

ERR γ Suppresses Cell Proliferation and Tumor Growth of Androgen-Sensitive and Androgen-Insensitive Prostate Cancer Cells and Its Implication as a Therapeutic Target for Prostate Cancer

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Abstract

Estrogen receptor-related receptors (ERR) are orphan nuclear receptors, which are constitutively activated without estrogen binding. Recent evidence indicates that the ligand-independent ERRs may be involved in similar ER-mediated regulatory pathways and modulate estrogen responsiveness in certain target cells. We recently showed that an ERR subtype, ERR γ , is coexpressed with ER β in normal human prostatic epithelial cells and exhibits reduced expression in many prostate cancer cell lines and clinical neoplastic prostate tissues. Based on this, we hypothesize that ERR γ may have growth regulatory roles in prostate and prostate cancer. We showed in this study that ERR γ was expressed in epithelial cell nuclei in fetal and pubertal human prostates, whereas its nuclear expression became reduced in advanced prostate cancer lesions. Stable ERR γ expression by retroviral transduction suppressed significantly both *in vitro* cell growth and *in vivo* tumorigenicity of two prostate cancer cell lines, LNCaP and DU145, as evidenced by a cell-cycle arrest at G₁-S transition and also induction of two cyclin-dependent kinase inhibitors p21^{WAF1/CIP1} and p27^{KIP1}. We further showed by reporter assay that induction of p21 and p27 by ERR γ was mediated through direct transactivation of their gene promoters. Moreover, we also showed that a selective ERR γ -agonist, DY131, could potentiate the ERR γ -induced growth inhibition in LNCaP-ERR γ and DU145-ERR γ cells in a dose-dependent manner compared with respective parental cells. Taken together, our results show that ERR γ may perform an antiproliferative or tumor-suppressing function in prostate cancer cells. More importantly, our results suggest that ERR γ could be a novel therapeutic target for prostate cancer treatment. [Cancer Res 2007;67(10):4904–14]

Introduction

Besides androgens, which play an important role in normal and neoplastic growths of prostate gland, estrogens have been suggested for a long time to play synergetic or distinct roles in the same processes (1, 2). It is generally believed that direct estrogenic effects in prostate or their possible roles in prostate cancer and benign prostatic hyperplasia are partially mediated through their specific receptors, estrogen receptor (ER) α and ER β , which bind directly to estrogen response elements (ERE) or other response sites via indirect interactions with other heterologous

transcription factors [e.g., Sp1 and activator protein 1 (AP-1)] in the target gene promoters to mediate transcriptional regulation. Both ER α and ER β are differentially expressed in adult prostatic tissues, with the epithelial cells expressing mainly ER β whereas the stromal cells expressing ER α at low levels (3). However, the exact roles of ERs and estrogen-signaling pathways in prostate still remain unclear. Previous studies of ER knock-out mice show that functional inactivation of ERs induces no apparent abnormality in both gross and microscopic structure of prostate (4–6), whereas estrogen-imprinting effects in neonatal prostate are mediated primarily through the stromal ER α and not epithelial ER β (7). However, some recent studies suggest that ER β may play an antiproliferative role in the regulation of prostatic epithelial differentiation, probably through activation by 5 α -androstane-3 β ,17 β -diol, a newly identified ER β ligand derived metabolically from dihydrotestosterone (8–10).

Recent advances in nuclear receptors show that members of the ligand-independent orphan nuclear receptors are important regulators of development and many cellular functions via their transcriptional regulations of gene expression. Among these orphan receptors, estrogen receptor-related receptors [estrogen related-receptors (ERR)], which are closely related to ERs and sharing high homology in their DNA-binding domains (DBD), are constitutively active without binding to natural estrogen (11, 12). Three different ERR subtypes (α , β , and γ) have been characterized thus far. All ERR members show considerable homology, especially in their DBD and the highly conserved activation function-2 (AF-2) domain, with the highest similarity between ERR β and ERR γ (13, 14). Like ERs, all ERRs bind to the classic ERE (AGGTCAn₃TGACCT), suggesting that ERRs may be involved in similar ER-mediated signaling pathways via regulation of similar target genes and competition for coregulatory proteins in same target cells. ERRs also bind to some ERE-related response elements with extended half-site core sequences (TNAAGGTCA; ERRE/SF-1RE; ref. 15), suggesting that ERRs may also have their own independent regulatory pathways or functions distinct from ERs. In addition, ERRs can regulate transcription in a similar manner to ER α via a tethered pathway by interacting with the Sp1 and AP-1 transcription factors at their respective DNA binding sites (16–18). Therefore, it is likely that ERRs may regulate a broad spectrum of genes in their target cells.

Human ERR γ (ERR3/NR3B3/ESRRG) was first cloned by rapid amplification of cDNA ends (RACE)-PCR from fetal brain (19) and subsequently from other organ cDNA libraries (13, 20), whereas mouse ERR γ was isolated by yeast two-hybrid screening of an embryo cDNA library using the transcriptional coactivator glucocorticoid receptor interacting protein 1 (GRIP1) as bait (21). Rat ERR γ has recently been cloned by yeast two-hybrid screening of a rat liver cDNA library with an orphan nuclear receptor, small heterodimer partner (SHP) as bait (15) and RACE-PCR from rat

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prostate (14). Multiple spliced ERR γ transcripts (ERR γ 1 and ERR γ 2), probably derived from alternative splicing at the 5'-end, have been identified from different cDNA libraries and show tissue-specific expression patterns (13, 20). Recently, a novel spliced variant ERR γ 3, which lacks the exon F encoding the second zinc finger motif in DBD, has been cloned, and interestingly, its transcript expression is confined to the prostate and adipocytes (22). However, their protein expressions in cells and tissues are not confirmed yet. Besides ERE and ERRE, ERR γ can also bind to a variety of ERRE-related sequences (15), suggesting that ERR γ may have its own distinct target genes, including the recently characterized *ERR α* , *DAX-1*, and *SHP* (23–26). A recent structural study indicates that ERR γ is a true orphan receptor as its ligand-binding domain (LBD) adopts a transcriptionally active conformation, which can recruit transcriptional coactivators in the absence of ligand (27). The ligand-independent transactivation of ERR γ depends on its binding via the AF-2 domain to some p160 transcriptional coactivators, including GRIP1, steroid receptor coactivator-1, amplified in breast cancer 2/ASC-2, and peroxisome proliferator-activated receptor γ coactivator-1 α /PGC-1 α , while repressed by receptor interacting protein 140/RIP140, which recruits histone deacetylases (21, 23, 24, 27, 28). However, it is shown that some synthetic ER agonist (diethylstilbestrol) and selective ER modulators (SERMs; tamoxifen, 4-hydroxytamoxifen) can bind to ERR β/γ as antagonists by disrupting the ERR-coactivator interactions (29, 30), whereas some synthetic acyl hydrazones can bind to ERR β/γ as agonists (31, 32). By Northern blot and reverse transcription-PCR (RT-PCR), ERR γ transcripts are detected in diverse fetal and adult human tissues and organs, including prostate, with particularly high levels in brain, gastrointestinal tract, heart, kidney, skeletal muscle, and placenta (13, 19, 20, 33). Similar expression patterns are also shown in mouse and rat adult tissues (14, 21), suggesting that ERR γ may play important roles in the differentiation and functions in these tissues and organs. However, *in vitro* or *in vivo* functional study of ERR γ is absent thus far.

Recently, we showed that ERR members (α , β , and γ) are coexpressed with ERs in normal prostatic epithelial cells, whereas they exhibit an apparent down-regulation in many prostate cancer cell lines and clinical neoplastic tissues (33), implying that the ligand-independent ERRs and ligand-dependent ERs may coregulate the growth and functions of prostatic cells. Among ERRs, ERR γ shows a down-regulation pattern in some androgen-independent prostate cancer lines and also clinical neoplastic tissues, whereas its transient ectopic expression inhibits the *in vitro* proliferation of PC-3 prostate cancer cells. To show whether ERR γ could be a useful marker for prostate cancer development, in this study, we investigated the roles of ERR γ in the growth regulation of prostate cancer cells by retroviral transducing ERR γ in both androgen-sensitive (LNCaP) and androgen-insensitive (DU145) prostate cancer cells. Moreover, the role of cell-cycle regulatory proteins, p21^{WAF1/CIP1} and p27^{KIP1} (hereafter referred to as p21 and p27), in the ERR γ -induced cell cycle arrest was also investigated. Finally, we also examined the efficacy of a selective ERR γ agonist on the ERR γ -positive prostate cancer cell growth.

Materials and Methods

Human Prostatic Tissues and Immunohistochemistry

Archival blocks of formalin-fixed fetal, pubertal, and neoplastic human prostates were used for immunohistochemistry (34). Immunohistoche-

mistry was done using a glucose-oxidase-diaminobenzidine-nickel procedure (33). All human tissues were obtained with informed consent and approval from Clinical Research Ethics Committee, the Chinese University of Hong Kong.

Cell Lines and Cell Culture

Human prostate cancer lines, LNCaP and DU145, cervical carcinoma line HeLa, and a mouse packaging cell line PA317 were obtained from the

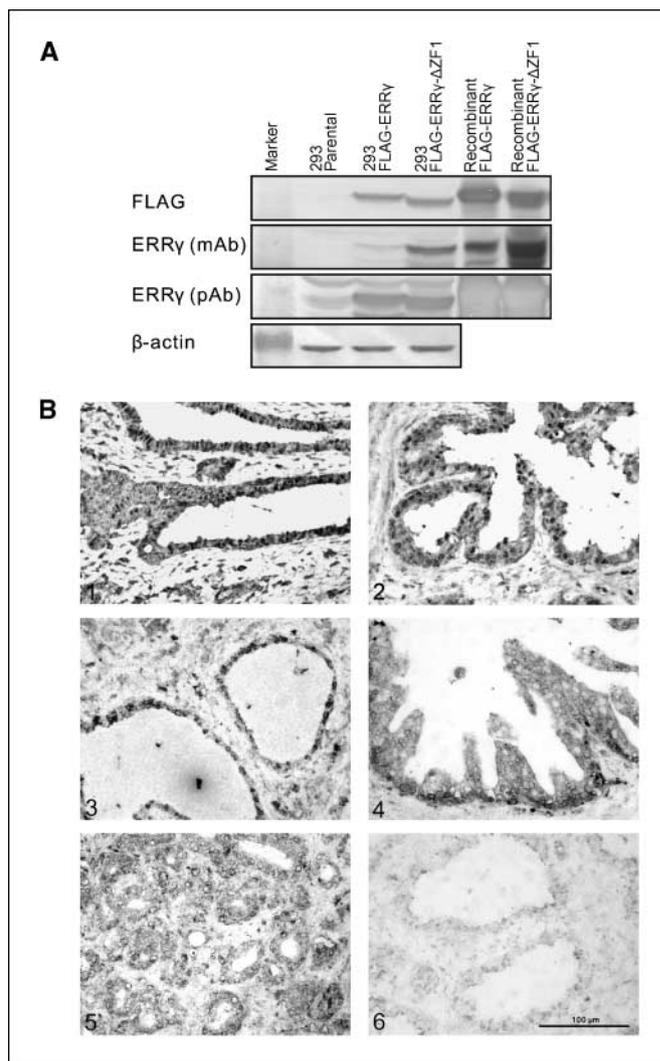


Figure 1. ERR γ expression in prostate. **A**, Western blot analysis of FLAG-ERR γ expressed in 293 cells and bacterial expressed recombinant FLAG-ERR γ fusion proteins. The anti-FLAG M2 monoclonal antibody recognized the FLAG-ERR γ and FLAG-ERR γ - Δ ZF1 expressed in transient transfected 293 cells and also the recombinant FLAG-ERR γ fusion proteins expressed in *E. coli*. Similarly, both the mouse monoclonal and rabbit polyclonal anti-ERR γ antibodies recognized specifically the FLAG-ERR γ and FLAG-ERR γ - Δ ZF1, the same as the anti-FLAG antibody. **B**, immunohistochemistry of ERR γ in prostate. **1**, fetal prostate. Positive ERR γ immunosignals were detected in the nuclei of developing prostatic epithelial and mesenchymal cells. **2**, pubertal prostate. The epithelial acinar cells showed positive immunoreactivity in their nuclei. **3**, aged normal prostate. The epithelial acinar cells showed reduced and variable nuclear immunoreactivity. **4**, high-grade PIN lesion. Most dysplastic epithelial cells in PIN lesions were negatively stained in their nuclei, whereas a few basally located epithelial cells showed positive nuclear staining. **5**, low-grade adenocarcinoma lesion. Malignant cells were weakly or negatively stained in their nuclei. **6**, control. Section was stained with secondary antibody only. No nuclear reaction was seen in the epithelial cells. Magnification, $\times 400$; bar, 100 μ m, applied to all figures shown.

American Type Culture Collection. All cell lines and derived clones were cultured according to the American Type Culture Collection recommendations. Total RNA and proteins were extracted from cells grown to 70% to 80% confluence.

Plasmid Construction

Mammalian expression vectors. FLAG-tagged or untagged full-length human ERR γ cDNAs were generated by PCR using pSG5-ERR γ (33; NM_001438) as template and subcloned into pBabe-puro for retroviral transduction

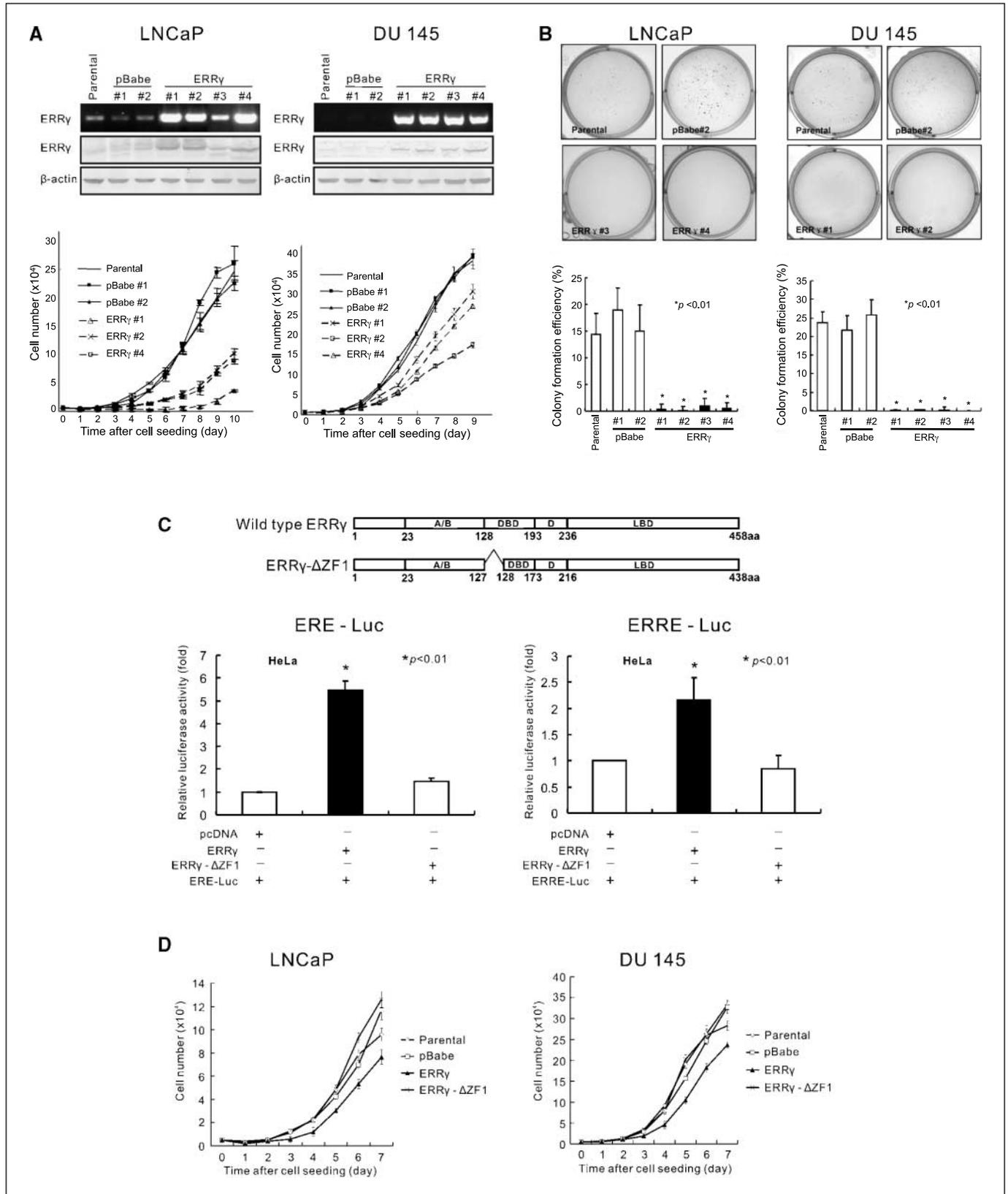
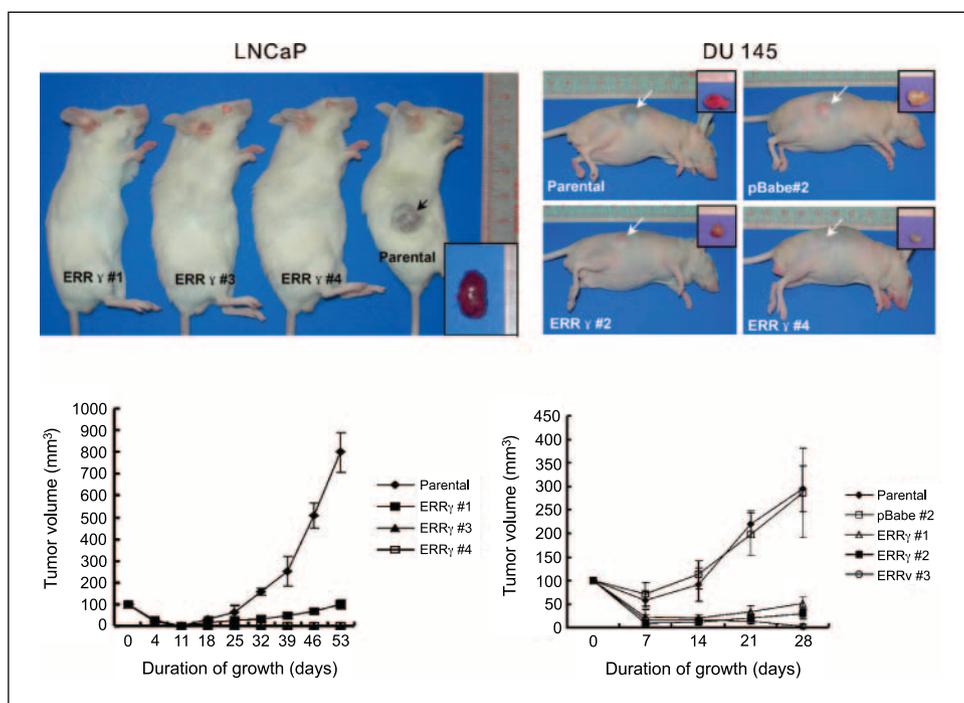


Figure 3. *In vivo* tumor growth of LNCaP-ERR γ clones in SCID mice and DU145-ERR γ clones in nude mice. *Top*, photographs show the representative mice bearing s.c. injected ERR γ clones and parental cells. Growth durations were 7.5 wks for LNCaP and 4 wks for DU145 cells. *Arrows*, tumors were grown in mice bearing the inoculated parental cells or pBabe clones (inserts at corners show the dissected tumors), whereas no tumors were grown or were growing very slowly in mice bearing the inoculated ERR γ clones. *Bottom*, growth curves of tumors growing in mice. The mean of tumor volumes was determined from tumors growing in three independent mice.



and pcDNA3.1 for transfections, at *Bam*HI and *Eco*RI sites, yielding pBabe-ERR γ , pBabe-FLAG-ERR γ , pcDNA3.1-ERR γ , and pcDNA3.1-FLAG-ERR γ , respectively. FLAG-tagged truncated mutant ERR γ - Δ ZFI with deletion of first zinc finger in DBD was generated by fusion-PCR method and subcloned into pcDNA3.1 and pBabe.

Reporter constructs. Luciferase reporter plasmids, pGL3-ERE₃-Luc and pGL3-ERRE₃-Luc containing three copies of ERE and ERRE, were constructed as described (33, 35). Human gene promoter sequences, p21 (-2,324 to +11) and p27 (-3,568 to +1), and their serial deletion constructs were amplified by PCR from human genomic DNA extracted from DU145 cells and cloned into pGL3 at *Xho*I and *Hind*III sites as pGL3-p21-Luc and pGL3-p27-Luc, respectively. All plasmid constructs were confirmed by DNA sequencing.

Transient Transfection and Luciferase Reporter Assay

HeLa cells were seeded at 5×10^4 cells/well in 24-well plates and grown for 24 h in MEM with 10% fetal bovine serum (FBS) before transfection. Cells were cotransfected with 0.2 μ g reporter plasmid (pGL3 or pGL3-ERE₃/ERRE₃/p21/p27), 0.2 μ g expression plasmid (pcDNA3.1, pcDNA3.1-FLAG-ERR γ /FLAG-ERR γ - Δ ZFI), and 0.04 μ g pRL-CMV using FuGENE6 transfection reagent. After 48 h post-transfection, cells were harvested for luciferase reporter assay using the Dual-Luciferase Reporter Assay System (Promega). The luciferase activation activity was normalized to that of the

internal control *Renilla* luciferase activity by pRL-CMV as relative luciferase unit and fold of activation of relative luciferase unit as compared with controls (transfection with pcDNA3.1) was determined. All assays were done in three separate experiments done in triplicate, and data were presented as mean \pm SD.

Retroviral Transduction and Generation of ERR γ -Stable Clones

pBabe-FLAG-ERR γ , pBabe-FLAG-ERR γ - Δ ZFI, or pBabe were transfected, respectively, into PA317 cells growing in DMEM and 10% FBS using FuGENE 6 reagent. Transfected cells were incubated in fresh medium for another 24 h. After 48 and 72 h, culture medium containing infectious virions was harvested for retroviral transduction. LNCaP and DU145 cells were incubated with the virus-containing medium mixed with 8 μ g/mL polybrene. After 48 h incubation, puromycin was added to eliminate the noninfected cells, whereas stable clones were isolated after 8-day drug selection. Vector control clones were generated by infecting cells with empty vector pBabe-puro.

In vivo Cell Growth Assays

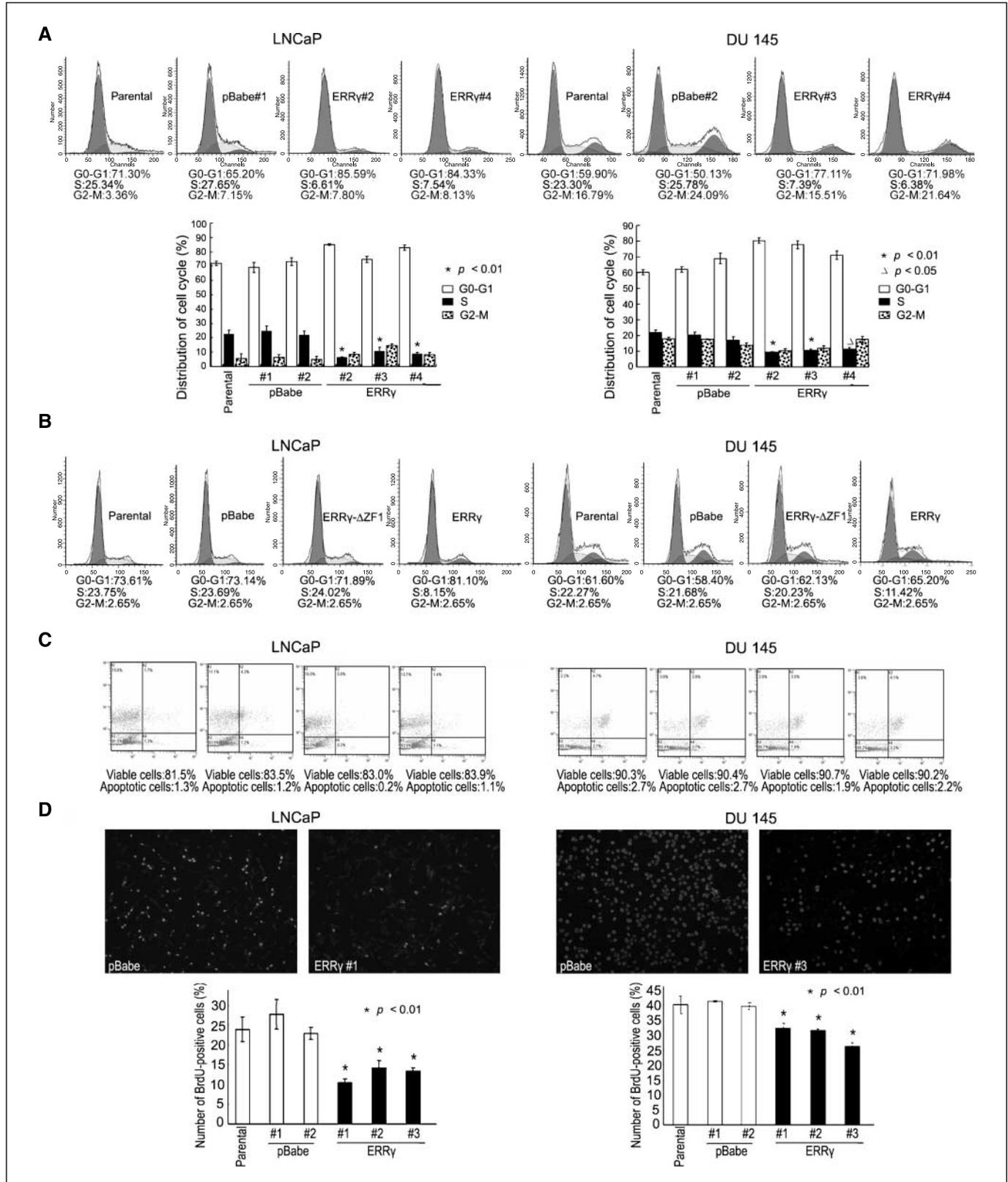
Cell counting. Stable ERR γ clones and their parental cells were plated at 5×10^3 per well in 24-well plates and cultured in growth media for 10 days, with fresh media replaced every 3 days. Viable cells were counted daily by

Figure 2. *In vitro* studies of LNCaP-ERR γ and DU145-ERR γ -transduced clones. *A*, characterization and cell proliferation of ERR γ -transduced clones. *Top*, by RT-PCR and immunoblot, ERR γ transcripts and proteins were detected at moderate to high levels in the selected representative LNCaP-ERR γ and DU145-ERR γ clones but weak (low endogenous expression in LNCaP and LNCaP-pBabe) or negative (DU145 and DU145-pBabe) in empty-vector pBabe clones and parental cells. *Bottom*, growth curves of the representative ERR γ clones, pBabe clones and parental cells. All LNCaP-ERR γ and DU145-ERR γ clones proliferated at significantly slower rates than their respective pBabe clones and parental cells (doubling time in hour: LNCaP, 40.59 ± 0.89 ; pBabe 1, 40.70 ± 0.48 ; pBabe 2, 38.50 ± 0.38 versus ERR γ 1, 127.08 ± 23.98 ; ERR γ 3, 56.59 ± 2.27 ; ERR γ 4, 58.44 ± 0.75 ; DU145, 31.35 ± 0.29 ; pBabe 1, 31.54 ± 0.33 ; pBabe 2, 31.29 ± 0.36 versus ERR γ 1, 34.02 ± 0.44 ; ERR γ 2, 39.52 ± 1.15 ; ERR γ 4, 35.17 ± 0.49). *B*, anchorage-independent growth of ERR γ clones in soft agar. *Top*, micrographs show the whole-well view of crystal violet-stained colonies that appeared as small spots. The representative LNCaP-ERR γ clones formed significantly fewer and smaller colonies, whereas DU145-ERR γ clones did not form colonies in soft agar, as compared with the pBabe clones and parental cells. *Bottom*, graphs show the colony formation efficiencies (determined by % of number of colonies formed from 5×10^3 seeded cells) obtained from three independent experiments. *, $P < 0.01$ versus parental cells. *C*, establishment and cell proliferation of mutant ERR γ - Δ ZFI infectants. *Top*, schematic representations of the wild-type ERR γ (458 aa) and the mutant ERR γ - Δ ZFI (438 aa) with truncation of the first zinc finger (deletion of 128–147 amino acids). The starting and ending amino acid residue numbers/numbers of the domains are indicated: A/B, activation function-1/AF-1; C or DBD, DNA-binding domain; D, hinge region; E/F or LBD, ligand-binding domain/AF-2. *Bottom*, transactivation of ERE- and ERRE-driven reporter luciferase gene by ERR γ and ERR γ - Δ ZFI transfected in HeLa cells in the absence of estradiol. Wild-type ERR γ induced a modest transactivation on ERE₃-Luc and ERRE₃-Luc reporters, whereas mutant ERR γ - Δ ZFI did not. *Columns*, mean of relative luciferase activities from three independent experiments; *bars*, SD. *, $P < 0.01$ versus empty vector pcDNA. *D*, growth curves of the pooled FLAG-ERR γ and FLAG-ERR γ - Δ ZFI infectants, pBabe infectants, and parental cells. All LNCaP-FLAG-ERR γ and DU145-FLAG-ERR γ infectants proliferated at significantly slower rates than their respective mutant FLAG-ERR γ - Δ ZFI infectants, pBabe infectants, and parental cells.

trypan blue exclusion assay. All counts were done on triplicate wells and repeated in three independent experiments, and mean \pm SD of cell number was plotted against time.

5-Bromodeoxyuridine incorporation assay. Cells at same density were plated on poly-lysine-coated coverslips placed in 24-well plates and

cultured for 24 h in growth media. After 24 h culture, cells were incubated with 10 μ mol/L 5-bromodeoxyuridine (BrdUrd) for 1 h and fixed in cold methanol-acetone (1:1) for 15 min. Fixed cells were stained with a mouse monoclonal anti-BrdUrd antibody (Becton Dickinson) followed by an anti-mouse immunoglobulin G-FITC. Stained cells were detected and counted



under a fluorescence microscope and analyzed with morphometry software (NeuroLucida-2000, Vermont). Percentages of BrdUrd-positive cells were determined after counting at least 500 cells in both parental cells and stable clones. Data were presented as mean \pm SD obtained from three independent experiments.

Soft agar assay for anchorage-independent growth. Cells were suspended in culture media with 0.3% agar and plated at 5×10^3 cells per well in six-well plates, which were precoated with 0.5% base agar in the same media. After culture for 28 days, cells were fixed in 4% formaldehyde, stained with 0.1% crystal violet for colony visualization, and counted under microscope. Colony-formation efficiency was determined as the number of colonies, with sizes more than 100 μ m in diameter, formed per total number of cells seeded. All experiments were done in triplicates.

Cell Cycle and Apoptosis Analyses

Parental cells and stable clones were cultured to 80% confluence and harvested. Trypsinized cells (1×10^6), suspended in PBS, were fixed in ice-cold 70% ethanol overnight at -20°C . Fixed cells were incubated in PBS containing 50 μ g/mL propidium iodide (PI) and 10 μ g/mL DNase-free RNase for 15 min. DNA flow cytometry was done in a flow cytometer (ALTRA Cell Sorting System, Coulter) using a total of 3×10^5 cells. Cell-cycle distribution was analyzed with commercial software (MoFit LT2). Apoptotic or necrotic cells were detected by a FITC-Annexin V-PI double staining method (ApopNexin Apoptosis detection Kit, Chemicon) and analyzed by bivariate DNA flow cytometry. By this staining method, the AV⁺/PI⁻ cell population represents the apoptotic cells, whereas AV⁺/PI⁺ and AV⁻/PI⁺ as the necrotic or late apoptotic cells.

In vivo Tumor Growth Assay

To evaluate the impact of ectopic ERR γ expression on *in vivo* tumorigenicity of prostate cancer cells, stable ERR γ clones or their parental cells (suspended in 100 μ L 1:1 growth medium–Matrigel mixture) were s.c. injected into the flanks of male non-obese diabetic/severe combined immunodeficient (SCID) mice (3×10^6 LNCaP cells per mouse) or athymic nude mice (5×10^6 DU145 cells per mouse) and allowed to grow for 4 to 8 weeks. All mice were housed in an air-filtered pathogen-free condition. Tumor growth was monitored weekly. Tumor volumes (mm^3) were measured using electronic calipers and calculated using the formula [(width) \times (length) \times (height)]/2. Growth of tumor volumes (mean \pm SD obtained from three independent animals) was plotted against time. All animal experiments were done with approval from the Animal Experimentation Ethics Committee, The Chinese University of Hong Kong, and in accordance with the LASEC Guidelines for laboratory animals.

RNA and Protein Analyses

RT-PCR analysis. Total RNA was extracted from cultured cells using TRIzol reagent. Approximately 2 μ g DNase I-treated RNA samples were reverse transcribed to cDNAs by M-MLV reverse transcriptase using oligo(dT) primer, and 1 μ L cDNA samples was used for PCR. Primers used are as follows: ERR γ : 5'-primer: 5'-ACCATGAATGGCCATCAGAA-3', 3'-primer: 5'-ACCAGCTGAGGGTTCAGGTAT-3'; and β -actin: 5'-primer: 5'-ATGGATGATGATATCGCCGCG-3', 3'-primer: 5'-CTCCATGTCGTCC-CAGTTGGT-3'.

Western blot analysis. Total cellular proteins were extracted from cultured cells grown to 70% to 80% confluence. Cells were lysed in 100 μ L ice-cold lysis buffer (33) supplemented with a protease inhibitor cocktail for 30 min at 4°C . Supernatants were collected after centrifugation at

11,625 \times g at 4°C . Protein concentration was determined by a modified Lowry method using bovine serum albumin as standard. Protein samples, loaded at 40 to 80 μ g per lane, separated by 10% to 15% SDS-PAGE gels and transblotted onto polyvinylidene difluoride membranes, were probed with primary antibodies at optimal dilutions, followed by an alkaline phosphatase-conjugated secondary antibody for colorimetric visualization. Primary antibodies used are as follows: (a) an affinity-purified rabbit polyclonal ERR γ antibody (14, 33), (b) a mouse monoclonal ERR γ antibody (R&D Systems), (c) p21 (Lab Vision), (d) p27 (Lab Vision), (e) p53 (Cell Signaling), (f) cyclin A (Santa Cruz), (g) cyclin B1 (Cell Signaling), (h) cyclin D1 (Lab Vision), (i) cyclin D3 (Lab Vision), and (j) anti-FLAG M2 (Sigma).

In vitro Treatments with ERR γ Agonist

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and BrdUrd incorporation. The efficacy of an ERR γ -specific agonist DY131 (31) on prostate cancer cell growth was evaluated by cell viability 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and BrdUrd incorporation. DY131 (Tocris) was dissolved in DMSO at 0.1 to 30 mmol/L as stock solutions and diluted (1:10³) in culture medium at different concentrations for *in vitro* treatments. For cell growth studies, ERR γ clones and parental cells were seeded at 2×10^3 per well onto 96-well plates in their growth media and allowed to adhere for 24 h before culturing in the same medium containing DY131 at doses ranging from 0.1 to 30 μ mol/L for 1 to 5 days. For controls, cells were treated with DMSO diluted (1:10³) in the same medium. Media with or without DY131 were renewed twice for treatments longer than 3 days. After treatments, the number of viable cells was determined by MTT assay. Experiments on each drug concentration were done in quadruplicate, and data as mean \pm SD were obtained from three independent experiments. DNA synthesis in DY131-treated cells was determined by BrdUrd incorporation. After 24-h culture, cells were treated with DY131 at various doses in the same medium and cultured for 3 to 4 days. After treatments, 10 μ mol/L BrdUrd was added to DY131-treated cells followed by BrdUrd staining procedure as mentioned above.

Luciferase assay. Human embryonic kidney epithelial cell line 293 was used for the study of DY131 on ERR γ -driven ERE/ERRE-Luc reporter activities. Cells were seeded at 7×10^4 cells per well in 24-well plates and cultured in DMEM medium with 10% FBS for 24 h before transfection. Cells were cotransfected with the reporter and ERR γ -expression plasmids as mentioned above. After 12 h post-transfection, cells were treated with DY131 at various doses. After treatments for 48 h, cells were harvested for luciferase reporter assay as mentioned above.

Statistical Analysis

All results are expressed as the mean \pm SD. Statistical analyses of data were done using two-tailed Student's *t* test, and differences were considered significant where $P < 0.01$.

Results

ERR γ expression is reduced in prostatic intraepithelial neoplasia and carcinoma. The specificities of newly generated ERR γ antibodies were verified and evaluated by blotting with FLAG-ERR γ expressed in transient transfected 293 cells and bacterial expressed recombinant FLAG-ERR γ fusion proteins (Fig. 1A). Immunohistochemistry of ERR γ revealed that in fetal

Figure 4. Cell-cycle analysis and BrdUrd incorporation in ERR γ -transduced prostate cancer cells. *A*, flow-cytometric analyses of the wild-type ERR γ clones, pBabe clones, and parental cells shown as DNA content histograms. Cell-cycle distribution is represented as the percentage of cells at each phase. In both LNCaP-ERR γ and DU145-ERR γ cells, the percentages of cells at S phase were significantly lower than their respective pBabe clones and parental cells. The ERR γ clones also had higher percentages of cells at G₁-G₀ phase than the pBabe clones and parental cells. *B*, flow-cytometric analysis of the pooled FLAG-ERR γ and FLAG-ERR γ - Δ ZFI infectants, pBabe infectants, and parental cells. Similar to the isolated ERR γ clones shown in (A), the pooled FLAG-ERR γ infectants had significantly lower percentages of cells at S phase and higher percentages of cells at G₀-G₁ and G₂-M phases than their respective pBabe infectants and parental cells. *C*, FACS analysis of viable and apoptotic cells in ERR γ clones, pBabe clones, and parental cells by FITC-AnnexV-PI staining. *Bottom left quadrants*, are viable cells (AV⁻/PI⁻); *bottom right quadrants*, apoptotic cells. Similar to pBabe clones and parental cells, the percentages of apoptotic cells were low and insignificant in ERR γ clones. *D*, *top*, representative results of BrdUrd staining; *bottom*, graphs show that the numbers of BrdUrd-positive cells were significantly reduced in the ERR γ clones, as compared with pBabe clones and parental cells. *, $P < 0.01$ versus parental cells.

prostate, the developing epithelial cells in proximal ducts extending from the urethra expressed moderate to intense ERR γ immunoreactivity in their nuclei, whereas epithelial cells in the developing acini and budding outgrowths of epithelial nests in distal region showed variable nuclear staining (Fig. 1B1). Mesenchymal cells in the stroma also showed positive nuclear staining. In pubertal prostate, both the basal and luminal epithelial cells exhibited moderate to intense nuclear immunoreactivity (Fig. 1B2). The stromal cells were variably stained at their nuclei. The aged normal prostate also showed similar staining pattern as in pubertal prostate but weaker nuclear immunoreactivities in the epithelial cells (Fig. 1B3), whereas the ERR γ immunoreactivity of stromal cells in aged prostate became markedly reduced or negative. The dysplastic epithelial cells in prostatic intraepithelial neoplasia (PIN) lesions showed reduced staining in their nuclei (Fig. 1B4). In well-differentiated adenocarcinoma lesions, the malignant cells showed significantly reduced or negative immunoreactivity in their nuclei (Fig. 1B5). The controls without primary antibody showed negativity in both epithelial and stromal cells (Fig. 1B6).

Ectopic ERR γ expression suppresses *in vitro* growth of prostate cancer cells, which is mediated through its intact DBD. To further elucidate the functional significance of ERR γ in prostate cancer cell growth, we generated ERR γ -stable clones in LNCaP and DU145 cells (LNCaP expresses low endogenous level of ERR γ , whereas DU145 expresses no ERR γ) by retroviral transduction and selected more than 10 stable clones for *in vitro* and *in vivo* growth studies (Fig. 2A). We did not observe any significant morphologic alterations in DU145-ERR γ clones, as compared with its parental line. However, we observed an induction of long dendritic cellular processes in LNCaP-ERR γ clones, as compared with their empty vector pBabe-infected clones and noninfected parental cells (data not shown). All generated LNCaP-ERR γ and DU145-ERR γ clones proliferated significantly slower than their corresponding pBabe clones and parental cells (Fig. 2A). Soft agar assay showed that colony formation efficiencies and colony sizes of LNCaP-ERR γ and DU145-ERR γ clones were significantly suppressed by stable expression of ERR γ , as compared with their pBabe clones and parental cells that formed colonies in soft agar (Fig. 2B).

To show whether the ERR γ -induced growth inhibition in prostate cancer cells were mediated through its intact DBD, we generated ERR γ - Δ ZFI mutant with deletion of the first zinc finger in its DBD (Fig. 2C). We found that the mutant was unable to transactivate the ERE/ERRE-Luc reporters (Fig. 2C) and did not show any inhibitory effect on the proliferation of LNCaP and DU145 cells (Fig. 2D).

ERR γ expression prevents *in vivo* tumor growth of prostate cancer cells. The effect of stable ERR γ expression on the tumorigenicity of LNCaP and DU145 cells was evaluated in immunodeficient mice. Significant suppression of tumor formation was observed in mice bearing the inoculated LNCaP-ERR γ and DU145-ERR γ clones, as compared with mice bearing the empty vector clones or parental cells, in which tumors were formed within 4 to 7 weeks after injection (Fig. 3).

Inhibition of prostate cancer cell proliferation by ERR γ is mediated through suppression of S-phase progression but not apoptosis. Because ectopic ERR γ expression suppressed both *in vitro* cell proliferation and tumor growth of prostate cancer cells, we next investigated the status of cell-cycle progression and cell proliferation in ERR γ clones by DNA flow cytometry and BrdUrd incorporation. The results of flow cytometry revealed

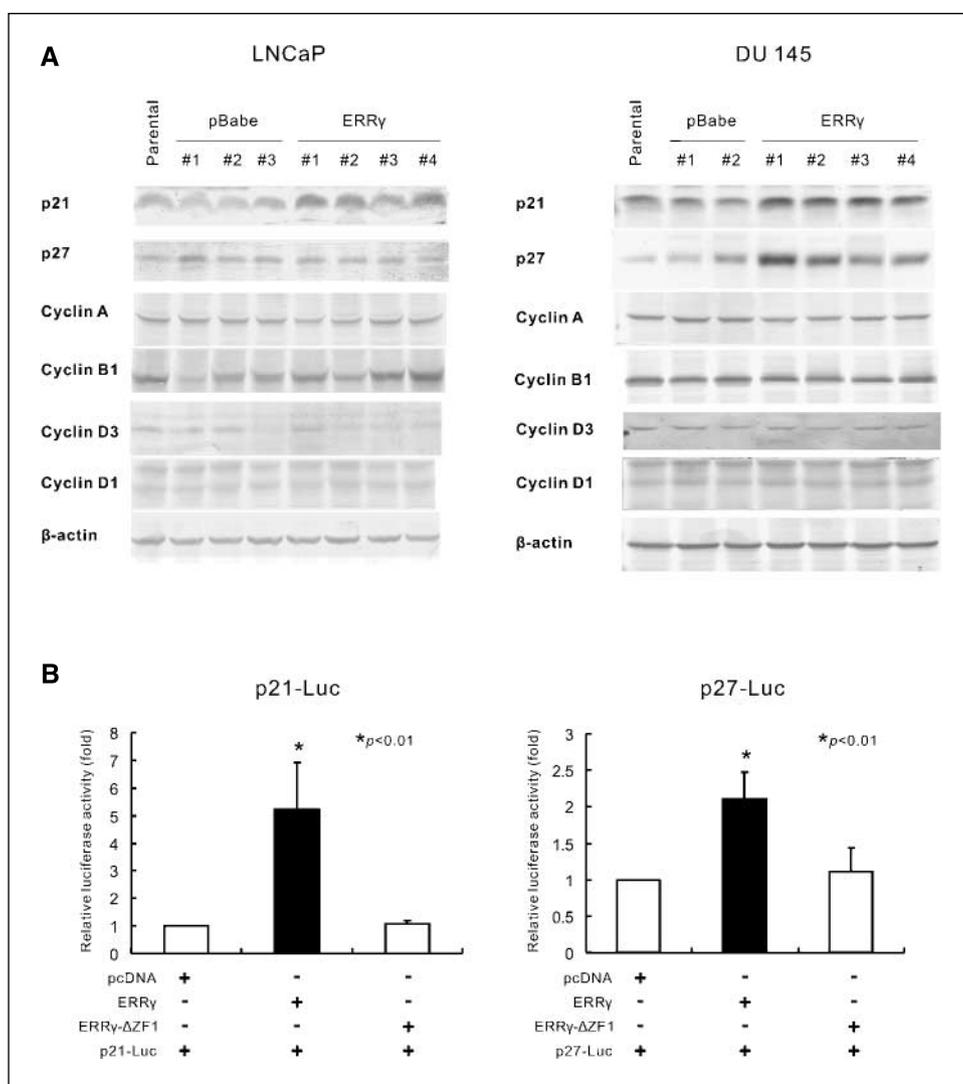
that the isolated ERR γ clones and the pooled ERR γ infectants of LNCaP and DU145 cells exhibited a significant decrease of cell populations at S phase (22–24% in parental cells versus 7–8% in infectants) and a concomitant moderate increase of cell fractions at G₀-G₁ phase, as compared with their respective pBabe infectants (isolated or pooled infectants) and parental cells (Fig. 4A and B). Similar to pBabe infectants and parental cells, the mutant ERR γ - Δ ZFI infectants showed no reduction of S-phase populations (Fig. 4B). Fluorescence-activated cell sorting (FACS) analysis of FITC-Annexin V-PI-labeled cells showed that there was no significant induction of apoptotic and necrotic cells in the LNCaP-ERR γ and DU145-ERR γ clones (Fig. 4C). The pattern of decreased cell populations at S phase was also consistent to the decreased BrdUrd incorporation in the ERR γ clones (Fig. 2D), suggesting that DNA replication rate was reduced in these clones. These results, together with the cell count study, suggest that the inhibition of cell proliferation and tumor growth by stable ERR γ expression in LNCaP and DU145 cells was mediated mainly through a blockage of S-phase entry and suppression of DNA replication.

Cell-cycle arrest in prostate cancer cells by ERR γ is accompanied with up-regulation of p21 or p27. We next investigated whether the arrest of cell-cycle progression at G₁-S transition in ERR γ -transduced prostate cancer cells could be associated with altered expression of cell-cycle regulators. Western blot analysis showed that there was an increased protein expression of cyclin-dependent kinase inhibitors p21 and p27 in DU145-ERR γ and p21 in LNCaP-ERR γ clones (Fig. 5A). A slightly decreased expression of cyclin A but no significant changes in cyclin B1, D1, and D3 were observed in the LNCaP-ERR γ and DU145-ERR γ clones.

Transactivation of p21 and p27 gene promoters by ERR γ . We next sought to find out whether the elevated protein expressions of p21 and p27 in ERR γ infectants was a direct transcription activation of their gene promoters by ectopic ERR γ or an indirect result mediated by other inhibitory growth-related signaling pathways or DNA damage (36). Luciferase reporter assay showed that the wild-type ERR γ but not ERR γ - Δ ZFI mutant was transcriptionally active because it could activate both ERE₃-Luc and ERRE₃-Luc reporters in transiently transfected ER-negative HeLa cells in the absence of ligand, showing that the exogenously expressed ERR γ was a functional protein (Fig. 2C). We further manifested by luciferase assay that ERR γ could activate the p21 gene promoter reporter (5-fold increase) and moderately on the p27 gene promoter reporter (2-fold increase) in HeLa cells (Fig. 5B). However, the ERR γ - Δ ZFI mutant was unable to activate any reporter constructs ERE₃/ERRE₃/p21/p27-Luc (Figs. 2C and Fig. 5B). Our results suggest that both p21 and p27 could be the target genes of ERR γ , whereas the increased p21 and p27 protein expressions in LNCaP-ERR γ and DU145-ERR γ infectants could be partially mediated through the stimulation of p21 and p27 gene promoter activities.

DY131 inhibits *in vitro* proliferation of prostate cancer cells. The effect of an ERR γ agonist DY131 on ERR γ -driven ERE₃- and ERRE₃-Luc reporter activities were evaluated. Administration of DY131 further enhanced ERR γ -activated ERE- and ERRE-driven luciferase transcription activities, with higher response on ERRE₃-Luc than ERE₃-Luc reporter, at concentrations >1 μ mol/L (Fig. 6A). DY131 at high concentrations also induced weak luciferase activities in 293 cells transfected with the mutant ERR γ - Δ ZFI (data not shown). We next investigated

Figure 5. Up-regulation of p21 and p27 in ERR γ -transduced prostate cancer cells. **A**, Western blot analysis of cell-cycle regulators in ERR γ -clones. Elevation of p21 and p27 from basal levels was seen in representative DU145-ERR γ and p21 in LNCaP-ERR γ clones. **B**, transcriptional activation of *p21* and *p27* gene promoters by ERR γ but not ERR γ - Δ ZF1 in transiently transfected HeLa cells. The results were expressed as fold of activation as compared with control (transfection with empty vector pcDNA). Columns, mean of triplicate samples repeated in three independent experiments; bars, SD.



whether DY131 could potentiate further inhibition in ERR γ clones than their parental cells. Cultured cells were exposed to DY131 at concentrations ranging from 0.1 to 30 μ mol/L. Under these conditions, DY131 inhibited cell proliferation and BrdUrd incorporation in LNCaP-ERR γ and LNCaP in a dose-dependent manner, with higher inhibition in LNCaP-ERR γ than LNCaP at lower concentrations of DY131 ($IC_{50} \cong 5$ μ mol/L for LNCaP; $IC_{50} \cong 2$ μ mol/L for LNCaP-ERR γ ; Fig. 6B). Similarly, DY131 also inhibited cell proliferation in DU145-ERR γ cells in a dose-dependent manner but only slight inhibition in DU145 cells ($IC_{50} \cong 26$ μ mol/L for DU145; $IC_{50} \cong 14$ μ mol/L for DU145-ERR γ ; Fig. 6C). No significant change in cellular morphology or induction of apoptotic cells was seen in DY131-treated cells (data not shown).

Discussion

In the present study, we reconfirmed our previous report that ERR γ is mainly expressed as a nuclear protein in epithelial cells in normal adult prostate, whereas its protein expression exhibits a negative pattern with the prostate cancer progression (33). By immunohistochemistry, we also observed that besides

epithelial localization, ERR γ was also localized to mesenchymal or stromal cells in fetal and pubertal prostates, whereas the stromal cell expression of ERR γ became markedly reduced in adult and aged prostates. The significance of stromal cell localization of ERR γ with reference to epithelial-stromal interaction is unclear. Interestingly, this expression pattern seems to be similar to ER β in developing and adult prostates (37). The colocalization of the ligand-independent ERR γ and ligand-dependent ER β in prostatic epithelial cells suggests that the two nuclear receptors may cross-talk with one another via the regulation of the same target genes or function independently.

We further investigated the functional role of ERR γ in cell growth regulation in prostate cancer cells and showed for the first time that ERR γ could suppress cell proliferation and tumorigenicity in both androgen-sensitive (LNCaP) and androgen-insensitive (DU145) prostate cancer cells, and the ERR γ -induced cell growth inhibitory effect was linked to a perturbation in cell-cycle progression with a significant suppression of S-phase fractions and decreased DNA replication, but with no apparent induction of apoptosis in ERR γ infectants. The results shown in isolated and pooled LNCaP-ERR γ and DU145-ERR γ infectants are consistent with our recent findings on rat ERR γ that the rat homologue also

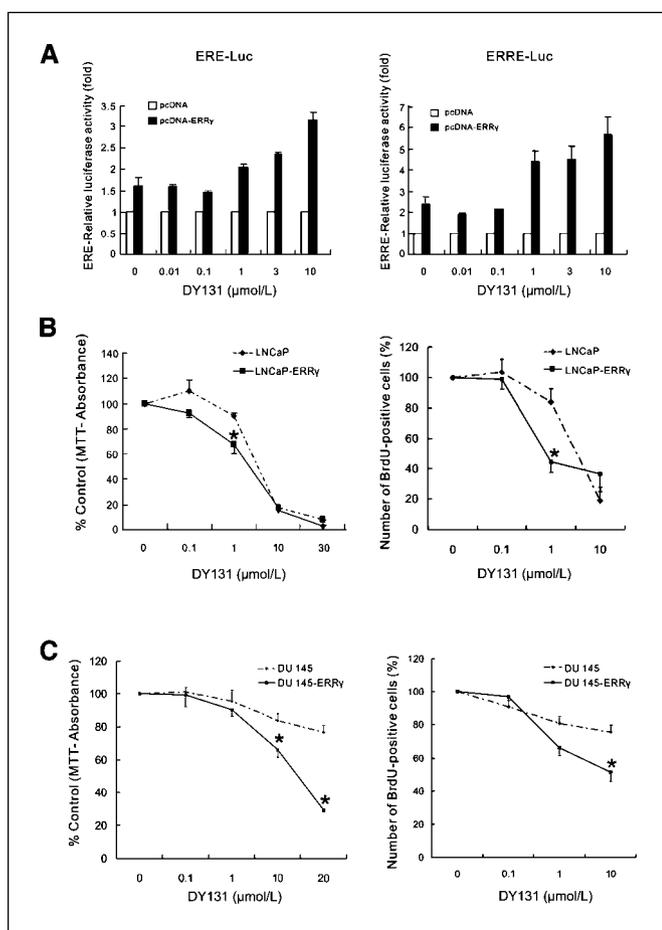


Figure 6. Inhibition on cell proliferation of $ERR\gamma$ -transduced prostate cancer cells by DY131. **A**, transactivation of ERE- and ERRE-driven reporter luciferase genes by $ERR\gamma$ in 293 cells in the presence of DY131. Wild-type $ERR\gamma$ induced a significant increase of transactivation on ERE_3 -Luc and $ERRE_3$ -Luc reporters in the presence of 1 μ mol/L or above DY131. Columns, mean of relative luciferase activities obtained from three independent experiments; bars, SD. *, $P < 0.01$ versus empty vector pcDNA. **B** and **C**, effect of DY131 on cell proliferation and BrdUrd incorporation in $ERR\gamma$ clones and their parental cells. Cells were treated with increasing concentrations of DY131 for 5 d. Viable cells were determined by MTT assay and expressed as percentage of untreated controls (A_{490} ; $n = 4$ and repeated from three independent experiments). DY131 suppressed cell proliferation and reduced BrdUrd-positive cells in both LNCaP- $ERR\gamma$ and LNCaP cells in a dose-dependent manner, with higher suppression in LNCaP- $ERR\gamma$ clone. Similarly, DY131 also induced higher inhibition in DU145- $ERR\gamma$ than its parental cells. *, $P < 0.01$ versus parental cells at the same dose.

exerts an antiproliferative action in immortalized rat prostatic epithelial cells and cancer cells stably transfected with $ERR\gamma$ (14), suggesting that the inhibitory function on cell proliferation or arrest of cell-cycle progression by ectopic $ERR\gamma$ is similar in both human and rat prostatic cells. We also asked whether this growth inhibitory effect was related to cell senescence or differentiation. Senescence-associated β -galactosidase staining revealed no significant difference between the $ERR\gamma$ clones and their parental cells, suggesting that the $ERR\gamma$ -induced cell growth inhibition in prostate cancer cells may not be associated with cell senescence (data not shown).

By immunoblot analysis, we further showed that the $ERR\gamma$ -induced suppression of cell cycle S phase (together with G_1 and G_2 -M arrest) in LNCaP and DU145 cells was accompanied with an increased protein expression of p21 and p27. Because p21 and p27

are key inhibitors of cell-cycle progression and play crucial role in the G_1 -S phase transition, it is believed that the blockage of G_1 -S transition in LNCaP- $ERR\gamma$ and DU145- $ERR\gamma$ cells may be partially exerted through the elevated levels of p21 or p27. In addition, the inhibition of DNA replication (BrdUrd incorporation) in LNCaP- $ERR\gamma$ and DU145- $ERR\gamma$ cells could also be a result of increased levels of p21 because it is well characterized that p21 can directly inhibit DNA replication by its interaction with proliferating cell nuclear antigen (PCNA) and blocking the PCNA-dependent DNA polymerase δ activity (38). On the other hand, it is also known that both p21 and p27 genes are estrogen responsive (39, 40) and $ER\alpha/\beta$ inducible in endometrial and breast cancer cells (41–43), whereas their transcriptional regulation by $ER\alpha$ is mediated through interaction with transcription factor Sp1 and histone deacetylase via the Sp1-binding sites in their promoters (44, 45). However, it is unclear whether p21 is cotargeted by ERs and $ERR\gamma$ in prostate cancer cells. In this study, we also showed by luciferase reporter assay that upon transient transfection, $ERR\gamma$ could stimulate the gene promoter activities of p21 and p27, suggesting that the elevation of p21 and p27 in $ERR\gamma$ -transduced prostate cancer cells might be attributed to direct transactivation of their gene promoters, in addition to other possible inhibitory cell growth signals (36). To map its binding sites, we have done a serial of 5'-deletion analysis on the p21 gene promoter and its activation by transiently expressed $ERR\gamma$ in HeLa cells. Our result showed that deletion of a region located between nucleotides $-2,324$ and $-2,132$ upstream of the p21 transcription start site resulted in a significant loss of activation (6-fold reduction). Chromatin immunoprecipitation assay also confirmed that $ERR\gamma$ bound directly to p21 promoter (data not shown). However, sequence information revealed that no canonical ERE or 1/2 ERE site was found within this region. Further detailed mapping and binding analysis is required to identify and confirm the putative $ERR\gamma$ -binding sites required for the positive regulation of p21 transcription by $ERR\gamma$ or whether its activation at these sites also involves indirect interactions with other transcription factors. In a recent study on the role of RIP140 on transcriptional regulation by ERs, Castet et al. (18) show that besides $ER\alpha$, $ERR\alpha$, and $ERR\beta$, $ERR\gamma$ can also weakly transactivate the p21 gene promoter through two proximal Sp1 response elements located at nucleotides between -82 and -69 in p21 promoter, whereas its transcriptional activity can be further enhanced by RIP140.

In this study, we also evaluated the *in vitro* efficacy of a selective $ERR\beta/\gamma$ -agonist DY131 (31) on prostate cancer cell growth. Our cell-based assay results showed that DY131 at micromolar levels could potentiate the $ERR\gamma$ -mediated growth inhibition in LNCaP- $ERR\gamma$ and DU145- $ERR\gamma$ clones compared with their parental cells (without significant induced apoptosis). It is also believed that the inhibitory effect of DY131 on the LNCaP and DU145 parental cells may be mediated through their endogenous low expression of $ERR\gamma$ and $ERR\beta$. By comparison, LNCaP/LNCaP- $ERR\gamma$ cells showed higher sensitivities to DY131 than DU145/DU145- $ERR\gamma$ cells, which may be attributed to the endogenous expression of $ERR\gamma$ in LNCaP but not DU145. Our encouraging results suggest that the orphan nuclear receptor $ERR\gamma$, which may have an antiproliferative function in prostate cancer, could be a novel therapeutic target for treatment of prostate cancer. However, further experiments are required to elucidate its pharmacology, including molecular mechanism of action besides targeting on $ERR\gamma$, *in vivo* tumor growth inhibition, efficacy in androgen-independent or metastatic

prostate cancer, and systemic cytotoxicity. It is worthy to further explore and develop selective ligands targeting to ERRs as new therapeutic drugs or anticancer agents supplementing the conventional hormonal therapies for prostate cancer. Previous studies in prostate cancer cell lines and animal models show that ER antagonists or SERMs, targeting on the ligand-dependent ER α and ER β , can inhibit growth of prostate cancer and its metastasis (46, 47), whereas it is claimed that these drugs may be useful as chemopreventive agents for prostate cancer (48). Characterization by binding affinity, reporter gene assays, and structural studies also reveal that some SERMs (tamoxifen, 4-hydroxytamoxifen) can act as ligands or antagonists to ERRs besides ERs (27, 29, 30, 49, 50). Thus, these SERMs, if they have any effects on the prostate cancer cells, may target on both ERRs and ERs.

In summary, we showed in this study that ERR γ inhibited the cell proliferation of androgen-sensitive and androgen-insensitive prostate cancer cells by arresting cell-cycle progression at the G₁-S phase transition, which was accompanied with an increased expression of two cyclin-dependent kinase inhibitors p21 and

p27, suggesting an antiproliferative role of ERR γ in prostate cancer. We further showed by reporter assay that the induction of p21 and p27 by ERR γ was mediated at transcriptional and gene promoter levels. Considering that ERR γ is down-regulated in clinical prostate cancer, our findings suggest that ERR γ may play a negative role in transformed prostatic cell growth and a tumor suppressor function in prostate cancer cells. Finally, we also showed that ERR γ could be useful as a potential therapeutic target for treating prostate cancer because a selective ERR γ agonist, DY131, could potentiate the growth inhibition of ERR γ -positive prostate cancer cells.

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ERR γ Suppresses Cell Proliferation and Tumor Growth of Androgen-Sensitive and Androgen-Insensitive Prostate Cancer Cells and Its Implication as a Therapeutic Target for Prostate Cancer

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