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Differential expression of estrogen receptor beta isoforms in prostate cancer through interplay between transcriptional and translational regulation

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Abstract

Estrogen receptor β (ER β) and its isoforms have different putative functions and expression patterns in prostate cancer. Current studies on 5'-most exons, 0K and 0N, show that their respective promoters are actively involved in transcription. These data, however, do not explain why ER β isoforms are differentially expressed in normal and cancerous tissues, since 0K and 0N transcripts are detectable in clinical specimens. Various combinations of 5' untranslated exons, termed exon 0Xs, associate with promoter 0K only and exon 0Xs accommodate upstream open reading frames (uORFs) reducing protein expression. Moreover, ER β 1, 2, and 5 are transcriptionally linked to promoter 0K; exon 0Xs are spliced only into ER β 2 and ER β 5 transcripts, suggesting that their expressions are regulated post-transcriptionally by exon 0Xs. This study reveals that expression of ER β 1 is regulated primarily at the transcriptional level, whereas that of ER β 2 and ER β 5 is controlled by the interplay between transcriptional and post-transcriptional regulation.

Keywords

ERbeta2; ERbeta5; upstream open reading frame; promoter; 5' untranslated region; post-transcriptional

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1. Introduction

Hormones are the indispensable factors in prostate carcinogenesis. Apart from androgen, estrogen also determines the risk of prostate cancer (PCa) (Ho et al., 2011). Most estrogenic actions are mediated by two estrogen receptor (ER) subtypes, α and β (Prins and Korach, 2008; Warner and Gustafsson, 2010). In humans, ER α is not expressed in the epithelial cells of the prostate where carcinogenesis takes place (Latil et al., 2001; Pasquali et al., 2001), whereas ER β (referred to as ER β 1) and its isoforms (Leung et al., 2006) are expressed at high levels in both basal and luminal epithelial cells in unique topographic patterns in normal glands (Leav et al., 2001; Leung et al., 2010). During the development and progression of PCa, ER β 1 expression is gradually lost at the primary site (Lai et al., 2004; Leung et al., 2010), supporting its proposed anti-proliferative or pro-apoptotic role in PCa cells (McPherson et al., 2010; Waldmann et al., 2010). ER β 1 also represses epithelial-mesenchymal transition (EMT) (Mak et al., 2010), an initial step in metastasis. ER β 2 and ER β 5, in contrast to ER β 1, are found consistently in high-grade PCa and may be involved in promoting progression and invasion (Leung et al., 2010). Thus, ER β 1 and its isoforms appear to be independently regulated by different mechanisms, including the alternative use of promoters and post-transcriptional regulation.

Studies of the prostate have revealed that ER β transcripts are derived primarily from two promoters, 0K and 0N (Zhang et al., 2007; Zhu et al., 2004), although additional promoters (E1 and M) have been identified in other tissues (Shoda et al., 2002; Smith et al., 2010a). The promoters were named by the different 5'-most untranslated exons (0K and 0N) upstream of exon 1 (Hirata et al., 2001; Zhao et al., 2003). Promoter 0N has higher transcriptional activity than 0K in the prostate, and the two promoters are regulated differently (Zhang et al., 2007). Promoter 0N can be silenced by DNA methylation (Zhao et al., 2003; Zhu et al., 2004) that targets a regulatory AP-2 site at its proximal CpG island (Zhang et al., 2007). Promoter 0K has a CpG-rich region but is not the target of DNA methylation (Zhang et al., 2007; Zhao et al., 2003). We showed that ER β 1 is transcribed predominantly from promoter 0N (Zhang et al., 2007; Zhu et al., 2004); however, the contribution from promoter 0K is unclear. Furthermore, the promoter dependency of other ER β isoforms in the prostate is currently unknown.

Differential expression of ER β 1 and its isoforms in the prostate may also be related to post-transcriptional regulatory mechanisms, such as inhibition of protein translation by upstream open reading frames (uORFs) (Calvo et al., 2009), as reported in a study of breast cancer cells (Smith et al., 2010a). An mRNA has multiple uORFs, defined as sequences flanked by a pair of in-frame start and stop codons and within 5' untranslated region (5' UTR). Despite the ability of ribosomes to initiate translation at the start codon of any ORF, attention was often paid to the protein translated from the longest ORF. In this regard, the ORFs located 5' of the coding sequence that do not contribute to the main protein expression are called uORFs. They have been shown to inhibit protein translation via mechanisms such as ribosome stalling, premature release of ribosome from the mRNA, and increased mRNA instability (Calvo et al., 2009; Morris and Geballe, 2000).

Our primary objective is to determine whether ER β 1 and its isoforms are differentially regulated in the normal and malignant prostatic specimens. Our data show that ER β 1 and 2 are transcribed from both promoter 0N and 0K, whereas ER β 5 is probably transcribed only from promoter 0K. In addition, we revealed that transcripts from promoter 0K harbor various combinations of untranslated exons (0X1-8), some named by Hirata *et al.* as 0Xs (Hirata et al., 2001). Exon 0Xs were shown to constitute a new regulatory mechanism of protein translation for various ER β s. In summary, our findings suggest that differential protein expression of ER β isoforms is a result of the alternative use of promoters at the transcriptional level and the regulation by untranslated exons at the post-transcriptional level.

2. Materials and Methods

2.1 Cell lines and culture conditions

BPH-1, C4-2, DU-145, HEK293, LNCaP, PC-3, RWPE-1, and WPWY-1 were cultured in ATCC-recommended medium and supplements (ATCC, Manassas, VA). Primary cell culture, PrEC, and immortalized NPrEC were maintained in keratinocyte serum-free medium (KSFM) (Life Technologies, Carlsbad, CA). All the cell lines were grown with 5% penicillin/streptomycin at 37 °C and in 5% CO₂.

2.2 5' Rapid amplification of cDNA ends (5' RACE)

Experiments were performed using GeneRacer (Life Technologies) and cDNA from PC-3 cells. The 5'RACE was performed using ER β exon 1-specific primers. Procedures were the same as described in the manual. The sequences of primers used in the experiment are described in Table 1.

2.3 Plasmid construction

The DNA fragments, which were isolated in 5' RACE experiments and encode exon 0N and different combinations of exon 0K and 0Xs, were gel-purified and subcloned to pCR2.1-TOPO vector (Life Technologies). DNA sequencing reactions were performed by Macrogen (Seoul, Korea). Amplicons generated from prostatic clinical specimens were also gel-purified, subcloned, and sequenced. Different combinations of exon 0N, 0K, and 0Xs sequences isolated from clinical specimens were subcloned into pGL3 -Promoter vector (Promega, Fitchburg, WI).

In vitro translation study was facilitated by adding T7 promoter to the 5' end of the untranslated exons, which were cloned upstream of the luciferase gene in pGL3-Promoter vector, through PCR reactions.

2.4 Normal and cancerous human prostate samples

Fourteen pairs of human cancerous and matched non-cancerous prostatic tissues collected previously (Ouyang et al., 2011) were used in the study.

2.5 In situ RNA-RNA hybridization (ISH)

In situ RNA-RNA hybridization was performed on human prostate formalin-fixed paraffin-embedded sections. Sections containing benign, prostatic intraepithelial neoplasia (PIN) or cancerous prostatic tissues of different Gleason grades from six patient samples were used. Oligonucleotides encoding the antisense sequence of exon 0K (35 bp) or 0N (36 bp) were annealed with T7 promoter sequence at 95 °C. Digoxigenin (DIG)-labeled cRNA probes were synthesized using T7 RNA polymerase according to the protocol (Roche Applied Science, Mannheim, Germany). Probe with scrambled sequence was used as negative control (Exiqon, Vedbaek, Denmark). Sequences of the probes are listed in Table 1. The detailed procedures were described in our previous study (Zhang et al., 2010). Positive signals were developed by incubating with BCIP/NBT substrate (Millipore, Billerica, MA); TSA DNP (AP) system (Perkin Elmer, Hebron, KY) was used for the amplification of positive signals. Methyl green (Sigma Aldrich, St. Louis, MO) was used for nuclear counterstaining. Pictures were captured with an Axiovert 200M fluorescent microscope and were analyzed with Axiovision 4.7 software (Carl Zeiss, Oberkochen, Germany).

2.6 PCR amplification

Expression of exon 0K- or 0N-initiated ER β transcripts in prostatic clinical samples was detected by PCR amplification. The primers used are listed in Table 1. RNAs were extracted by TRIZOL reagent and were treated with DNase I (Life Technologies). cDNAs of prostatic clinical samples were synthesized using Superscript III reverse transcriptase with oligo d(T) primers according to the manufacturer's protocol (Life Technologies). PCR reactions were performed using Platinum Taq polymerase (Life Technologies).

Nested PCR was used to amplify the regions between 5' UTR and ER β isoforms. The cDNAs were synthesized with oligo d(T) primer and PCR reactions were performed with Platinum Taq polymerase. Touchdown PCR with annealing temperature dropping gradually from 68 °C to 55 °C was performed in the first and second (nested) round of PCR reactions. Primers used in the experiments are listed in Table 2.

2.7 Quantitative reverse transcription (RT)-PCR

The cDNAs were synthesized with random primers and Superscript III reverse transcriptase according to the manufacturer's protocol (Life Technologies). Quantitative RT-PCR reactions were performed with SYBR Green qPCR SuperMix and ABI7900 real-time PCR system (Life Technologies). GAPDH was used as the housekeeping gene. The C_t method was used to calculate the relative expression levels. Primers used in quantitative RT-PCR are summarized in Table 1. The specificity of primers used in the amplification was determined by the presence of single-band products in gel electrophoresis. Moreover, a discrete peak for each primer pair was observed in melting curve analysis after quantitative RT-PCR.

2.8 Prediction of the secondary structure and stability of mRNAs

The modeling program RNA Folding Form (version 2.3 energies) from mfold web server (RNA Institute, State University of New York at Albany) was used to predict the secondary

structure and stability during mRNA folding by thermodynamic methods (Zuker, 2003). The algorithm predicts the stability of mRNA through the free-energy change (ΔG) during base pairing and structural folding (Zuker, 2003).

2.9 Luciferase reporter assay

HEK293 and PC-3 cells were seeded in 24-well plates at 2.8×10^5 and 0.9×10^5 , respectively. The pGL3-Promoter vectors containing full-length exon 0N, 0K or different combinations of 0K and 0Xs together with β -galactosidase (CMV- β -gal) were transfected using Lipofectamine2000 (Life Technologies) for HEK293 cells or XtremeGene HP (Roche Applied Science) for PC-3 cells. After 48-h of transfection, cells were lysed with Glo lysis buffer (Promega) and luciferase activities were measured with the Bright-Glo luciferase kit (Promega). Normalization of the transfection efficiency was done by measuring β -galactosidase activity with the β -galactosidase assay kit (Promega). Each experiment was carried out in technical triplicates.

2.10 In vitro translation and western blotting

Plasmids containing T7 promoter at the 5' end of exon 0N, 0K, and different combinations of 0K and 0Xs were respectively translated *in vitro* by the TNT T7-ribulocyte system (Promega). The pGL3-Promoter vector was used as a positive control. Three microliters of reaction products were subjected to SDS-PAGE and western blotting. Mouse monoclonal anti-luciferase (Sigma Aldrich) was used at a 1:1000 dilution. The protein bands were detected with IRDye secondary antibody, and the signals were obtained with the Odyssey Infrared Imaging System (LiCor Bioscience, St. Lincoln, NE). Band intensities of luciferase in different lanes were measured by ImageJ analysis (NCBI, Bethesda, MD).

2.11 Site-directed mutagenesis

Seven single-site mutations and one double-site mutation on the 0K0X2'-pGL3-Promoter construct were generated with the QuikChange lightning site-directed mutagenesis kit as described in the manufacturer's protocol (Agilent Technologies, Santa Clara, CA). Primers for mutagenesis were designed through the QuikChange primer design program (Agilent Technologies) (Table 2). Briefly, the mutant-strand synthesis was done by PCR reaction with high-fidelity DNA polymerase and mutagenesis primers. The PCR product was treated with restriction endonuclease *DpnI* to digest the parental DNA template. The mutated single-strand DNA was transformed into competent cells and converted to duplex form *in vivo*.

2.12 Statistical analysis

Student's t-test was applied for statistical analysis with the use of QuickCalcs (GraphPad Software, La Jolla, CA). All calculated P-values were two-sided, and $P < 0.05$ was considered to be statistically significant.

3. Results

3.1 Different combinations of exon 0Xs exist between exon 0K and exon 1 of ER β in the prostate

So far, four promoters (0N, 0K, and E1) have been shown to be associated with ER β expression (Hirata et al., 2001; Smith et al., 2010b). To investigate whether any new promoters are responsible for ER β expression in the prostate, we performed 5' RACE with PC-3 cells, which is the PCa cell line with the highest expression of ER β transcript (Fig. 1A) (Leung et al., 2006). Intriguingly, 5' RACE products of various sizes were amplified with the primers homologous to oligo adapter at 5' end and the RACE primers complementary to exon 1 of ER β . The 5' RACE products were subjected to DNA sequencing (Fig. 1B). Exon 0K and 0N, but not E1, were identified at the 5' end of the ER β transcripts (Fig. 2A). To investigate the promoter usage of ER β in PCa clinical specimens, we measured the expression of exon 0N and 0K using qualitative PCR. Exon 0N was amplified in the majority of normal and cancerous tissues of 14 prostatic samples (Fig. 1C, lower panel), whereas exon 0K was less commonly amplified (Fig. 1C, upper panel), revealing that the transcripts from both promoters had different expression patterns. In some normal and cancerous samples, PCR products larger than the predicted size (300 bp) between exon 0K and exon 1 were observed (Fig. 1C, upper panel).

The amplicons obtained in 5' RACE experiments and amplification from clinical specimens were cloned and subjected to DNA sequencing. Sequencing data revealed that eight untranslated exons, termed exon 0Xs, were inserted between exon 0K and exon 1 (Fig. 2A and B). Surprisingly, there were many different combinations of alternative splicing between exon 0K and 0Xs. Incomplete exon 0Xs, termed 0Xs', were formed by alternative splicing within exons and found in some promoter 0K-initiated transcripts (Fig. 2A). Moreover, the alternative splicing leading to various combinations of exon 0K and 0Xs was present in cancerous tissues (3 of 14) and adjacent normal counterparts (8 of 14) (Fig. 1C, upper panel).

3.2 Promoter 0K and 0N are active in the transcription of ER β in both normal and cancerous prostatic tissues

Next, we used *in situ* RNA-RNA hybridization to determine the cellular localization of exon 0N and 0K's expressions. By using oligonucleotide probes specific to exon 0K and 0N, we showed that both were expressed abundantly in the benign region, PIN lesion, and Gleason grade 3 cancer foci. Their expression decreased when PCa progressed to the higher grade (Gleason grade 5) (Fig. 3). These results suggest that both promoter 0K and 0N actively contribute to the transcription of ER β in normal and cancerous prostatic tissues.

3.3 Promoter 0K and 0N differentially regulate the transcription of different ER β isoforms

To investigate whether promoter 0K and 0N are responsible for the transcription of ER β and its isoforms in prostatic cells, we first determined the expression of exon 0K, 0N, and ER β 1, 2 and 5 among different prostate cell lines. Quantitative RT-PCR was performed to measure their relative expression in normal prostate cells, such as PrEC, NPrEC, RWPE-1, BPH-1, and WPMY-1, and in PCa cell lines, such as DU-145, PC-3, LNCaP, and C4-2 (Fig. 4A).

Expression of exon 0N was relatively higher in PC-3, LNCaP, and C4-2 (Fig. 4A, upper panel, left), whereas expression of exon 0K was relatively higher in RWPE-1, LNCaP, and C4-2 (Fig. 4A, upper panel, right). ER β 1, 2, and 5 were also differentially expressed in different cell lines. Expression of ER β 1 was relatively higher in NPrEC, RWPE-1, and PC-3 (Fig. 4A, lower panel, left), whereas expression of ER β 2 was highest in PC-3 (Fig. 4A, lower panel, middle). ER β 5 were expressed strongest in BPH-1 (Fig. 4A, lower panel, right). These data demonstrated that ER β 1, 2, and 5 had differential expression patterns, revealing that the extent of their use of the two promoters in the prostate may differ.

To determine which promoter regulates the transcription of ER β 1, 2, and 5, we performed nested long-range PCR with primer pairs specific to exon 0K or 0N and exon 8 of each ER β isoform (Fig. 4B), because ER β 1, 2 and 5 possess unique sequences only in exon 8. The cDNA of PC-3 was used, as it exhibits significant expression of these isoforms. The schematic diagram of the experiments is shown in Fig. 4B. PCR products of the expected sizes were formed in the amplification of the sequences between exon 0N and exon 8 of ER β 1 or ER β 2 but not ER β 5 (Fig. 4C, lower panel, left). The absence of amplification was not due to poor primer design because the primer pair was able to amplify promoter 0N-initiated ER β 5 transcript in the breast cancer cell line MDA-MB-231 (Fig. 4C, lower panel, right), a finding consistent with that of another group (Smith et al., 2010a). Moreover, BPH-1, which has high expression of ER β 5 (Fig. 4A, lower panel, right), was used to demonstrate that promoter 0N-initiated ER β 5 transcript is absent in prostate cells (Fig. 4C, lower panel, left), suggesting that the splicing of exon 0N into the ER β 5 transcript is tissue-specific.

In contrast, DNA fragments of ER β 1, 2, and 5 were amplified with primer sets specific to exon 0K and respective ER β isoforms in PC-3 (Fig. 4C, upper panel). A single band was detected for promoter 0K-initiated ER β 1 transcript, whereas multiple bands were amplified using exon 0K primer and isoform-specific primers of ER β 2 and 5, respectively (Fig. 4C, upper panel). Multiple PCR products were also observed for promoter 0K-initiated ER β 5 transcripts using cDNA of BPH-1, but the number and size were different from those obtained in PC-3 (Fig. 4C, upper panel). Since some of the promoter 0K-initiated ER β 2 or 5 transcripts were larger than expected, we next investigated whether exon 0Xs were present in those amplicons. Sequencing revealed no extra sequence was present between exon 0K and exon 1 in ER β 1 transcript (Fig. 4D, upper panel). Interestingly, different combinations of exon 0Xs were present between exon 0K and exon 1 in some ER β 2 and 5 transcripts. Exon 0X2, 0X2', 0X5, 0X6, 0X7, and 0X8 were found in ER β 2 transcripts (Fig. 4D, middle panel), whereas exon 0X2, 0X2', 0X4, and 0X4' were present in ER β 5 transcripts (Fig. 4D, lower panel). These data suggested that promoter 0K and 0N differentially regulate the transcription of different ER β isoforms in prostate cells. Both promoters transcribe ER β 1 and 2, although it is not clear which promoter is predominant. ER β 5 may be transcribed preferentially by promoter 0K in the prostate. Moreover, specific combinations of exon 0Xs are only present in ER β 2 and 5 transcripts transcribed by promoter 0K.

3.4 The 5' UTRs of ER β inhibit the translational efficiency to different extents

To investigate whether these newly discovered sequences (ie, 5' UTRs) formed by different combinations of exon 0Xs may play a role in regulating ER β expression, we performed the following studies. Since 5' UTRs have been shown to regulate the translational efficiency of downstream mRNAs (Calvo et al., 2009; Meijer and Thomas, 2002) through ribosome stalling, premature release of ribosome from the mRNA, and increased mRNA instability (Calvo et al., 2009; Morris and Geballe, 2000), we first analyzed sequences to identify any known features that would modulate ribosome loading during protein translation. Sequence analysis revealed the presence of various elements possibly affecting the translational efficiency in exon 0N, 0K, and 0Xs (Supplementary Fig. 1). The putative regulatory elements include the start and stop codons. A "strong start codon" represents a start codon flanked by a sequence similar to the Kozak consensus sequence (Kozak, 1986), while we defined the region as "similar Kozak consensus sequence" by the presence of three or more identical nucleotides apart from ATG start codon. Start codon and in-frame stop codon separated by at least nine nucleotides constitute uORF (Kozak, 1986; Kozak, 1987). The uORFs are present in all untranslated exons except 0X3 and 0X8. An internal ribosomal entry site (IRES) was predicted present only in exon 0X8 (Supplementary Fig. 1). Moreover, we used the computer modeling program mfold to predict the secondary structure and stability of 5' UTRs (Zuker, 2003). Their stabilities were represented by the free-energy change (ΔG). The ΔG value of exon 0X4 is the highest among exon 0K and 0Xs (Supplementary Fig. 1), suggesting that exon 0X4 forms a more stable structure that releases higher energy during mRNA folding and thus possesses a larger ΔG value.

To determine whether exon 0Xs are able to regulate the translational efficiency of their downstream coding sequence, we selected eight combinations of exon 0K and 0Xs isolated from the clinical specimens (Fig. 1C, upper panel). The number of start codons, stop codons, and uORF were analyzed (Fig. 5A). We subcloned these exon 0K-0X combinations, as well as full-length exon 0K and 0N, to the upstream of luciferase-coding sequence, and transfected plasmids into HEK293 or PC-3 cells to determine the luciferase activity (Fig. 5B). The mRNA expression of luciferase was measured by quantitative RT-PCR. The translational efficiency was determined by normalizing the luciferase activity with its mRNA expression. The inhibitory effect of exon 0K is 3.7-fold and 2.2-fold stronger than that of exon 0N in HEK293 and PC-3 cells, respectively (Fig. 5B). Exon 0K in combination with different 0X sequences exerted more remarkable repressive effects (>90% reduction in both cell lines, except for exon 0K-0X4' in PC-3 cells) (Fig. 5B). We also used a cell-free system to transcribe and translate luciferase *in vitro* to investigate the post-transcriptional regulation of protein expression by these untranslated exons. Since luciferase mRNAs of all constructs are transcribed *in vitro* by T7 promoter with equal amounts of plasmids, perturbation of protein expression could only be due to untranslated exons. Relative expression of luciferase was determined by western blotting and densitometric analysis (Fig. 5C). The expression of luciferase with exon 0N at the 5' end was higher than that with exon 0K, whereas the presence of different exon 0Xs greatly reduced luciferase expression (Fig. 5C). The results were consistent with those from luciferase reporter assays (Fig. 5B), implying that the post-transcriptional regulation by different 5'UTRs was independent of the

cell context. These data demonstrate that exon 0K together with exon 0Xs significantly inhibits protein expression through the post-transcriptional mechanism.

3.5 uORFs in exon 0K and 0Xs are responsible for the inhibition of translational efficiency

The uORFs of untranslated exons play an important role in inhibiting protein translation (Calvo et al., 2009; Morris and Geballe, 2000). We investigated whether the exon 0Xs repress the translation via uORFs by mutating all the start codons, which possess in-frame stop codons, present in the exon 0K-0X2' construct. We chose this 5' UTR because the suppressive effect of this simple exon combination was consistently high in both cell-based and cell-free studies. More importantly, it was identified in the clinical specimens (Fig. 1C, upper panel) and was also present in both ER β 2 and 5 transcripts (Fig. 4D, middle and lower panel). The positions of uORF's start codon are shown in Fig. 6A. The start codons were mutated from AUG to UUG; the numbers indicate the position of adenine of the start codon. We determined the effect of different uORFs on the translational efficiency by luciferase assay and western blotting of *in vitro* translated luciferase. Luciferase expression of all mutants increased compared with that of the wild-type, although the mutations had different effects on translation (Fig. 6B). Most of the mutations in exon 0K and 0X2' increased translation by ~1.5- to 3-fold (Fig. 6B). Again, *in vitro* translation confirmed that luciferase expression of the mutants was generally higher than that of the wild-type (Fig. 6C), although the extent differed. Our data suggest that uORF is one of the key features in exon 0K and 0Xs responsible for the inhibition of ER β translation.

4. Discussion

Our laboratory previously showed that the temporal expression patterns of ER β 1 and its isoforms during PCA progression differ (Leav et al., 2001; Leung et al., 2010). ER β 1 is strongly expressed in the normal prostate epithelium but is lost in high-grade cancer. In contrast, both ER β 2 and 5 continue to be expressed in high-grade cancer foci (Leung et al., 2010). Moreover, these ER β isoforms are differentially localized among various cell types in the normal prostate (Leav et al., 2001; Leung et al., 2010). We thus have hypothesized that ER β 1 and its isoforms differentially utilize different promoters for their regulation in the prostate, which is supported by our findings.

Multiple putative promoters (0N, 0K, and E1) have been reported in the literature (Hirata et al., 2001; Smith et al., 2010b). To the best of our knowledge, the promoters used in the transcription of ER β 1 and its isoforms in the prostate have not been identified with certainty. In this study, using an unbiased 5' RACE analysis, we showed that only promoter 0K and 0N are used for the transcription of all ER β transcripts. Previous cell-based studies suggested that promoter 0N is the dominant promoter for ER β transcription (Smith et al., 2010a; Zhang et al., 2007). Using quantitative PCR and *in situ* hybridization, we found that transcripts from promoter 0K and 0N are expressed at comparable levels in normal and cancerous epithelial cell lines and in tissue specimens. The data contrast with previous findings demonstrating that exon 0N is found more consistently than 0K in multiple normal and malignant tissues (Smith et al., 2010a). Collectively, these data indicate that both promoter 0K and 0N are regulatory promoters of ER β expression in the prostate.

Our understanding of ER β functions has broadened since the discovery of ER β isoforms (Leung et al., 2010; Leung et al., 2006; Moore et al., 1998), yet little is known about how these isoforms are regulated in the prostate. We used long-range nested PCR to determine the promoter usage by different isoforms and found that ER β 1 and 2 were transcribed from both promoters, 0K and 0N, in PC-3 cells whereas ER β 5 was transcribed only from promoter 0K in PC-3 and BPH-1 cells. Of interest is that in benign and cancerous mammary epithelial cells these isoforms are transcribed from both promoters (Smith et al., 2010a). Alternative promoter usage is therefore a mechanism for regulating ER β -isoform transcription and thus explains, in part, the discordance between the expression patterns of ER β 1 and its isoforms during the progression of PCa. With the knowledge that ER β 1 is anti-proliferative whereas 2 and 5 are potentially pro-metastatic, future investigation may focus on factors determining promoter usage, such as *cis*-regulatory elements and *trans-acting* factors.

Discrepancies between abundance of mRNA and the actual expression of protein point to regulatory mechanisms at work aside from transcriptional control (Foley et al., 2000; Greenbaum et al., 2003; Vogel and Marcotte, 2012). Our current study found that ER β expression is under post-transcriptional control that uses a mechanism involving multiple 5' untranslated exons. In the 5' RACE analysis, we identified, in addition to the exon 0K and 0N, non-coding sequences between exon 0K and exon 1 only in transcripts from promoter 0K. Some of these sequences match exon 0Xs (0X1 to 0X5) first described in the human testis (Hirata et al., 2001). Moreover, we found three additional sequences, which we named exon 0X6, 0X7, and 0X8, located 3' of exon 0X5. These transcripts from promoter 0K contain highly variable combinations of exon 0Xs. The expression of exon 0Xs may be restricted to the male reproductive tract, as they have not been found in many other tissues examined (Hirata et al., 2001; Smith et al., 2010a). In this regard, the addition of 0Xs, in various combinations, to the ER β transcripts from promoter 0K in the prostate would change the sequence context of 5' UTR and may be the mechanism influencing ER β translation.

Two commonly reported mechanisms of post-transcriptional regulation are aberrant secondary and tertiary mRNA structure 1) affecting transcript stability and 2) affecting the availability and residence time of ribosomes to protein-coding ORF due to uORFs and IRES (Gilbert, 2010; Morris and Geballe, 2000). Prediction analysis of secondary mRNA structure suggested that the stability of transcript initiated from promoter 0N is higher than that initiated from promoter 0K, implying that inhibition of protein translation by exon 0N may be stronger than that by exon 0K. This prediction did not support our results from the luciferase assay and western blot analysis. Thus, we postulate the stability of mRNA structure may not be the most important feature in the post-transcriptional regulation exerted by untranslated exons.

We performed bioinformatic analyses and identified different numbers of uORF in the transcripts with various combinations of 0Xs, but only one IRES was predicted in the exon 0X8. Because none of the transcripts from clinical specimens contained exon 0X8, we chose to focus on the relationship between uORFs and protein translation. We made artificial constructs according to the sequences identified from clinical specimens to carry different combinations of exon 0Xs with varying numbers of uORF. Both luciferase assay and

western blot analysis showed an inverse correlation between ER β translational efficiency and the number of uORFs in constructs; thus, the construct with exon 0K, 0X1, 0X2', and 0X4' (9 uORFs) was found to have the lowest translational efficiency. The direct involvement of uORFs was further illustrated by site-directed mutagenesis experiments. Abolishment of the start codon in uORFs by mutation restored translation efficiency in all constructs. In the cell-free translation assay, the restoration of translational efficiency was consistent among different constructs; however, in the cell-based assay, we observed more variations. One possible explanation is the presence of cellular factors such as eIF4E and miRNAs, which have been reported to affect the inhibitory action of uORFs in protein translation (Farazi et al., 2011; Lee and Gorospe, 2011; Smith et al., 2010a). In summary, these data support an inhibitory role of uORFs in the various exon 0Xs in ER β protein translation, a finding that has not been reported previously. Therefore, uORFs in exon 0Xs (0X-uORFs) could be an important mechanism in post-transcriptional regulation of ER β .

The long-range nested PCR study showed exon 0Xs only in ER β 2 and 5 transcripts — not in ER β 1 transcripts. We anticipate that 0X-uORFs can suppress protein expression of ER β 2 and 5 but not that of ER β 1. Our previous study showed that ER β 2 and 5 proteins are expressed in both normal tissues and high-grade cancer (Leung et al., 2010). However, ER β 2 can be transcribed from both promoters 0K and 0N but that ER β 5 is transcribed only from promoter 0K, and yet the activities of these promoters declined in high-grade cancer as compared with that in benign tissues as suggested by our *in situ* hybridization data. In addition, exon 0Xs were detected more frequently in cancerous tissues than in normal tissues. Therefore, the persistence of expression of ER β 2 and 5 in cancerous tissues can be explained in part by the mode of action 0X-uORFs. In contrast, ER β 1 does not have exon 0Xs and will not be under the influence of 0X-uORFs. In this regard, transcriptional control may be the key mechanism of ER β 1 regulation. Apropos to this suggestion is the finding that the loss of ER β 1 expression is highly correlative with the hypermethylation of promoter 0N during PCa progression. On the basis of our current and previous findings, we propose that the expression of ER β 1 is primarily under transcriptional and epigenetic regulation, whereas the expression of ER β 2 and 5 are controlled by complex interplay between transcriptional and post-transcriptional regulation (Fig. 7). In this model, alternative use of promoter is the first level of regulation. If transcription utilizes promoter 0K, the ultimate level of protein expression is subjected to 0X-uORFs modulation. However, promoter 0N is used primarily for transcriptional control that is influenced by DNA methylation.

In conclusion, the expression of ER β 1 and that of its isoforms are differentially regulated during PCa progression. Whereas the expression of ER β 1 is controlled predominantly by transcriptional regulation, expression of ER β 2 and 5 is tightly regulated at both transcriptional and post-transcriptional levels. Further research will focus on the identification of the factors determining the promoter usage of different ER β isoforms. Moreover, the mechanism incorporating exon 0Xs into transcripts of ER β isoforms will be dissected. The molecular events that lead to sustainable expression of ER β 2 and ER β 5 in high-grade PCa will also be investigated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Promoter 0K and 0N are active in the ER β transcription in the prostate.
- Various combinations of exon 0Xs are associated only with promoter 0K of ER β .
- ER β 1, 2 and 5 differentially utilize promoter 0K and 0N for transcription.
- Exon 0Xs are present in 5' untranslated regions of ER β 2 and 5 but not ER β 1.
- Exon 0Xs possess upstream open reading frames to reduce protein expression.

Fig. 1.

ER β transcripts possess 5' untranslated regions (5' UTRs) of different sizes in prostate cell line and clinical specimens. (A) Schematic diagram shows the location of 5' RACE primer sets and 5' UTRs of ER β . (B) A representative result of 5' RACE using PC-3 cDNA reveals 5' UTRs of different sizes are present. The 5' end of ER β transcripts were amplified using primers specific to the oligo adapters and exon 1 of ER β as shown in panel A. Several nested PCR reactions with different conditions were performed to enhance the specificity of amplifications. The gel photo shown is an example of a single reaction. Arrows indicate the 5' RACE products of different sizes. (C) Promoter OK- or ON-initiated ER β transcripts were detected in 14 matched pairs of prostate cancerous (T) and adjacent normal (N) tissues. PCR reactions were performed by primers specific to exon OK/ON and exon 1 of ER β . PCR fragments less than predicted size of 300 bp were considered to be non-specific products.

Fig. 2.

Schematic diagram summarizes the location of exon 0N, 0K and different combinations of exon 0K and 0Xs in the 5' UTRs of ER β in the prostate. (A) Two transcription start sites of promoter 0K and 0N are present in exon 0K and 0N respectively. Amplicons from the 5' RACE experiment (Fig. 1B), together with PCR products amplified from the regions between exon 0K/0N and exon 1 in clinical specimens (Fig. 1C), were subjected to DNA sequencing. Multiple untranslated exons, called exon 0Xs, were located uniquely between exon 0K and exon 1. Three new exon 0Xs (0X6, 0X7, 0X8) were discovered in this study. Various combinations of exon 0K and 0Xs are shown by the lines connecting exons in the diagram. Incomplete exon 0Xs were formed by alternative splicing within exons and are indicated by lines connecting the middle part of exons. (B) A table shows the length of each untranslated exon and the intronic distance between different untranslated exons.

Fig. 3.

Promoter OK- or ON-initiated ER7 β transcripts are expressed in normal and cancerous tissues. Both transcripts were expressed in benign prostate, prostatic intraepithelial neoplasia (PIN), Gleason grade 3 and 5 prostate cancer in *in situ* RNA-RNA hybridization experiments. Digoxigenin (DIG)-labeled exon OK- or ON-specific oligonucleotide was used to detect the expression of respective transcripts. Positive signals are shown in purple; the nuclei were counterstained with methyl green. Right panels represent the magnified view ($\times 630$) of the corresponding boxed region from the left panels ($\times 100$) for either exon OK or ON-specific probe. Each panel shows the representative picture of six patient samples. The probe with scrambled sequence was used as negative control and no positive signal was shown in different magnifications ($\times 100$ or $\times 320$).

Fig. 4. Promoter 0K and 0N differentially regulate the transcription of different ERβ isoforms. (A) Relative expression of exon 0K, 0N, and ERβ1, 2, and 5 was determined in different prostate cell lines. cDNAs of PrEC, NPrEC, RWPE-1, BPH-1, DU-145, PC-3, LNCaP, C4-2 were used in quantitative RT-PCR. Three consecutive passages of cell lines were used. The relative expression of different targets was normalized to their expression levels in PrEC. Experiments were carried out in technical triplicates, and results were the average of two independent experiments. Data are represented as mean $\pm$ SD. (B) Schematic diagram shows the promoter 0K/0N and ERβ-isoform-specific nested PCR amplification. Exon 0K- and 0N-specific forward primers and isoform-specific reverse primers were designed for nested PCR amplification. (C) Nested PCRs of promoter 0K/0N-initiated ERβ-isoform transcripts reveal the differential usage of promoters in the transcription of ERβ isoforms. The amplicons marked with asterisks (*) contained transcripts of ERβ2 or 5 as indicated. Other bands were products of non-specific amplification. (D) Schematic diagram shows the presence of exon 0K, 0N and 0Xs in the transcripts of ERβ1, 2, and 5. After nested PCR analyses stated in

panel D, amplicons of different sizes were sequenced. ER β 1, 2, and 5 transcripts which were identified in the current study are displayed diagrammatically.

Fig. 5.

The 5' UTRs of ER β inhibit the translational efficiency to different extents. (A) A table shows the sequence analysis of exon ON, OK, and various combinations of exon OK and OXs. The combinations of exon OK and OXs were isolated in the clinical specimens in Fig. 1C. The number of start codons, stop codons, and upstream open reading frame (uORF) in different 5' UTRs is shown. Strong ATG contains the flanking sequence similar to the Kozak consensus sequence as described in the result section. uORF is composed of in-frame start and stop codons. ΔG is the free energy change during mRNA folding. (B) 5' UTRs of ER β inhibited the translational efficiency of luciferase in different cell lines. pGL3-Promoter plasmids containing different 5' UTRs were transfected into HEK293 (upper panel) or PC-3 (lower panel) cells together with pCMV- β -gal. The translational efficiency was defined as the relative luciferase activity per unit of mRNA. Results are the average of three independent experiments. Data are represented as mean $\pm$ SD. The statistical significance of the difference in translational efficiency between exon OK and combinations of exon OK and OXs is shown as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. (C) 5' UTRs of ER β inhibit the translational efficiency of luciferase *in vitro*. Plasmids containing T7 promoter were translated *in vitro* and the lysates were subjected to western blotting. Band intensities were measured and normalized to the value of control vector. Two independent experiments were performed. Results are the average of three measurements in a representative experiment. Data are presented as mean $\pm$ SD.

Fig. 6. uORFs in exon 0K and 0Xs are responsible for inhibition of translational efficiency. (A) Schematic diagram shows the position of uORFs' start codons in the construct of exon 0K-0X2'. The start codons were mutated to non-functional codon UUG. Seven constructs with a single mutation and one construct with double mutation (134, 138th) were made. (B) uORFs on the exon 0K-0X2' differentially inhibited the translational efficiency of luciferase in cells. Eight mutants mentioned in panel A and the wild-type of exon 0K-0X2' (wt) on pGL3-Promoter plasmids were transfected into HEK293 cells with pCMV- β -gal. The translational efficiency was determined similar to that in Fig. 5B. Results are the average of three independent experiments. Data are presented as mean $\pm$ SD. The statistical significance of the difference in translational efficiency between exon 0K-0X2' wt and each mutant is shown as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. (C) uORFs on the exon 0K-0X2' differentially inhibit the translational efficiency of luciferase *in vitro*. Exon 0K-0X2' wt and the eight mutants on pGL3-Promoter plasmids possessing T7 promoter were translated *in vitro*. The lysates were subjected to western blotting. Band intensities were measured and normalized to the value of exon 0K-0X2' wt. Two independent experiments were performed. The results are the average of three measurements in a representative experiment. Data are presented as mean $\pm$ SD.

Fig. 7.

Schematic diagram shows the transcriptional and post-transcriptional regulation of ERβ-isoform expression lead to the different outcomes of prostate cancer progression. The alternative use of promoter 0K or 0N acts as the first step of controlling the amount of ERβ transcripts. In normal cells, promoter 0N contributes to the high level of ERβ1 to maintain the regular cell proliferation, whereas it is methylated in cancer cells causing the loss of ERβ1 expression. On the other hand, promoter 0K transcribes ERβ2 and ERβ5 in both normal and cancer cells. Promoter 0K-initiated ERβ2 and 5 transcripts are more likely to be incorporated with exon 0Xs in normal cells than in cancer cells. Translation of the transcripts composed of exon 0K and 0Xs is significantly inhibited due to the presence of uORFs. This is the second step of regulating the protein expression of ERβ2 and 5. In cancer cells, lower proportion of ERβ2 and 5 transcripts consists of exon 0Xs. Their expressions are higher than those in normal cells, resulting in promoting PCa progression, increasing cell invasiveness and metastasis.

Table 1

Primers used in different experiments

Primers for	Sequences
GeneRacer 5' RACE	
5' RACE	CAGGTAAGGTGTGTTCTAGCGATCTTG
5' RACE nested primer	GAAAGGTGCCCAGGTGTGG
Amplification of 0K, 0N in clinical samples	
ER β -0K-F	CTGAATCTGACGCTCAGCAG
ER β -0K-R	GTCTTGAGATAACAGCTGAGAAAACA
ER β -0N-F	AGCCTGAGCTGCAGGAGGTG
ER β -0N-R	AGCCGTGCTCCAGGGGTAA
In situ hybridization RNA-RNA	
0K antisense+T7 RNA polymerase	ACAGTCCGAAGGGTCCGTTAGCGGGTAGAATAAGGACCCTATAGTGAGTCGTATTACTG
0N antisense+T7 RNA polymerase	GGACGAGAAGCGGGACGTTCAAAGTTCTCCGTCAACCCTATAGTGAGTCGTATTACTG
Scrambled	GTGTAACACGTCTATACGCCCA
Quantitative RT-PCR	
ER β 1-RT-F	TGGCTAACCTCCTGATGCTC
ER β 1-RT-R	TCCAGCAGCAGGTCATACAC
ER β 2-RT-F	AGGCATGCGAGGGCAGAA
ER β 2-RT-R	GGCCACCGAGTTGATTAGAGG
ER β 5-RT-F	CGGAAGCTGGCTCACTTGCT
ER β 5-RT-R	CTTACCCTCCGTGGAGCAC
Luciferase-1-F	ATCCATCTTGCTCCAACACC
Luciferase-1-R	TTTTCCGTCATCGTCTTTCC
GAPDH-RT-F	GAAGGTGAAGGTCGGAGTCA
GAPDH-RT-R	GACAAGCTTCCCCTTCTCAG

Table 2

Primers used in different experiments

Primers for	Sequences
1st- and 2nd-round amplification of sequence between ERβ isoforms	
0K-1st-F	CGTCCACACTGGAGAAGGA
0K-2nd-F	TGGCCCCTTGAGTTACTGAG
ER β 1-0K-R	TCAGCTTGTGACCTCTGTGG
ER β 2-0K-R	CTTTGCCTTCCAGGTTCAAG
ER β 5-0K-R	TTGCAGACACTTTTCCCAA
0N-1st-F	GGCTTTTGGACACCCACT
0N-2nd-F	CCCCTAATGCGGGAAAAG
ER β 1-0N-R	GGGACCACATTTTGCACCT
ER β 2-0N-R	TGATCCCAGAGGGAAATTGA
ER β 5-0N-R	CATTCCAAATGAGGCATTCA
Mutagenesis of uORFs start codon on 0K0X2' (only shows the sense primer)	
96th	GCGGCAGCTGGGTTGCTGGAGAGGA
134th	TTGAGTTACTGAGTCCGTTGAATGTGCTTGCTCTG
138th	TGAGTTACTGAGTCCGATGATTGTGCTTGCTCTGC
134138th	TTGAGTTACTGAGTCCGTTGATTGTGCTTGCTCTGCTGG
188th	CTCAGGTTACAGTCATCCAATTTGGTTCTGAAGACATC
216th	GGTTCTGAAGACATCCAAGTGGAGATTGGCATTAAATTC
231st	GGAGATATGGCATTAAATTCCTTGAGATTGGATGAGATCCAC
241st	CATTAAATTCATGAGATTGGTTGAGATCCCACCAAAGGAACA