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Positive Inter-Regulation between β-Catenin/T Cell Factor-4 Signaling and Endothelin-1 Signaling Potentiates Proliferation and Survival of Prostate Cancer Cells

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ABSTRACT

Both malignant and normal prostate epithelial cells produce endothelin-1 (ET-1), a critical factor in prostate cancer (CaP) progression. β-Catenin (β-cat), a key component of the Wnt signaling pathway, is also implicated in CaP progression via β-cat/T cell factor (Tcf) signaling. We recently demonstrated that β-cat/Tcf-4 regulates transcription of *ET-1* in colon cancer cells. In the present study, we found that Tcf-4 specifically bound to and activated the *ET-1* promoter in vivo in human CaP cells and mouse prostate tissue. Expression of ET-1 in DU145 CaP cells was down-regulated by knocking down endogenous β-cat or Tcf-4. Ectopic activation of β-cat/Tcf-4 signaling significantly elevated expression of ET-1 in LNCaP cells. In addition, genetic ablation of β-cat significantly inhibited transcription of *ET-1* in primary prostate epithelial cells. Meanwhile, exogenous ET-1 enhanced β-cat/Tcf signaling and ET-1 expression in DU145 cells, which was blocked by both selective phosphatidylinositol 3-kinase (PI3K) inhibitor 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY294002) and endothelin-A receptor antagonist cyclo(L-Leu-D-Trp-D-Asp-L-Pro-D-Val) (BQ123). Furthermore, knockdown of either β-cat or Tcf-4 substantially reduced cell proliferation and potentiated paclitaxel-induced apoptosis in DU145 cells, which largely were rescued by treatment with exogenous ET-1. Together, our results suggest that β-cat/Tcf-4 signaling transcriptionally activates ET-1 in CaP cells; meanwhile, ET-1 enhances β-cat/Tcf-4 signaling and in turn further increases ET-1 expression in a PI3K-dependent manner. The positive inter-regulation between β-cat/Tcf-4 signaling and ET-1 signaling potentiates proliferation and survival of CaP cells, thereby representing a novel mechanism that contributes to CaP progression.

Prostate cancer (CaP) is the most commonly diagnosed malignancy and the second leading cause of cancer death in American men. Although our ability to detect early stage CaP has increased, predictive diagnosis and effective tumor therapy remain limited. Advanced CaP brought to remission with androgen ablative therapy often relapses, leading to hormone-refractory tumors for which only experimental treatments exist (Culig et al., 1998). Hence, understanding the molecular basis of CaP progression is paramount for broadening current diagnostic and therapeutic modalities.

β-Catenin (β-cat), originally identified as an essential regulator for E-cadherin-mediated cell-cell interaction, is also a key component of the Wnt signaling pathway (Zhang et al., 1998; Chesire and Isaacs, 2003). Wnt signals are transduced via specific cell surface receptors to activate a series of biochemical reactions involving a large protein complex consisting of β-cat, adenomatous polyposis coli (APC), glycogen synthase kinase-3β (GSK-3β), β-transducin repeat-containing protein (β-Trcp) and axin, resulting in stabilization of β-cat protein and an increase in the soluble pool of β-cat (Wodarz and Nusse, 1998; Nusse, 1999). The soluble β-cat interacts with the T cell factor (Tcf) family of transcription factors and transactivates a number of downstream target genes, leading to increased proliferation and decreased differentiation of

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ABBREVIATIONS: CaP, prostate cancer; β-cat, β-catenin; APC, adenomatous polyposis coli; GSK-3β, glycogen synthase kinase-3β; β-Trcp, β-transducin repeat-containing protein; Tcf, T cell factor; ET-A, endothelin-A; CRT, β-cat/Tcf-related transcription; AR, androgen receptor; PI3K, phosphatidylinositol 3-kinase; ETP, *ET-1* promoter reporter; muETP, *ET-1* promoter reporter (ETP) and its mutant control; LY294002, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one; BQ123, cyclo(L-Leu-D-Trp-D-Asp-L-Pro-D-Val); FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; CMV, cytomegalovirus; PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ChIP, chromatin immunoprecipitation; EGFP, enhanced green fluorescent protein; TBE, Tcf binding elements; siRNA, small interfering RNA; RT, reverse transcription; PrEC, prostate epithelial cell; ELISA, enzyme-linked imunosorbent assay; bp, base pair(s).

epithelial cells (Chesire et al., 2000; de la Taille et al., 2003). Pathological mutation of β-cat, APC, β-Trcp, axin, and Tcf all lead to activation of β-cat/Tcf signaling and are found in a variety of cancers, including CaP (Barker and Clevers, 2000). In all, 5 to 7% of CaP carry mutations in β-cat (Chesire et al., 2000). One study showed that 32% of CaP samples carried mutations in β-cat signaling pathway components, including β-cat, APC, or β-Trcp (Gerstein et al., 2002). In cases of advanced CaP, nuclear localization of β-cat is significantly increased despite the absence of detectable β-cat mutations (Chesire et al., 2002). A recent study reported that high levels of expression of Wnt-1 and cytoplasmic/nuclear β-cat were observed in situ in 77% of lymph node metastases and 85% of bone metastases of CaP (Chen et al., 2004). In addition, abnormal cytoplasmic/nuclear β-cat expression is associated with high Gleason scores in CaP patients (de la Taille et al., 2003; Chen et al., 2004). These findings indicate that abnormal activation of β-cat is associated with CaP progression, particularly the development of advanced, hormone-refractory CaP.

Endothelin-1 (ET-1) is a potent vasoconstrictor initially isolated from endothelial cells (Nelson et al., 2003a). Both malignant and normal prostate epithelial cells (PrEC) produce ET-1 (Nelson et al., 1996; Pirtskhalaishvili and Nelson, 2000). Although its physiological function in the normal prostate remains unclear, ET-1 has been shown to promote CaP cell proliferation in vitro and CaP bone metastasis in vivo through the endothelin-A (ET-A) receptor (Nelson et al., 1996). Compelling evidence suggests that ET-1 plays an important role in CaP progression. ET-1 levels are evidently higher in advanced, metastatic CaP specimens than in primary lesions (Nelson et al., 1996). Expression of ET-1 in CaP cells is markedly up-regulated by factors involved in cancer progression (Granchi et al., 2001). Furthermore, plasma ET-1 levels are abnormally elevated in 58% of men with advanced, hormone-refractory CaP (Nelson et al., 2001). The importance of ET-1 in CaP progression is further emphasized by results of clinical trials, which indicate that a selective ET-A receptor antagonist is beneficial in controlling progression of hormone-refractory CaP and in suppressing CaP-induced bone remodeling (Fisher, 2002; Nelson et al., 2003b).

Multiple observations suggest that β-cat/Tcf-related transcription (CRT) is correlated with expression of ET-1 in CaP cells. For instance, activation of androgen/androgen receptor (AR) signaling down-regulates both CRT activity and expression of ET-1 in CaP cells (Grant et al., 1997; Granchi et al., 2001; Chesire and Issacs, 2002; Mulholland et al., 2003). We have recently demonstrated that β-cat/Tcf-4 regulates *ET-1* transcription in colon cancer cells (Kim et al., 2005). In this study, we show that β-cat/Tcf-4 transcriptionally activates ET-1 expression in both malignant and normal PrECs; meanwhile, ET-1 stimulates β-cat/Tcf-4 signaling via a phosphatidylinositol 3-kinase (PI3K)-dependent pathway. Thus, β-cat/Tcf-4 signaling and ET-1 signaling forms a positive feedback loop to enhance both signaling pathways and potentiate proliferation and survival of CaP cells.

Materials and Methods

Plasmids and Reagents. The TOPflash and FOPflash plasmids and plasmids encoding human β-cat or Tcf-4 were kindly provided by Dr. Hans Clevers (Netherlands Institute for Developmental Biology, Utrecht, The Netherlands). To generate retroviral expression of *β-cat*, human β-cat cDNA was subcloned into retroviral vector pLX-SHD (Miller and Rosman, 1989). Human *ET-1* promoter reporter (ETP) and its mutant control (muETP) were generated as described previously (Kim et al., 2005). To construct retroviral-RNA interference plasmids, gene-specific oligonucleotides (Table 1) were phosphorylated, annealed, and ligated into pRetro-Super vector (Brummelkamp et al., 2002) digested with BglII and HindIII. The Cre-expressing adenovirus was kindly provided by Dr. Gen-Sheng Feng (Burnham Institute for Medical Research, La Jolla, CA). Dual-luciferase reporter assay system was purchased from Promega (Madison, WI). TRIzol reagent for RNA isolation, the SYBR Green I kit, the Annexin V-EGFP apoptosis detection kit and the ET-1 ELISA kit were from Invitrogen (Carlsbad, CA), Roche (Indianapolis, IN), BioVision (Mountain View, CA), and R&D Systems (Minneapolis, MN), respectively. Anti-Pan-cytokeratin antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Tcf-4 antibody was from Upstate (Lake Placid, NY). Anti-GSK-3β and Anti-phospho-GSK-3β (serine 9) antibodies were from Cell Signaling Technology (Beverly, MA). All secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA). C57BL/6J mouse with floxed β-cat alleles were purchased from The Jackson Laboratory (Bar Harbor, ME). LY294002, BQ123, synthetic ET-1, thiorphan, and anti-β-cat antibody were purchased from Sigma.

Cell Lines and Mouse Primary PrEC Culture. DU145 and LNCaP cells were acquired from American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). Primary culture of mouse PrECs was prepared as described previously (Ravindranath and Dym, 1999; Sawicki and Rothman, 2000). In brief, pieces of prostate tissue dissected from 10-week-old male mice with floxed *β-cat* alleles were incubated in DMEM/Ham's F-12 medium containing type I collagenase (1 mg/ml) at 37°C for 20 min on a rotator. The sediment was resuspended in DMEM/Ham's F-12 containing collagenase (1 mg/ml) and hyaluronidase (1 mg/ml) at 37°C for 30 min on a rotator. The cell aggregates were then cultured in DMEM/Ham's F-12 containing 10% FBS in 5% CO_2 at 37°C overnight. On the following morning, floating epithelial clumps were collected and further digested with 0.05% trypsin-EDTA at 37°C for 10 min, followed by passage through a 40-μm cell strainer. Approximately 5×10^4 cells were inoculated to each well of a 24-well plate in 1 ml of DMEM/Ham's F-12, 4% FBS, 10 ng/ml cholera toxin, 1 μM dexamethasone, and ITS supplement (insulin/transferrin/sodium selenite). The cells were maintained in 5% CO_2 at 37°C and used within 1 week.

TABLE 1
Gene-specific oligonucleotides used to construct the retroviral-RNA interference vectors

Target	Oligonucleotides
β-cat	5′-GATCCCCGACTCCAGTGGTAATCTACTTCAAGAGAGTAGATTACCACTGGAGTCTTTTTGGAAA-3′
	5′-AGCTTTTCCAAAAAGACTCCAGTGGTAATCTACTCTCTTGAAGTAGATTACCACTGGAGTCGGG-3′
Tcf-4	5′-GATCCCCGGAGCGACAGCTTCATATGTTCAAGAGACATATGAAGCTGTCGCTCCTTTTTGGAAA-3′
	5′-AGCTTTTCCAAAAAGGAGCGACAGCTTCATATGTCTCTTGAACATATGAAGCTGTCGCTCCGGG-3′
Scramble	5′-GATCCCCCAGTAACTGAATAGCTACCTTCAAGAGAGGTAGCTATTCAGTTACTGTTTTTTGGAAA-3′
	5′-AGCTTTTCCAAAAACAGTAACTGAATAGCTACCTCTCTTGAAGGTAGCTATTCAGTTACTGGGG-3′

Luciferase Assay. Cells were transfected with reporter plasmids combined with plasmids encoding β-cat or Tcf-4 using lipofectamine 2000 (Invitrogen). Plasmid PRL-CMV encoding *Renilla reniformis* luciferase (at one fifth molar ratio to test plasmids) was cotransfected with test plasmids in each transfection as an internal control for data normalization. The luciferase assays were performed 24 h after transfection with a dual-luciferase reporter assay system (Promega) according to the manufacturer's instructions. Each experiment was repeated for three times in duplicates and results are shown as mean ± S.E..

Retroviral Infection. Retrovirus was produced by transfecting phoenix packaging cells with retrovirus-based plasmids. Tissue culture supernatants were collected 48 h after transfection and filtered through 0.45-μm filters. Infection was performed overnight with the retrovirus-containing media and polybrene (8 μg/ml). Pools of stable infectants were generated via selection with puromycin (5 μg/ml).

Real-Time Quantitative Reverse Transcription PCR. RNA were prepared using TRIzol reagent followed by purification with TURBO DNA-free System (Ambion, Austin, TX). The cDNAs were synthesized using SuperScript II reverse transcriptase (Invitrogen). Real-time quantitative PCR was performed on the LightCycler thermal cycler system (Roche Diagnostics) using SYBR Green I kit (Roche) as described by the manufacturer. The results were normalized against that of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) in the same sample. The primers used are as follows: for human *ET-1*, 5′-TCCTCTGCTGGTTCCTGACT-3′ (forward) and 5′-CAGAAACTCCACCCCTGTGT-3′ (reverse); for human cyclin D1, 5′-ACGCTTTGTCTGTCGTGATG-3′ (forward) and 5′-AACCAGAAATGCACAGACCC-3′ (reverse); for human *GAPDH*, 5′-GTCAGTGGTGGACCTGACCT-3′ (forward) and 5′-TGCTGTAGCCAAATTCGTTG-3′ (reverse); for mouse *ET-1*, 5′-TGGACATCATCTGGGTCAACA-3′ (forward) and 5′-GACCTGGAAGAACCTCCCAATC-3′ (reverse); for mouse cyclin D1, 5′-TACCGCACAACGCACTTTCT-3′ (forward) and 5′-TCCACATCTCGCACGTCGGT-3′ (reverse); for mouse *GAPDH*, 5′-TTCACCACCATGGAGAAGGC-3′ (forward) and 5′-GGCATGGACTGTGGTCATGA-3′ (reverse). Each experiment was repeated for two times in triplicates and results are expressed as mean ± S.E.

Immunoassays. The secreted ET-1 levels in cell culture supernatants were determined using an ET-1 ELISA kit. In brief, cells were grown to confluence in 10-cm dishes in RPMI 1640 medium supplemented with 10% FBS, followed by replacing the medium with serum-free medium and further incubation for 16 h. The cell culture supernatants were collected for ELISA according to the manufacturer's instructions (R&D Systems). ELISA-detected ET-1 concentrations were normalized against cell number (per 10^6 cells). Each ELISA experiment was repeated for three times in duplicates and results are expressed as mean ± S.E. Immunoblotting and immunocytochemistry were performed as described previously with respective antibodies (Hanazono et al., 1998; Zhang et al., 1998). To prepare the soluble cell lysate fraction, DU145 cells were lysed in 0.1% Nonidet P-40 lysis buffer (0.1% Nonidet P-40, 10 mM HEPES, pH 7.5, 142.5 mM KCl, 5 mM $MgCl_2$, and 1 mM EGTA). The lysates were centrifuged at 13,000 rpm for 10 min, and the supernatants were saved as soluble cell lysate. The pellets representing the insoluble fraction of cell lysates were lysed in radioimmunoprecipitation assay buffer.

Chromatin Immunoprecipitation. Tissue and cell ChIPs were performed as described previously (Sun and Loh, 2001, 2002) except that protein G Sepharose beads were used instead of Staph A cells for binding the immune complexes in these experiments. The primers used are as the following: for human *ET-1* promoter (−290/−65 fragment) amplification, 5′-CAGCTTGCAAAGGGGAAGCG-3′ (forward) and 5′-TCCGACTTTATTCCAGCCCC-3′ (reverse); for mouse *ET-1* promoter (−290/−65 fragment) amplification, 5′-CAGCTAGCAAAAGGGGAAGC-3′ (forward) and 5′-TCTGACTTTATTCCAGCCCT-3′ (reverse); for human/mouse *GAPDH* exon 8 amplification, 5′-ATCACTGCCACCCAGAAGACTGTGGA-3′ (forward) and 5′-TCATACCAGGAAATGAGCTTGACAAA-3′ (reverse).

[^{3}H]Thymidine Incorporation Assay. Cells were seeded at 2×10^5 cells per well in a 24-well plate. The cells were placed in serum-free medium for 10 h followed by incubation in nucleotide-free medium containing 10% dialyzed FBS and 1 μCi/well [^{3}H]thymidine (GE Healthcare, Little Chalfont, Buckinghamshire, UK) in the presence or absence of 5 pM synthetic ET-1 for 12 h. Quantitation was performed as described previously (Coward et al., 1998). Each experiment was repeated for three times in triplicates and results are expressed as mean ± S.E.

Apoptosis Analysis. Cells were seeded at 5×10^5 per well in six-well plates. After cells attached to the dishes (approximately 2 h after seeding), cells were treated with paclitaxel (5 nM) in the presence or absence of ET-1 (5 or 100 pM) for 12 h. Cell apoptosis was detected with an Annexin V-EGFP Apoptosis Detection kit (BioVision) coupled with flow cytometry analysis.

Statistical Analysis. Statistic tests were performed using analysis of variance followed by post hoc comparisons of means by the least-significance differences method. The statistical significance level was set at 0.05 ($\alpha = 0.05$) for each analysis.

Results

β-Cat/Tcf-4 Directly Activates the ET-1 Promoter in DU145 CaP Cells. The human *ET-1* promoter contains multiple potential Tcf binding elements (TBE) (Kim et al., 2005). In this study, we identified a functional TBE located at −73 to −67 bp relative to the transcription initiation site (TIS, designated as +1) in the human *ET-1* promoter in DU145 CaP cells (Fig. 1A). The *ET-1* promoter sequence spanning from −1300 to +230 bp surrounding the TIS was inserted into a luciferase reporter plasmid to make an *ET-1* promoter reporter. A mutated *ET-1* promoter reporter was generated by deleting the functional TBE from the *ET-1* promoter reporter (Fig. 1B). Deletion of the functional TBE (−73/−67) markedly decreased the *ET-1* promoter activity and abolished the synergistic effect of β-cat and Tcf-4 on transactivation of the *ET-1* promoter (Fig. 1C). To verify that Tcf-4 binds to the *ET-1* promoter in vivo, we performed ChIP assays. As shown in Fig. 1D, *ET-1* promoter fragments containing the putative TBE (−73/−67) were specifically precipitated from cross-linked DU145 chromatin extracts with an anti-Tcf-4 antibody. In contrast, no detectable *ET-1* promoter fragment was precipitated with an anti-tubulin antibody. Together, these results indicate that β-cat/Tcf-4 specifically binds to and transactivates the *ET-1* promoter in DU145 CaP cells.

Knockdown of β-Cat/Tcf-4 Signaling Inhibits Expression of ET-1 in DU145 Cells. To investigate the role of β-cat/Tcf-4 in regulating ET-1 expression in CaP cells, DU145 cells stably expressing small interfering RNA (siRNA) against *β-cat* or *Tcf-4* were generated using a retroviral vector (Brummelkamp et al., 2002). Immunoblotting analysis revealed that more than 70% of endogenous β-cat or the two isoforms of Tcf-4 present in DU145 cells (Chesire et al., 2002) were knocked down by expressing corresponding siRNA. In contrast, an siRNA with scrambled sequence showed no effect on expression of either β-cat or Tcf-4 in DU145 cells (Fig. 2A). The transcriptional activity of β-cat/Tcf-4 in the β-cat- or Tcf-4-knockdown DU145 cells was also significantly reduced compared with that in the scrambled-siRNA-expressing cells, as measured by the luciferase activities of TOPflash, a synthetic β-cat/Tcf-dependent luciferase reporter (Fig. 2B). In contrast, little change was observed

with FOPflash, a negative control reporter with mutated TBEs. Real-time quantitative RT-PCR assays showed a 65% decrease at the ET-1 mRNA level (Fig. 2C), and ELISA assays detected a 55% reduction of secreted ET-1 in the β-cat- or Tcf-4-knockdown DU145 cells compared with control cells expressing scrambled siRNA (Fig. 2D). As a positive

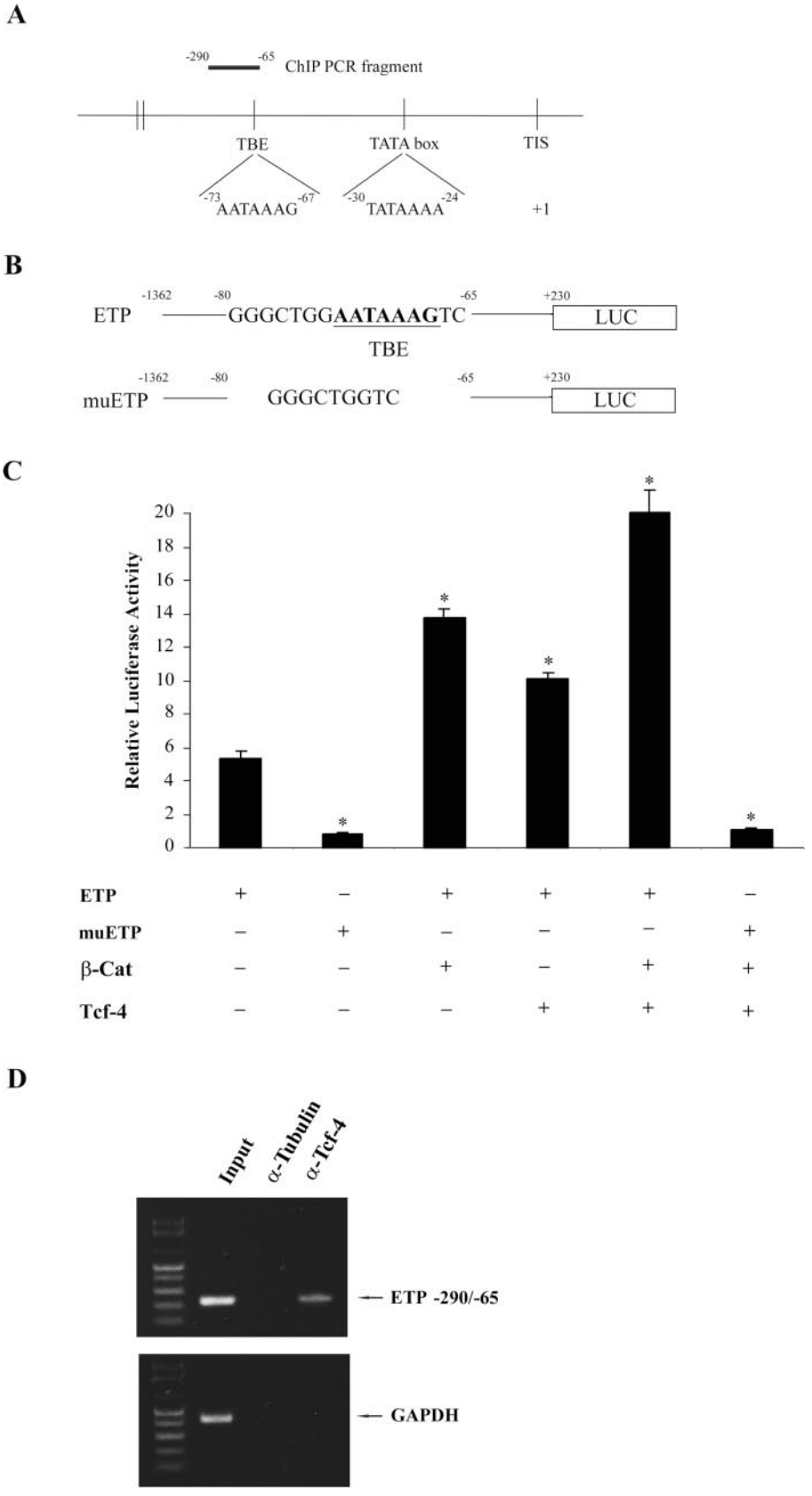


Fig. 1. β-Cat/Tcf-4 directly activates the *ET-1* promoter in DU145 CaP cells. A, schematic representation of the human *ET-1* promoter. The relative position of the TATA box, the functional TBE, and the TIS (designated as +1) are shown. The *ET-1* promoter fragment amplified in ChIP is indicated (black bar). B, illustration of the *ET-1* promoter reporter constructs. The human *ET-1* promoter sequence spanning from −1362 to +230 bp surrounding the TIS was inserted into a luciferase (LUC) reporter plasmid to make the ETP reporter. A muETP reporter was generated by deleting the functional TBE from ETP. C, regulation of the *ET-1* promoter activity in DU145 cells. ETP or muETP was cotransfected with plasmids encoding *β-cat* or *Tcf-4* into DU145 cells. Luciferase assays were performed 24 h after transfection. Normalized luciferase activities are expressed as mean ± S.E. (*, $P <$ 0.05 versus ETP). The empty expression vector (pcDNA3.1; Invitrogen) was added to make an equal amount of DNA for each transfection. D, binding of Tcf-4 to the *ET-1* promoter in vivo. Cross-linked DU145 chromatin extracts were immunoprecipitated with an anti-Tcf-4 antibody (α-Tcf-4) or an anti-tubulin antibody (α-tubulin). The *ET-1* promoter fragment covering −290 to −65 bp (ETP −260/−65) and a DNA fragment from exon 8 of human *GAPDH* gene were PCR-amplified from the immunoprecipitated and the input chromatin, respectively.

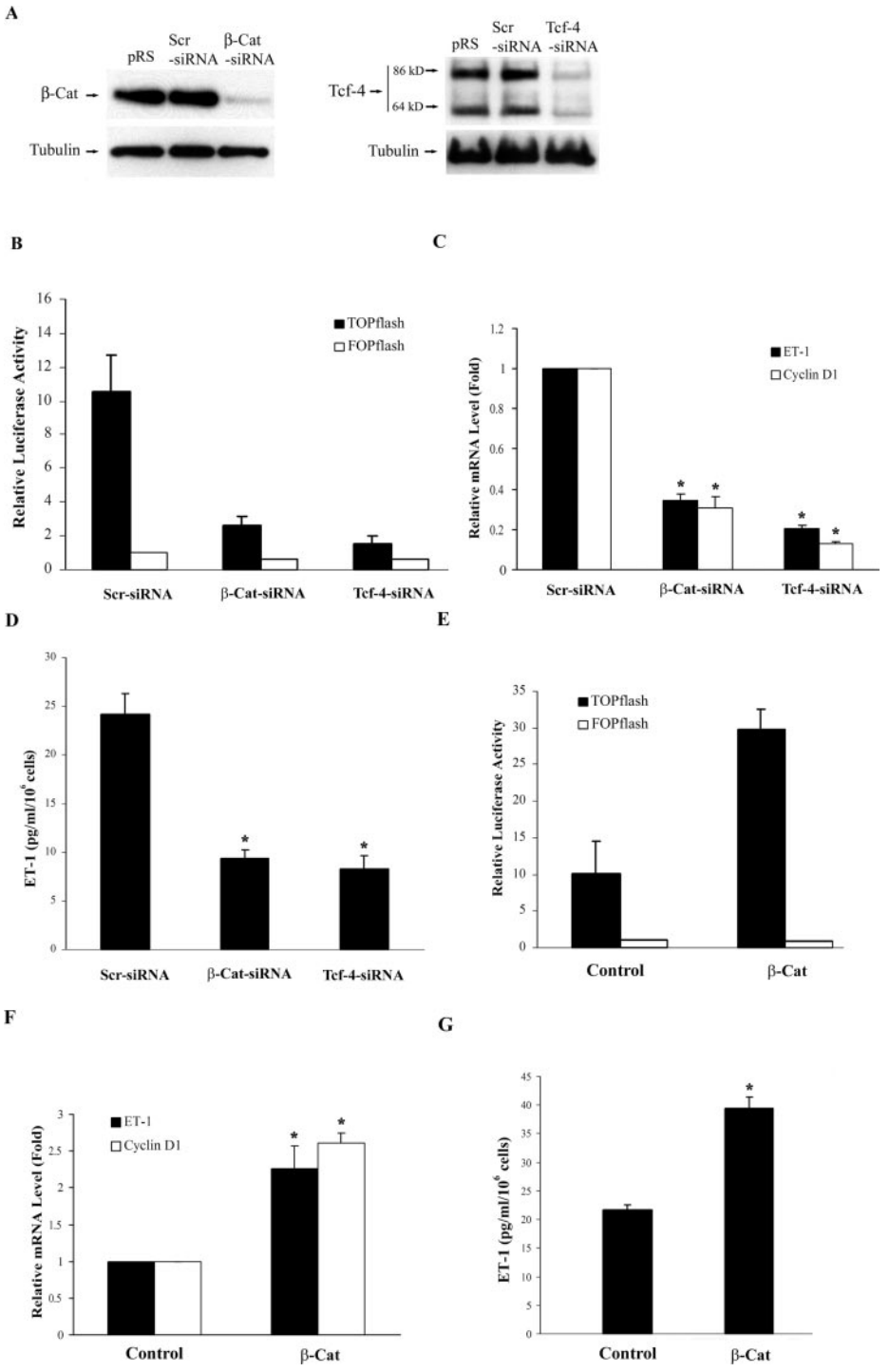


Fig. 2. Knockdown of endogenous β-Cat or Tcf-4 decreases expression of ET-1 in DU145 cells. A, knockdown of endogenous β-cat or Tcf-4 by gene specific retroviral siRNA. Lysates from DU145 cells stably expressing scrambled-siRNA (Scr-siRNA), siRNA against *β-cat* (β-Cat-siRNA), or siRNA against *Tcf-4* (Tcf-4-siRNA) as well as lysates from cells infected with an empty control retrovirus (pRS) were analyzed for β-cat or Tcf-4 expression by immunoblotting. The same membrane was blotted with an anti-tubulin antibody as the loading control. B, transcriptional activities of β-cat/Tcf in the β-cat- or Tcf-4-knockdown DU145 cells. The TOPflash (filled bar) or FOPflash (empty bar) reporter was transfected into DU145 cells expressing Scr-siRNA, β-Cat-siRNA, or Tcf-4-siRNA. Luciferase assays were performed 24 h after transfection. Normalized luciferase activities were expressed as fold activation to the FOPflash luciferase activity of cells expressing Scr-siRNA (arbitrarily defined as 1). C, ET-1 mRNA levels in the β-cat- or Tcf-4-knockdown DU145 cells. The ET-1 mRNA levels in DU145 cells expressing Scr-siRNA, β-Cat-siRNA, or Tcf-4-siRNA were determined by real-time quantitative RT-PCR. The mRNA level of cyclin D1 was included as a positive control. Results were normalized as -fold increase to the ET-1 (filled bar) or the cyclin D1 (empty bar) mRNA level of cells expressing Scr-siRNA (designated as 1) (*, $P < 0.05$). D, levels of secreted ET-1 in the β-cat- or Tcf-4-knockdown DU145 cells. Secretion of ET-1 by DU145 cells expressing Scr-siRNA, β-Cat-siRNA, or Tcf-4-siRNA was quantitated using ELISA and normalized against cell number (*, $P < 0.05$). E, transcriptional activities of β-cat/Tcf in DU145 cells overexpressing *β-cat*. The TOPflash (filled bar) or FOPflash (empty bar) reporter was cotranfected with a β-cat-encoding plasmid (β-Cat) or an empty plasmid (Control) into DU145 cells. Luciferase assays were performed 24 h after transfection. Luciferase activities were expressed as -fold activation to the FOPflash luciferase activity of the Control (arbitrarily defined as 1). F and G, ET-1 and cyclin D1 mRNA levels (F) and levels of secreted ET-1 (G) in DU145 cells transiently transfected with *β-cat* (*, $P < 0.05$).

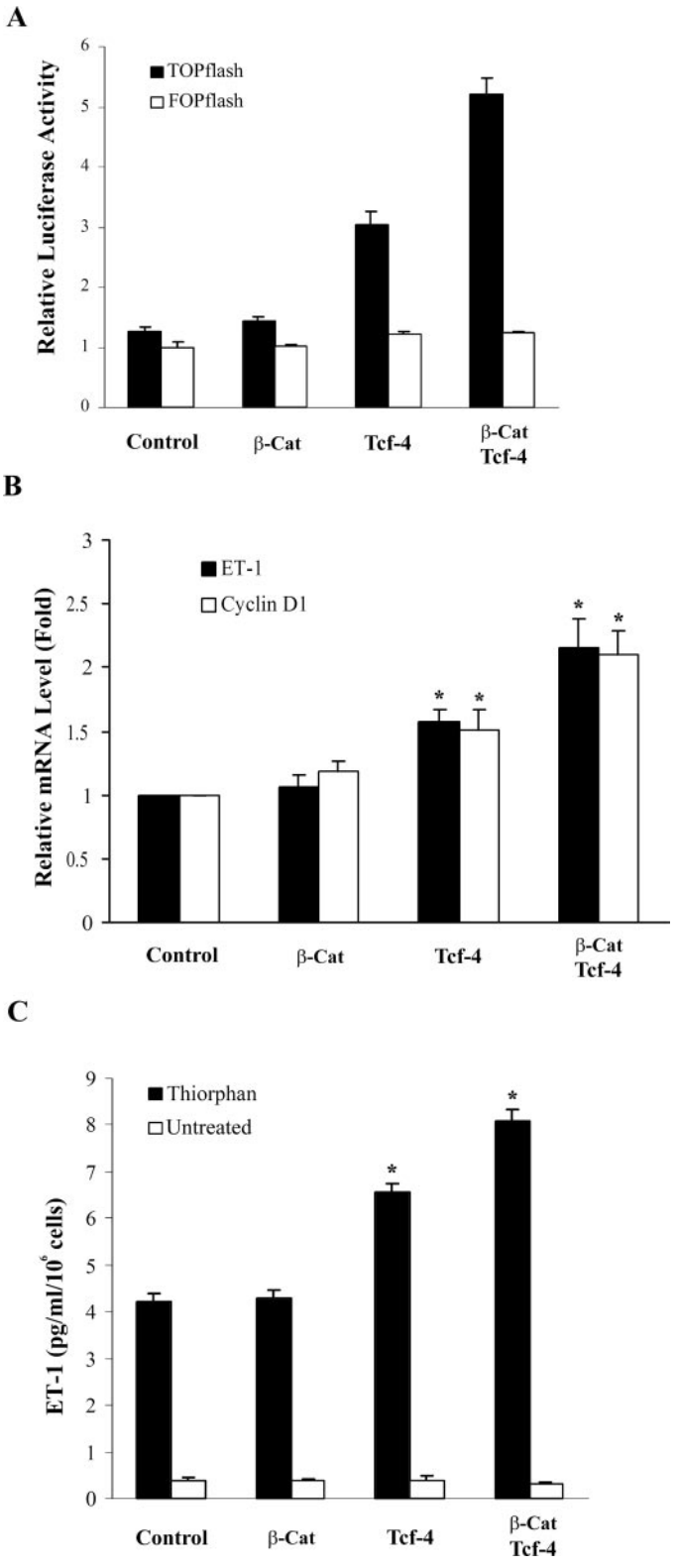


Fig. 3. Activation of β-Cat/Tcf-4 signaling increases expression of ET-1 in LNCaP cells. A, induction of transcriptional activities of β-cat/Tcf-4 in LNCaP cells. The TOPflash (filled bar) or FOPflash (empty bar) reporter was cotransfected with the β-cat and/or the Tcf-4 expression plasmids into LNCaP cells. Cells cotransfected with the empty pcDNA3.1 vector and the TOPflash or FOPflash were taken as the control. Luciferase assays were performed 24 h after transfection. Luciferase activities were expressed as -fold activation to the FOPflash luciferase activity of the control (designated as 1). B, ET-1 mRNA levels in LNCaP cells overexpressing β-cat and/or Tcf-4. The ET-1 (filled bar) and the cyclin D1 (empty bar) mRNA levels in LNCaP cells overexpressing β-cat and/or Tcf-4 were determined by real-time quantitative RT-PCR. Results were normalized as fold increase to the ET-1 or the cyclin D1 mRNA level of the control, which is arbitrarily defined as 1 (*, $P < 0.05$). C, secretion of ET-1 in LNCaP cells overexpressing β-cat and/or Tcf-4 in the presence (filled bar) or absence (empty bar) of thiorphan (1 μM) (*, $P < 0.05$).

control, the mRNA level of cyclin D1, an established downstream target of β-cat/Tcf signaling in CaP cells (Chesire and Isaacs, 2002), was also significantly reduced by knockdown of β-cat or Tcf-4 (Fig. 2C). In addition, overexpression of β-cat significantly raised the TOPflash activity (Fig. 1E), the ET-1 and the cyclin D1 mRNA levels (Fig. 1F), and the secreted ET-1 level (Fig. 1G) in DU145 cells. Together, these results indicate that endogenous β-cat/Tcf-4 signaling plays an essential role in regulating ET-1 expression in DU145 cells.

Activation of β-Cat/Tcf-4 Signaling Increases Expression of ET-1 in LNCaP Cells. The LNCaP cell is an androgen-dependent human CaP cell line that minimally expresses ET-1 (Nelson et al., 2001). In agreement with a previous report that LNCaP cells exhibit poor CRT activity because of a low expression level of Tcf-4 (Chesire et al., 2002), overexpression of β-cat in LNCaP cells showed little effect on the constitutively low TOPflash luciferase activity, whereas transfection of Tcf-4 alone or cotransfection of β-cat and Tcf-4 augmented the TOPflash activity by approximately 1.5- and 3-fold, respectively (Fig. 3A). In accordance with the results, overexpression of Tcf-4 alone or with β-cat increased the ET-1 mRNA level as well as the cyclin D1 mRNA level to approximately 1.5- and 2-fold of the control, respectively (Fig. 3B). ELISA detection of secreted ET-1 in the presence of neutral endopeptidase 24.11 inhibitor thiorphan (Dawson et al., 2004) showed that overexpression of Tcf-4 alone or coexpression of β-cat and Tcf-4 increased the ET-1 protein secretion to approximately 1.5- and 1.9-fold of the control, respectively (Fig. 3C). Thiorphan was used in the culture because LNCaP cells highly express neutral endopeptidase 24.11, a cell-surface enzyme that cleaves and inactivates ET-1 (Sumitomo et al., 2000; Sumitomo et al., 2001). Together, the results indicate that activated β-cat/Tcf-4 signaling is capable of up-regulating ET-1 expression in LNCaP cells, although ET-1 seems not responsible for the tumorigenicity of LNCaP cells. Together with the findings in DU145 cells, these results suggest that activation of β-cat/Tcf-4 signaling is required to enhance expression of ET-1 in both androgen-independent and -dependent CaP cells.

Knockdown of Endogenous β-Cat Inhibits Transcription of ET-1 in Mouse Primary PrECs. As both malignant and normal PrECs produce ET-1 (Nelson et al., 2001), we next examined whether β-cat/Tcf-4 regulates expression of ET-1 in normal PrECs. Sequence alignment showed that the mouse *ET-1* promoter shares an overall 70% sequence homology with its human counterpart (Harats et al., 1995). It is noteworthy that approximately 90% sequence identity is present in the 400-bp region immediately upstream of the TIS. In addition, the −73/−67 TBE, the TATA consensus sequences, and the TIS are conserved both in sequences and in relative locations between the human and the mouse *ET-1* promoters (Fig. 4A) (Harats et al., 1995). ChIP assays using an anti-Tcf-4 antibody specifically precipitated DNA fragments containing the −73/−67 TBE from the mouse prostate tissue (Fig. 4B), suggesting in vivo binding of Tcf-4 to the mouse *ET-1* promoter via the particular TBE.

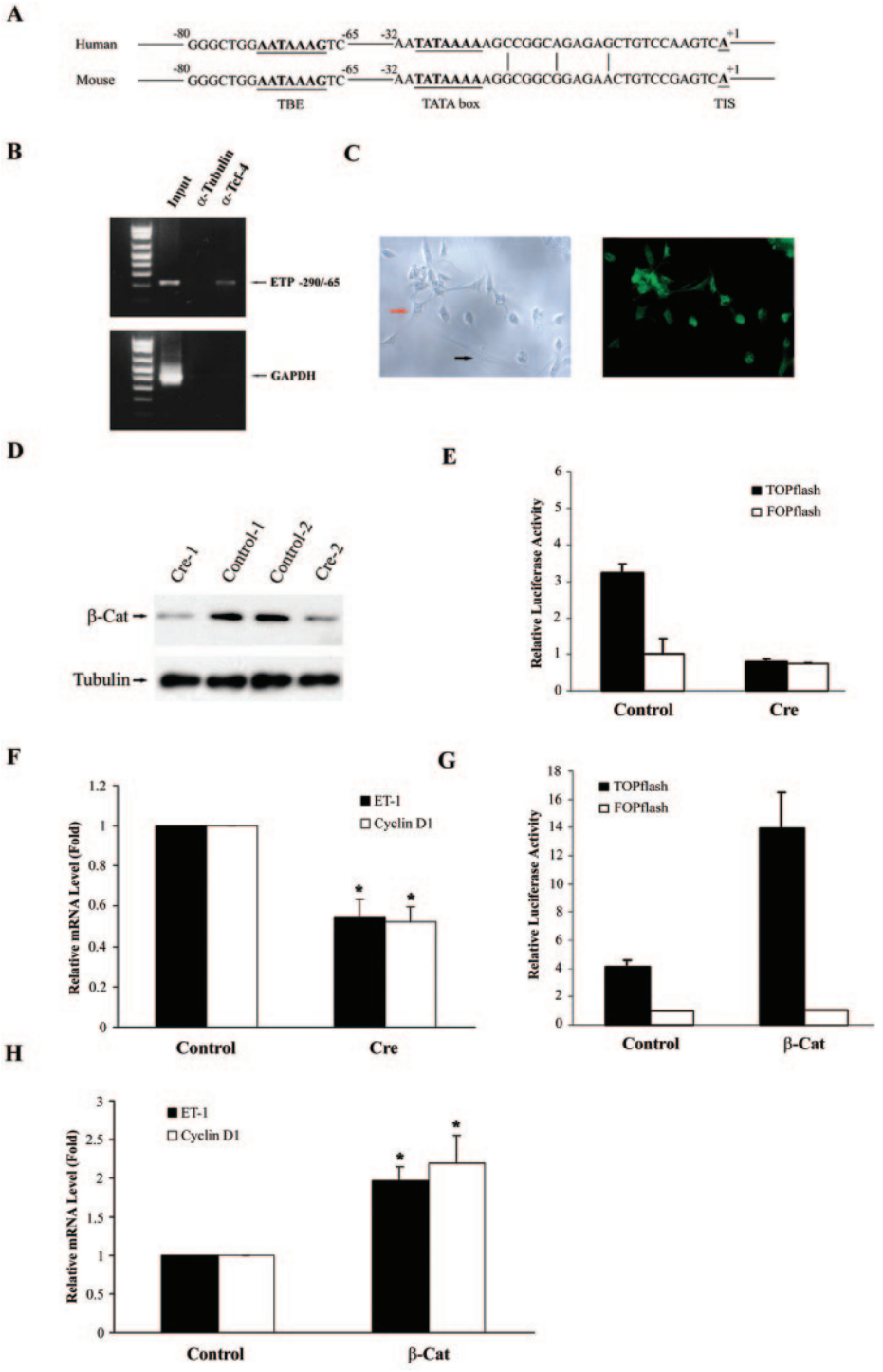


Fig. 4. Knockdown of endogenous β-Cat inhibits transcription of *ET-1* in primary PrECs. A, partial sequence alignment of the promoter region of human and murine *ET-1* genes. The −73/−67 TBE, the TATA box and the TIS are bold and underscored. The unmatched nucleotides are marked with vertical lines. B, binding of Tcf-4 to the mouse *ET-1* promoter in vivo. Cross-linked chromatin extracts from mouse prostate tissue were immunoprecipitated with an anti-Tcf-4 antibody (α-Tcf-4) or an anti-tubulin antibody (α-tubulin). The *ET-1* promoter fragment covering −290 to −65 bp (ETP −260/−65) and a DNA fragment from exon 8 of mouse *GAPDH* gene were PCR-amplified from the immunoprecipitated and the input chromatin, respectively. C, primary culture of mouse PrECs harboring floxed *β-cat* alleles. PrECs were obtained from mice carrying homozygous floxed *β-cat* alleles. Primary PrECs on day 2 of culture were immunostained with an anti-pan-cytokeratin antibody. Images of phase contrast (left) and immunofluorescence staining for cytokeratin (right) of the same view field are shown. Epithelial cells (red arrow) and stromal cells (black arrow) are indicated. D, knockdown of β-cat in mouse PrECs with a Cre recombinase-encoding adenovirus. Lysates from PrECs infected with a Cre recombinase-encoding adenovirus (Cre) or an empty control adenovirus (control) were analyzed for β-cat expression by immunoblotting 72 h after infection. The same membrane was blotted with an anti-tubulin antibody as the loading control. E and F, deletion of β-cat down-regulates transcription of *ET-1* in mouse primary PrECs. Mouse PrECs infected with an adenovirus encoding Cre recombinase (Cre) or an empty control adenovirus (Control) were analyzed for transcriptional activities of β-cat/Tcf (E) and levels of ET-1 mRNA and cyclin D1 mRNA (F) (∗, $P < 0.05$). G and H, overexpression of β-cat increases transcription of *ET-1* in mouse primary PrECs. Mouse PrECs were infected with a retrovirus encoding β-cat or an empty control retrovirus (Control). Transcriptional activities of β-cat/Tcf (G) and levels of ET-1 mRNA and cyclin D1 mRNA (H) are shown (∗, $P < 0.05$).

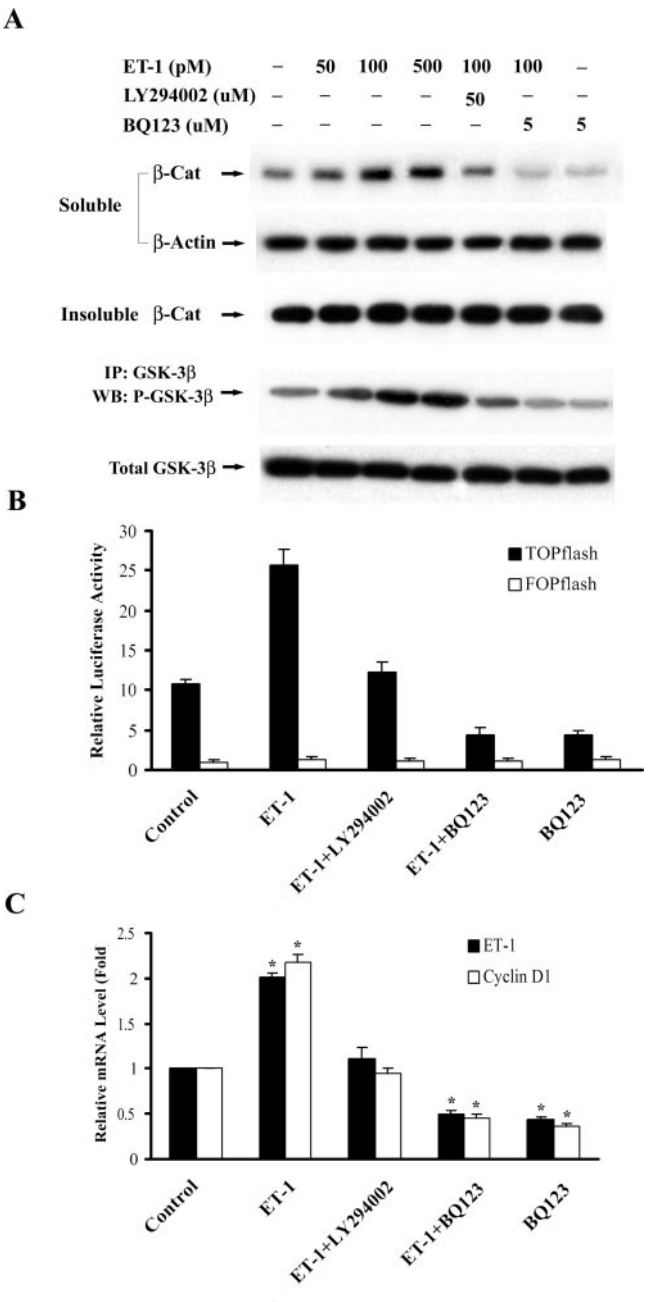


Fig. 5. ET-1 enhances β-Cat/Tcf signaling via a PI3K-dependent mechanism. A, effects of ET-1 on β-cat level and GSK-3β phosphorylation status in DU145 cells. DU145 cells were treated with ET-1 in the presence or absence of PI3K inhibitor LY294002 or ET-A receptor antagonist BQ123 at indicated concentrations for 2 h. Soluble and insoluble cell lysate fractions were prepared as described under *Materials and Methods* and immunoblotted for β-cat. The same membrane was blotted with an anti-β-actin antibody as the loading control. The whole-cell lysate was immunoprecipitated with an anti-GSK-3β antibody and blotted with an anti-phospho-GSK-3β (serine 9) antibody. The same membrane was blotted for total GSK-3β as the loading control. B, effects of ET-1 on transcriptional activities of β-cat/Tcf in DU145 cells. The TOPflash (filled bar) or FOPflash (empty bar) reporter was transfected into DU145 cells treated with ET-1 (100 pM) and/or BQ123 (5 μM). Normalized luciferase activities were expressed as -fold activation to the FOPflash luciferase activity of untreated control cells (arbitrarily defined as 1). C, effects of ET-1 on transcription of ET-1 in DU145 cells. The ET-1 mRNA levels (filled bar) in DU145 cells treated with ET-1 (100 pM) and/or BQ123 (5 uM) were determined by real-time quantitative RT-PCR 24 h after treatment. The mRNA level of cyclin D1 (empty bar) was included as a positive control. Results were normalized as fold increase to the ET-1 or the cyclin D1 mRNA level of untreated control cells (designated as 1) (*, $P < 0.05$).

To investigate the role of β-cat/Tcf-4 in regulating ET-1 expression in normal PrECs, we made primary PrEC cultures from mice with floxed *β-cat* alleles. As shown in Fig. 4C, the majority of the cultured PrECs (~80%) displayed typical epithelial morphology and immunofluorescence staining for epithelial marker cytokeratin (Hanazono et al., 1998). In contrast, stromal cells in the same view field showed no cytokeratin staining. These results verify the epithelial nature of the primary cultures as well as the specificity of the immunostaining performed. To ablate expression of β-cat, the mouse PrEC cultures were infected with an adenovirus encoding Cre recombinase. β-Cat expression and TOPflash activities were markedly decreased in PrECs infected with the adenoviral-Cre but not in cells infected with a control adenovirus (Fig. 4, D and E). Quantitative real-time RT-PCR showed a 45% down-regulation at the ET-1 mRNA level as well as at the cyclin D1 mRNA level in PrECs with knockdown of β-cat, compared with that of the control cells (Fig. 4F). Thus, β-cat/Tcf signaling seems responsible for a substantial part of ET-1 expression in normal prostate epithelium. Furthermore, overexpression of β-cat by infecting PrECs with a β-cat-encoding retrovirus elevated the TOPflash activity and the ET-1 mRNA level to approximately 3.5- and 1.9-fold of the control, respectively (Fig. 4, G and H). Together, these results manifest a potent role of β-cat/Tcf signaling in regulating transcription of the *ET-1* gene in normal prostate epithelium.

ET-1 Enhances β-Cat/Tcf Signaling via a PI3K-Dependent Mechanism. Having established ET-1 as a downstream target of the β-cat/Tcf pathway in CaP cells as well as in normal prostate epithelium, we sought to determine whether ET-1 could reciprocally affect β-cat/Tcf signaling. Previous studies had demonstrated that ET-1 stimulates the PI3K/Akt pathway via the ET-A receptor (Shi et al., 2004) and that stimulation of the PI3K/Akt pathway increases the stability of soluble β-cat by phosphorylation and inactivation of GSK-3β in CaP cells (Sharma et al., 2002). To examine the effects of ET-1 on β-cat/Tcf signaling in CaP cells, we first

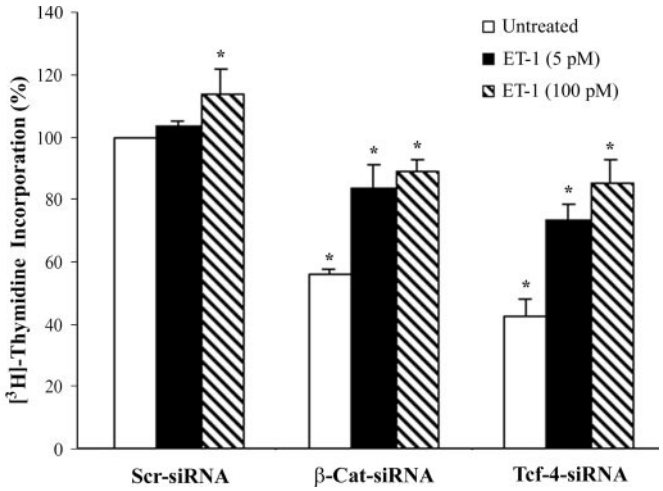
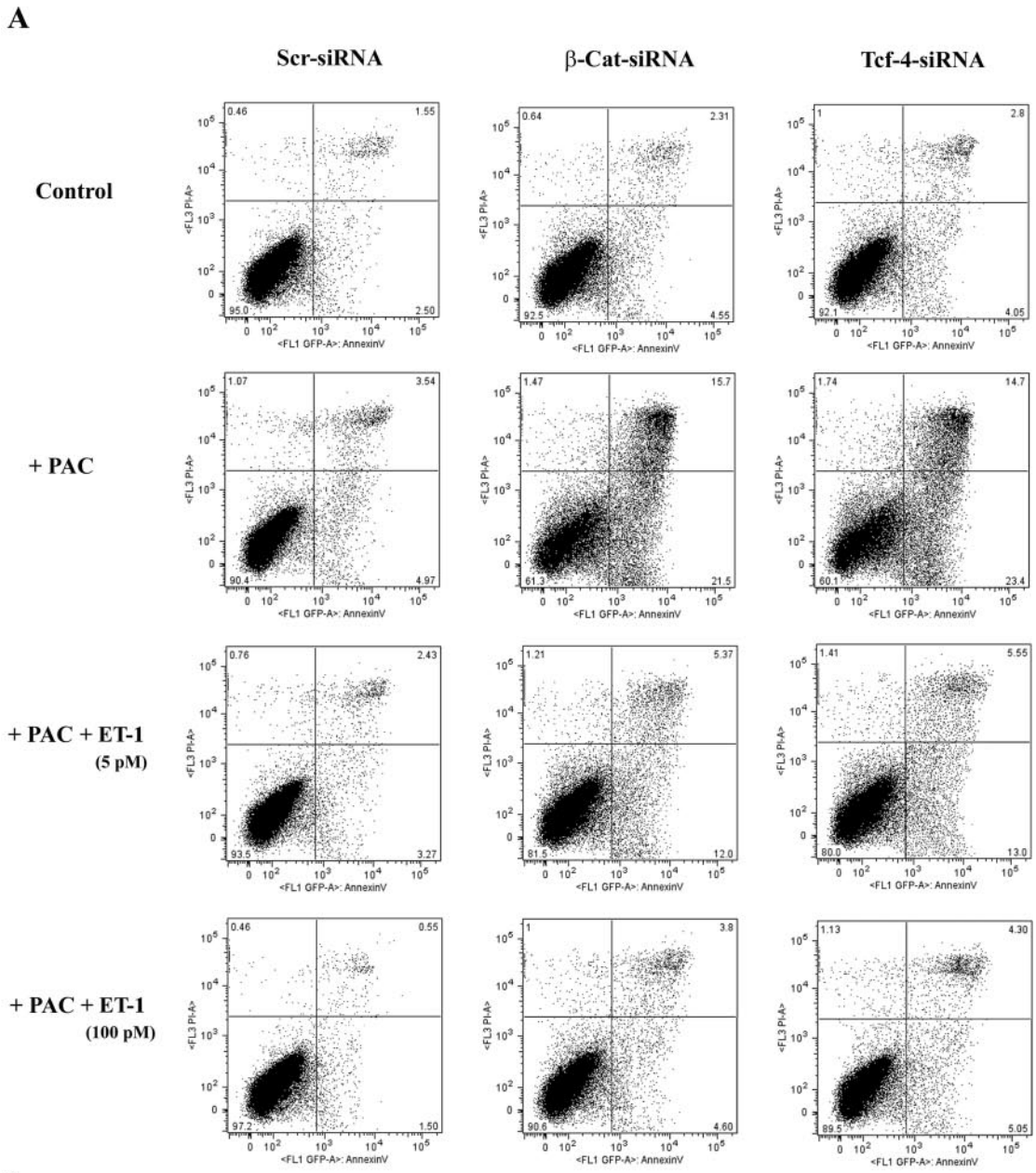
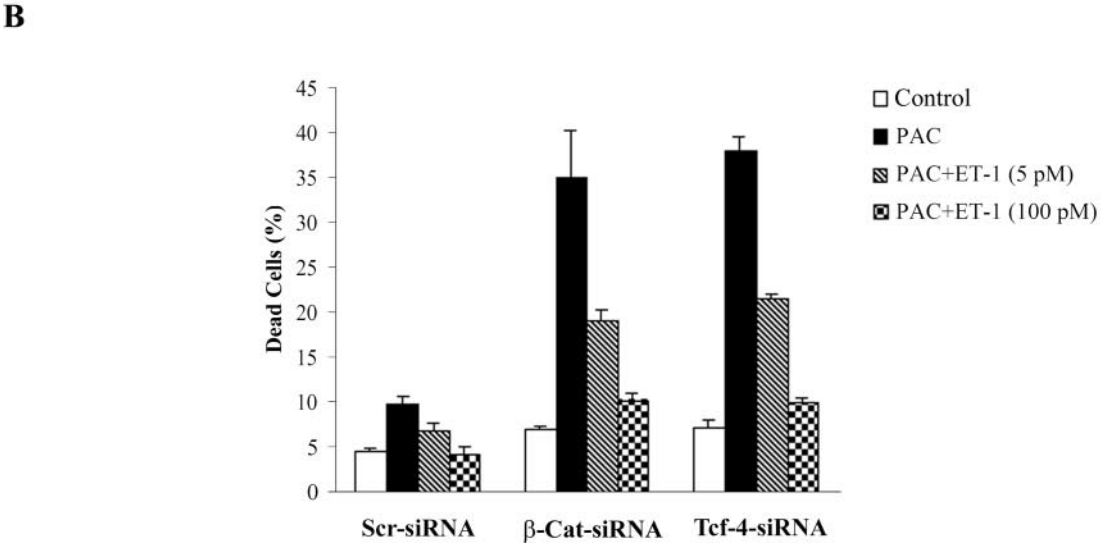


Fig. 6. β-Cat/Tcf-4 promotes [^{3}H]thymidine incorporation in DU145 cells partially via ET-1. DU145 cells stably expressing scrambled-siRNA (Scr-siRNA), siRNA against β-cat (β-Cat-siRNA), or siRNA against Tcf-4 (Tcf-4-siRNA) were metabolically labeled with [^{3}H]thymidine in the presence or absence of 5 or 100 pM ET-1. The results are expressed as percentages of the [^{3}H]thymidine incorporation of untreated Scr-siRNA-expressing cells, which is arbitrarily defined as 100% (*, $P < 0.05$).





determined the soluble β-cat level in DU145 cells treated with ET-1 in the presence or absence of a PI3K-specific inhibitor. As shown in Fig. 5A, exogenous ET-1 (≤100 pM) concentration-dependently increased the soluble β-cat level in DU145 cells until a plateau was reached. Treatment with selective PI3K inhibitor LY294002 abolished the increase of soluble β-cat induced by exogenous ET-1. In contrast, β-actin and insoluble pool of β-cat showed no detectable change. These results suggest that ET-1 increases soluble β-cat in DU145 cells through a PI3K-dependent manner. To determine the effect of endogenous ET-1 on soluble β-cat, a selective ET-A receptor antagonist BQ123 was employed to block endogenous ET-1 function in DU145 cells, because ET-A receptor is the predominant type of ET receptors in CaP cells (Nelson et al., 1996). Densitometry showed that treatment of DU145 cells with BQ123 decreased the soluble β-cat level by approximately 50% of the control in the presence or absence of exogenous ET-1 (Fig. 5A), which indicates that endogenous ET-1 constitutively maintains a part of the soluble β-cat pool in DU145 cells.

GSK-3β is a major downstream target of the PI3K/Akt pathway. Phosphorylation of GSK-3β at serine 9 by PI3K/Akt inactivates it and results in subsequent stabilization and accumulation of soluble β-cat (Sharma et al., 2002). To verify that ET-1 increases soluble β-cat through the PI3K/Akt pathway, we examined serine 9-phosphorylation status of GSK-3β in DU145 cells treated with ET-1 in the presence or absence of a PI3K-specific inhibitor. To manifest the phosphorylation status of GSK-3β, DU145 cell lysates were precipitated with an anti-GSK-3β antibody and blotted with an antibody that specifically recognized the serine 9-phosphoylated GSK-3β. As expected, exogenous ET-1 (≤100 pM) concentration-dependently enhanced phosphorylation of GSK-3β, which was blocked by LY294002. BQ123 further reduced the phosphorylation level of GSK-3β below that of the untreated control, with or without the presence of exogenous ET-1 (Fig. 5A).

Consistent with the above findings, treatment of DU145 cells with exogenous ET-1 increased the TOPflash activities to 2.3-fold of the control and raised the ET-1 and cyclin D1 mRNA levels to approximately 2-fold of the control. LY294002 completely blocked this effect, whereas BQ123 further decreased the TOPflash activity level by approximately 60% and reduced the ET-1 and cyclin D1 mRNA levels by 50% compared with those of the untreated control (Fig. 5, B and C). Together, these results indicate that ET-1 enhances β-cat/Tcf signaling in DU145 cells by increasing the soluble pool of β-cat through a PI3K-dependent pathway and that endogenous ET-1 maintains part of the constitutive β-cat/Tcf signaling in DU145 cells. Together with our findings that β-cat/Tcf-4 transcriptionally activates ET-1 in CaP cells, these results suggest that a positive feedback loop between β-cat/Tcf-4 signaling and ET-1 signaling may function to potentiate both signaling pathways in CaP cells.

β-Cat/Tcf-4 Promotes [^{3}H]Thymidine Incorporation in DU145 Cells Partially via ET-1. Both β-cat/Tcf signaling and ET-1 signaling are known to promote proliferation and survival of CaP cells (de la Taille et al., 2003; Nelson et al., 2003a). To determine functional significance of the β-cat/Tcf-4-regulated expression of ET-1 in CaP cells, [^{3}H]thymidine incorporation assays were performed to examine the proliferation potential of the β-cat or Tcf-4 knockdown DU145 cells. [^{3}H]Thymidine incorporation in DU145 cells expressing scrambled siRNA was taken as a control. As shown in Fig. 6, knockdown of β-cat or Tcf-4 in DU145 cells reduced the [^{3}H]thymidine incorporation to approximately 60% and 45% of the control, respectively. Treatment with exogenous ET-1 at a concentration (5 pM) that is comparable with the level of secreted ET-1 in normal DU145 cells recovered [^{3}H]thymidine incorporation to approximately 85% and 75% of the control, respectively ($P < 0.05$). In contrast, little change of [^{3}H]thymidine incorporation was observed in control cells treated with 5 pM ET-1. Higher concentration of ET-1 at 100 pM significantly increased [^{3}H]thymidine incorporation in control cells but failed to further rescue [^{3}H]thymidine incorporation in the β-cat or Tcf-4 knockdown cells, suggesting that ET-1 mediates proliferation of CaP cells partially through β-cat/Tcf-4 signaling and that other β-cat/Tcf-4 target genes play irreplaceable roles in β-cat/Tcf-mediated CaP cell growth. Annexin V/PI staining in parallel of the [^{3}H]thymidine incorporation assays showed little difference in apoptosis among the β-cat or Tcf-4 knockdown cells and the control cells (data not shown), indicating that the changes observed in the [^{3}H]thymidine incorporation assays resulted directly from cell proliferation. In addition, the observed cell proliferation-promoting effect of ET-1 seems to be independent of enhancing β-cat/Tcf-4 signaling, because exogenous ET-1 barely rescued β-cat/Tcf signaling in the β-cat or Tcf-4 knockdown DU145 cells (data not shown). Taken together, these results indicate that ET-1 partially mediates β-cat/Tcf-4–regulated proliferation of DU145 cells as a downstream target of β-cat/Tcf-4 signaling.

ET-1 Suppresses Paclitaxel-Induced Apoptosis That Is Sensitized in the β-Cat or Tcf-4 Knockdown DU145 Cells. We next examined whether β-cat/Tcf-4 signaling regulates survival of CaP cells via ET-1. Under normal culture conditions, the β-cat or Tcf-4 knockdown DU145 cells displayed no excessive apoptosis compared with control cells expressing scrambled siRNA (Fig. 7). However, treatment with a low concentration (5 nM) of paclitaxel, an apoptosis-inducing drug used to treat a variety of cancers, including CaP (Kim et al., 2004), led to approximately 36% cell death in the β-cat or Tcf-4 knockdown DU145 cells, whereas only approximately 10% cell death was induced in the control cells. Treatment with exogenous ET-1 significantly ($P < 0.05$) reduced the cell death to approximately 20% at 5 pM and nearly completely suppressed the paclitaxel-induced apoptosis at 100 pM in the β-cat or Tcf-4 knockdown DU145 cells.

Fig. 7. ET-1 suppresses paclitaxel-induced apoptosis that is sensitized in the β-cat- or Tcf-4-knockdown DU145 cells. A, effects of ET-1 on paclitaxel-induced apoptosis in the β-cat or Tcf-4 knockdown DU145 cells. DU145 cells stably expressing scrambled-siRNA (Scr-siRNA), siRNA against *β-cat* (β-Cat-siRNA), or siRNA against *Tcf-4* (Tcf-4-siRNA) were treated with 5 nM paclitaxel (PAC) in the presence or absence of ET-1 (5 or 100 pM) for 12 h. The untreated cells were analyzed as control. Apoptosis/cell death was quantitated using annexin V/PI staining coupled with flow cytometry analysis. The *number* in each quadrant of the dot figures refers to the percentage of positive cells in total cells. Data shown are representative of at least two independent experiments. B, percentages of dead cells. Sums of percentages of early apoptotic cells (left lower quadrant), late apoptotic cells/dead cells (right upper quadrant) and necrotic cells (right left quadrant) are shown as percentages of dead cells in histograms (mean ± S.D.).

These results indicate that endogenous β-cat/Tcf-4 signaling maintains a state of apoptosis resistance in DU145 cells essentially via ET-1.

Discussion

We have demonstrated in this study that β-cat/Tcf-4 signaling regulates ET-1 expression in both malignant and normal PrECs via direct modulation of the *ET-1* promoter activity. Meanwhile, ET-1 stimulates β-cat/Tcf-4 signaling via a PI3K-dependent pathway. The positive inter-regulation between β-cat/Tcf-4 and ET-1 signaling plays an important role in promoting proliferation and survival of CaP cells.

Both β-cat and ET-1 are associated with CaP progression, particularly the development of advanced, metastatic CaP (Pirtskhalaishvili and Nelson, 2000; Chesire et al., 2002). Significantly higher levels of ET-1 or cytoplasmic/nuclear β-cat were detected in situ in advanced, hormone-refractory CaP than in the primary disease (Nelson et al., 1996; Chen et al., 2004). In addition, abnormally elevated plasma ET-1 levels were detected in 58% of men with hormone-refractory CaP (Nelson et al., 2001). The proportion roughly matches that of high-level cytoplasmic/nuclear β-cat detected in CaP metastatic sites (Chen et al., 2004). Our results are consistent with the previous reports and uncover a mechanistic link between the activation of nuclear β-cat signaling and the high levels of ET-1 expression detected in advanced, hormone-refractory CaP.

Previous studies indicate that nuclear β-cat signaling is involved in CaP progression mainly through two pathways, namely, the β-cat/Tcf pathway and the β-cat/AR pathway (Chesire et al., 2002). Activation of the β-cat/Tcf pathway promotes cell proliferation and suppresses apoptosis (de la Taille et al., 2003). On the other hand, β-cat interacts with AR in a ligand-dependent manner and potentiates the androgen signaling pathway, which also increases cell proliferation and survival. In androgen-dependent CaP cells, activation of the β-cat/AR pathway inhibits the β-cat/Tcf pathway by competing away nuclear β-cat (Chesire et al., 2002; Mulholland et al., 2003). Consistent with these reports, our results show that the β-cat/Tcf pathway is constitutively active in androgen-independent DU145 CaP cells (Fig. 2). It is noteworthy that the β-cat/Tcf pathway is also constitutively active in mouse primary PrECs, though at a relatively low level (Fig. 4E). It is likely that a reciprocal balance between the β-cat/Tcf and the β-cat/AR pathways contributes to the homeostasis of normal prostate epithelium. Disruption of the balance may lead to abnormal cell proliferation and survival, which eventually promotes tumorigenesis.

Our results revealed that ET-1 stimulates β-cat/Tcf-4 signaling in a PI3K-dependent manner in DU145 cells, as a positive feedback to β-cat/Tcf-4-mediated transcription. As a downstream target of the β-cat/Tcf-4 pathway, ET-1 may regulate proliferation and survival of CaP cells via traditional ET-1 signaling mechanisms (Nelson et al., 2003a). On the other hand, by enhancing β-cat/Tcf-4 signaling, ET-1 may also regulate proliferation and survival of CaP cells through other downstream targets of β-cat/Tcf-4 in addition to ET-1 itself, such as *c-myc* and cyclin D1 (de la Taille et al., 2003). Consistent with this notion, we found that exogenous ET-1 could only partially rescue DU145 cell proliferation reduced by knocking down β-cat or Tcf-4 (Fig. 6). However, the β-cat/Tcf-4–regulated expression of ET-1 serves as a major contributor to the survival of DU145 cells under severe stress (Fig. 7). The apoptosis-protective effect of ET-1 on DU145 cells seems to be independent of enhancing β-cat/Tcf-4 signaling, because endogenous β-cat/Tcf-4 signaling in the β-cat or Tcf-4 knockdown DU145 cells was irreversibly abrogated and recalcitrant to rescue by exogenous ET-1 (data not shown). The underlying mechanism remains to be determined.

Previous studies demonstrated the presence of high levels of cytoplasmic/nuclear β-cat in advanced, hormone-refractory CaP (Chen et al., 2004). It is postulated that a portion of hormone-refractory CaP containing cytoplasmic/nuclear β-cat staining bears elevated CRT because of relief of AR-mediated CRT repression (Chesire and Isaacs, 2003). We propose that the elevated CRT promotes expression of ET-1, which in turn further enhances β-cat/Tcf-4 signaling and ET-1 expression in a PI3K-dependent manner. The positive feedback loop between β-cat/Tcf-4 signaling and ET-1 signaling enhances both signaling pathways to potentiate proliferation and survival of CaP cells against severe stress such as androgen ablation and chemotherapeutic agents, thereby promoting the progression and malignancy of CaP. Hence, the newly uncovered positive inter-regulation between β-cat/Tcf-4 signaling and ET-1 signaling is likely to represent a novel mechanism that contributes to the progression of at least a subset of androgen-independent CaP that possesses active β-cat/Tcf signaling. Moreover, our results suggest that a combination of ET-A receptor antagonist and appropriate chemotherapeutic agent(s) could prove effective in treatment of advanced CaP.

Acknowledgments

We thank Dr. Hans Clevers for the human *β-cat* and *Tcf-4* expression plasmids and Dr. Gen-Sheng Feng (Burnham Institute for Medical Research, La Jolla, CA) for the Cre recombinase-encoding adenovirus.

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Correction to "Positive Inter-Regulation between β-Catenin/T Cell Factor-4 Signaling and Endothelin-1 Signaling Potentiates Proliferation and Survival of Prostate Cancer Cells"

In the above article [Sun P, Xiong H, Kim TH, Ren B, and Zhang Z (2006) *Mol Pharmacol* **69:**520–531], the small interfering RNA sequences for β-catenin shown in Table 1 are incorrect. The correct sequences are as follows:

5′-GATCCCGTGGGTGGTATAGAGGCTCTTCAAGAGAGAGCCTCTATACCACCCAC-TTTTTGGAAA-3′

5′-AGCTTTTCCAAAAAGTGGGTGGTATAGAGGCTCTCTCTTGAAGAGCCTCTATA-CCACCCACGG-3′

The authors regret this error and apologize for any confusion or inconvenience it may have caused.