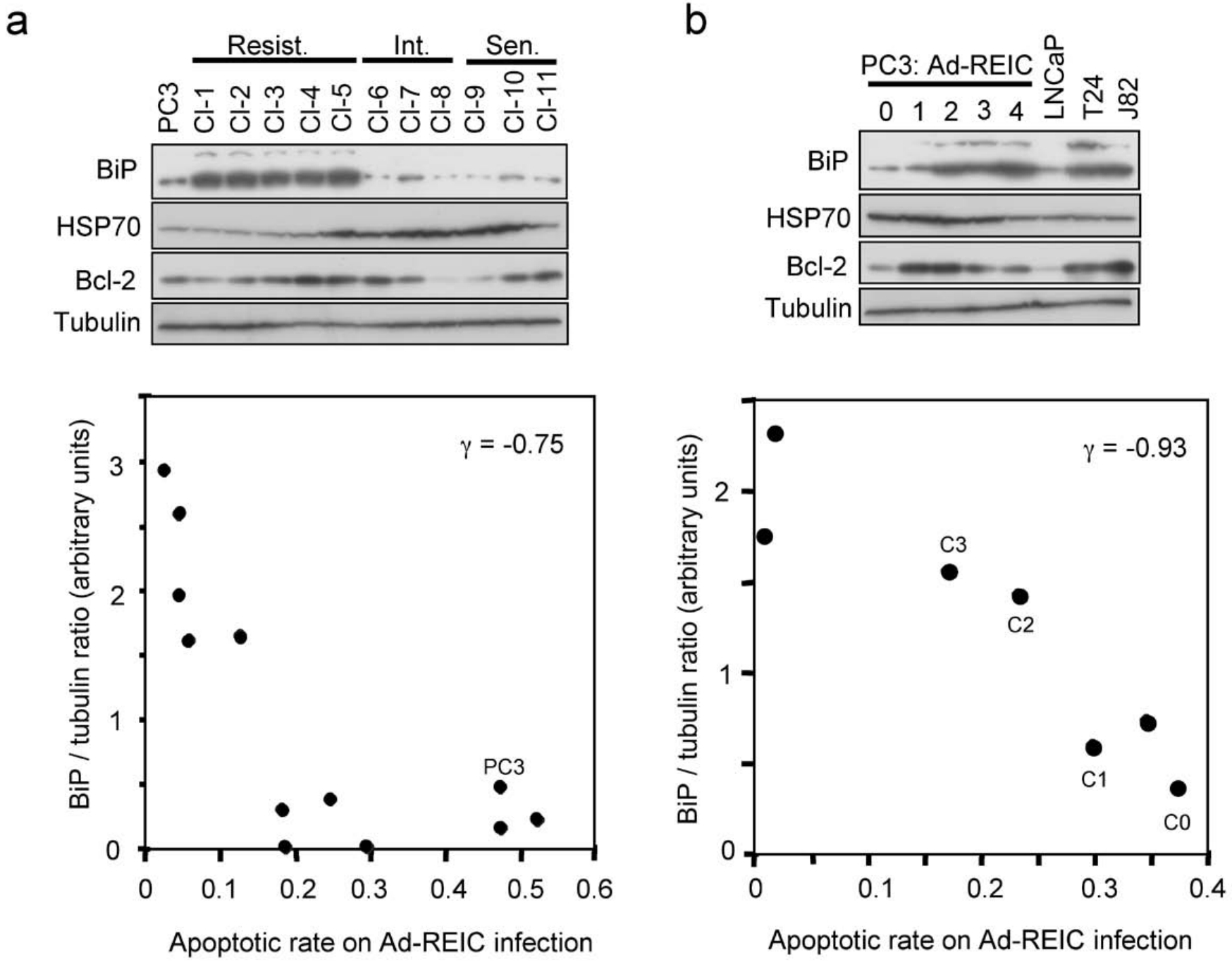
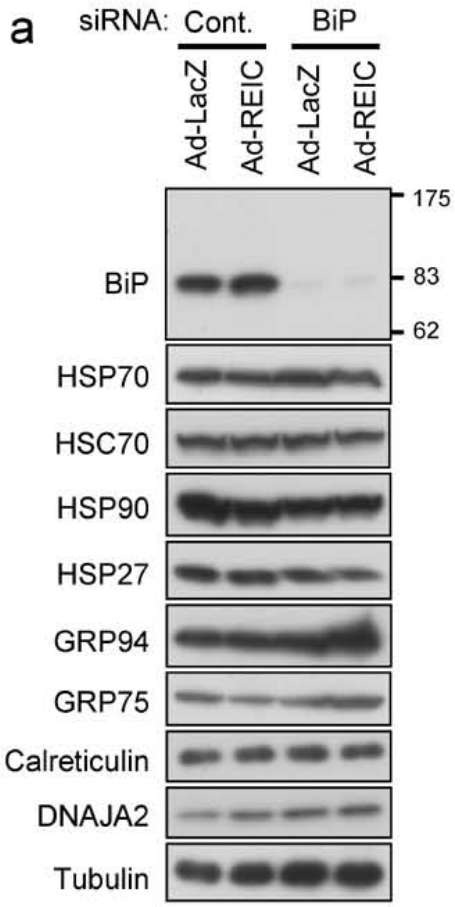
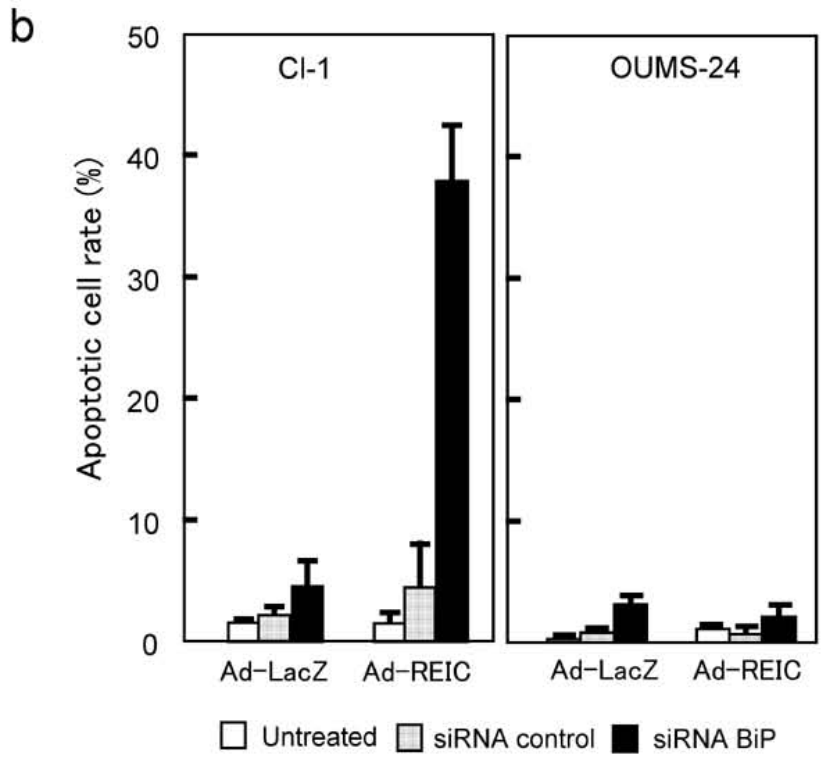
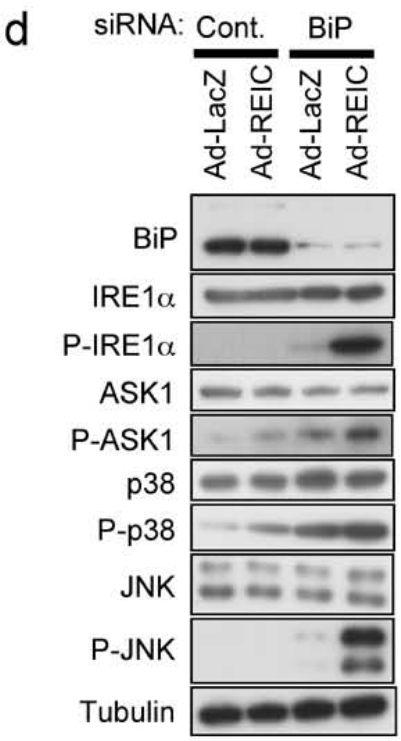

a
Resist.
Int.
Sen.
PC3
Cl-1
Cl-2
Cl-3
Cl-4
Cl-5
Cl-6
Cl-7
Cl-8
Cl-9
Cl-10
Cl-11
BiP
HSP70
Bcl-2
Tubulin
b
PC3: Ad-REIC
0 1 2 3 4
LNCaP
T24
J82
BiP
HSP70
Bcl-2
Tubulin
γ = -0.75
BiP / tubulin ratio (arbitrary units)
PC3
Apoptotic rate on Ad-REIC infection
γ = -0.93
C3
C2
C1
C0
BiP / tubulin ratio (arbitrary units)
Apoptotic rate on Ad-REIC infection

Figure 4


a
siRNA: Cont. BiP
Ad-LacZ
Ad-REIC
Ad-LacZ
Ad-REIC
175
83
62
BiP
HSP70
HSC70
HSP90
HSP27
GRP94
GRP75
Calreticulin
DNAJA2
Tubulin


b
Cl-1
OUMS-24
Apoptotic cell rate (%)
50
40
30
20
10
0
Ad-LacZ
Ad-REIC
Ad-LacZ
Ad-REIC
Untreated
siRNA control
siRNA BiP


d
siRNA: Cont. BiP
Ad-LacZ
Ad-REIC
Ad-LacZ
Ad-REIC
BiP
IRE1α
P-IRE1α
ASK1
P-ASK1
p38
P-p38
JNK
P-JNK
Tubulin

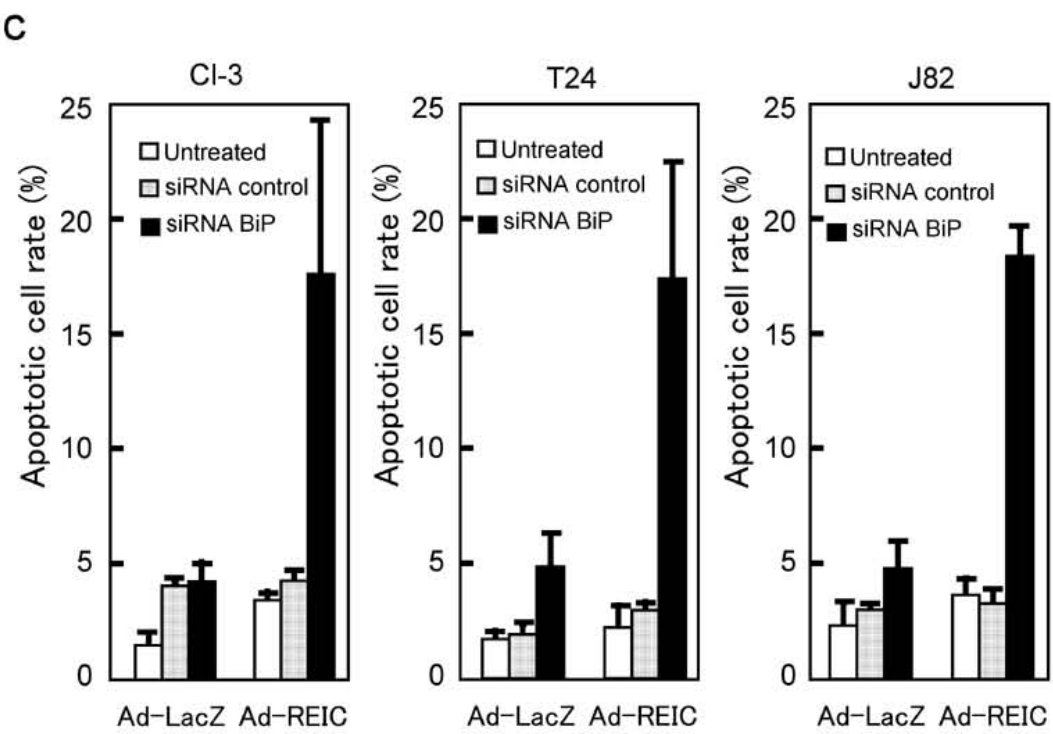
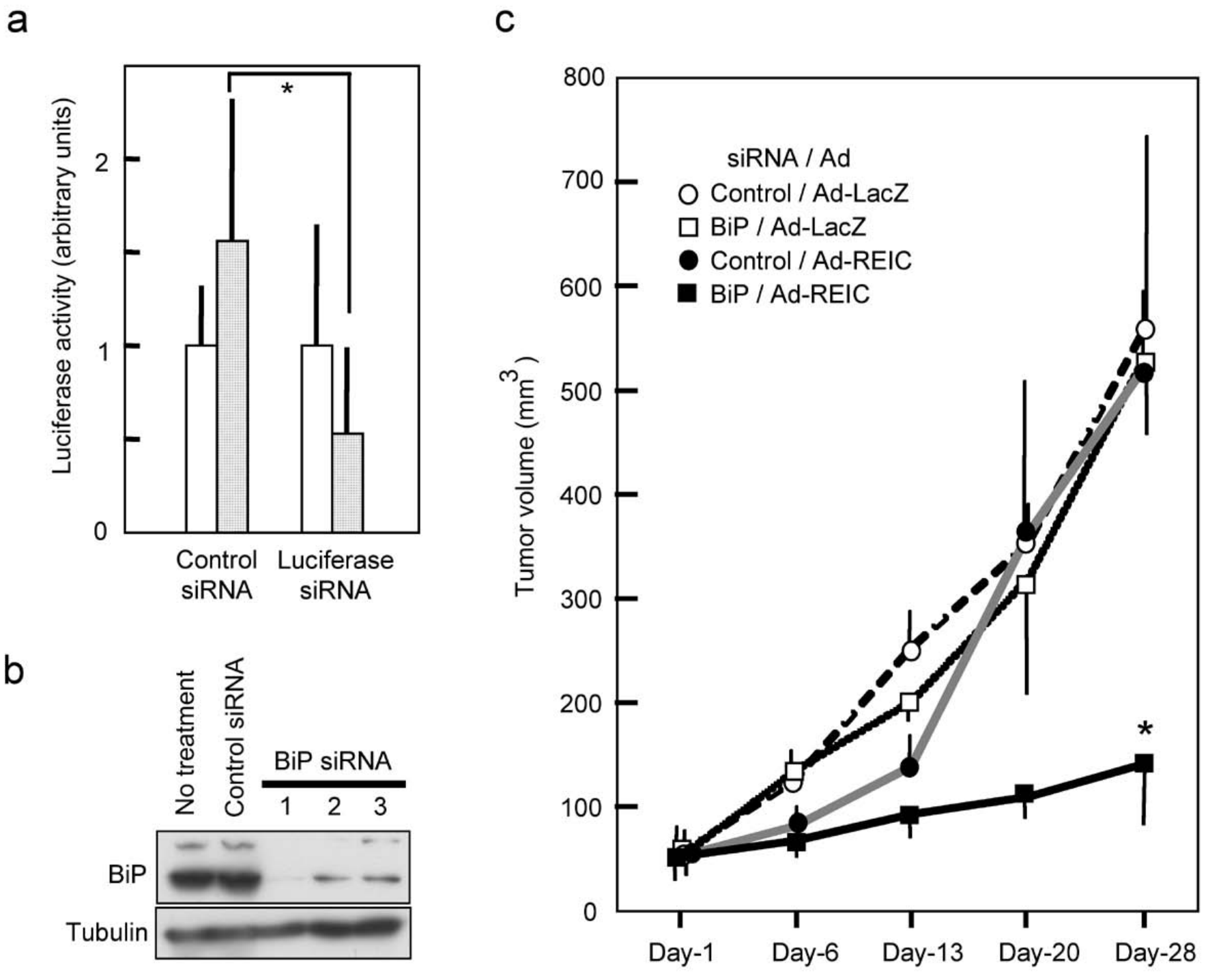

c
Cl-3
T24
J82
Apoptotic cell rate (%)
25
20
15
10
5
0
Untreated
siRNA control
siRNA BiP
Ad-LacZ
Ad-REIC

Figure 5


a
Luciferase activity (arbitrary units)
0
1
2
*
Control siRNA
Luciferase siRNA
b
No treatment
Control siRNA
BiP siRNA
1
2
3
BiP
Tubulin
c
Tumor volume (mm3)
0
100
200
300
400
500
600
700
800
siRNA / Ad
Control / Ad-LacZ
BiP / Ad-LacZ
Control / Ad-REIC
BiP / Ad-REIC
*
Day-1
Day-6
Day-13
Day-20
Day-28