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

Ming-Tsung Lee [a], Bin Ouyang [a], Shuk-Mei Ho [a,b,c,d,*], Yuet-Kin Leung [a,b,c,*]

[a] Division of Environmental Genetics and Molecular Toxicology, Department of Environmental Health, Cincinnati, OH, United States
[b] Center for Environmental Genetics, Cincinnati, OH, United States
[c] Cancer Institute, University of Cincinnati College of Medicine, Cincinnati, OH, United States
[d] Cincinnati Veteran Affairs Medical Center, Cincinnati, OH, United States

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ABSTRACT

Estrogen receptor β (ERβ) 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 ERβ 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, ERβ1, 2, and 5 are transcriptionally linked to promoter 0K; exon 0Xs are spliced only into ERβ2 and ERβ5 transcripts, suggesting that their expressions are regulated post-transcriptionally by exon 0Xs. This study reveals that expression of ERβ1 is regulated primarily at the transcriptional level, whereas that of ERβ2 and ERβ5 is controlled by the interplay between transcriptional and post-transcriptional regulation.

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, ERα is not expressed in the epithelial cells of the prostate where carcinogenesis takes place (Latil et al., 2001; Pasquali et al., 2001), whereas ERβ (referred to as ERβ1) 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, ERβ1 expression is gradually lost at the primary site (Zhu et al., 2004; 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). ERβ1 also represses epithelial-mesenchymal transition (EMT) (Mak et al., 2010), an initial step in metastasis. ERβ2 and ERβ5, in contrast to ERβ1, are found consistently in high-grade PCa and may be involved in promoting progression and invasion (Leung et al., 2010). Thus, ERβ1 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 ERβ 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 ERβ1 is transcribed predominantly from promoter 0N (Zhang et al., 2007; Zhu et al., 2004); however, the contribution

* Corresponding authors. Address: Room 130, Kettering Laboratory Complex, 3223 Eden Avenue, Cincinnati, OH 45267, United States. Tel.: +1 (513) 558 5701; fax: +1 (513) 558 5155 (S.-M. Ho). Address: Room 331, Kettering Laboratory Complex, 3223 Eden Avenue, Cincinnati, OH 45267, United States. Tel.: +1 (513) 558 5181; fax: +1 (513) 558 5155 (Y.-K. Leung).
E-mail addresses: shuk-mei.ho@uc.edu (S.-M. Ho), ricky.leung@uc.edu (Y.-K. Leung).

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from promoter 0K is unclear. Furthermore, the promoter dependency of other ERβ isoforms in the prostate is currently unknown.

Differential expression of ERβ1 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 ERβ1 and its isoforms are differentially regulated in the normal and malignant prostatic specimens. Our data show that ERβ1 and 2 are transcribed from both promoter 0N and 0K, whereas ERβ5 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 ERβs. In summary, our findings suggest that differential protein expression of ERβ 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% CO_2.

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 ERβ 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.

Table 1
Primers used in different experiments.

Primers for	Sequences
GeneRacer 5′ RACE	
5′ RACE primer	CAGGTAAGGTGTGTTCTAGCGATCTTG
5′ RACE nested primer	GAAAGGTGCCCAGGTGTTGG
Amplification of 0K, 0N in clinical samples	
ERβ-0K-F	CTGAATCTGACGCTCAGCAG
ERβ-0K-R	GTCTTGAGATAACAGCTGAGAAAACA
ERβ-0N-F	AGCCTGAGCTGCAGGAGGTG
ERβ-0N-R	AGCCGTGCTCCAGGGGTAA
In situ RNA-RNA hybridization	
0K antisense + T7 RNA polymerase	ACAGTCCGAAGGGTCCGTTAGCGGGTAGAATAAGGA CCCTATAGTGAGTCGTATTACTG
0N antisense + T7 RNA polymerase	GGACGAGAAGCGGGACGTTCAAAGTTCTCCGTCAA CCCTATAGTGAGTCGTATTACTG
Scrambled	GTGTAACACGTCTATACGCCCA
Quantitative RT-PCR	
ERβ1-RT-F	TGGCTAACCTCCTGATGCTC
ERβ1-RT-R	TCCAGCAGCAGGTCATACAC
ERβ2-RT-F	AGGCATGCGAGGGCAGAA
ERβ2-RT-R	GGCCACCGAGTTGATTAGAGG
ERβ5-RT-F	CGGAAGCTGGCTCACTTGCT
ERβ5-RT-R	CTTCACCCTCCGTGGAGCAC
Luciferase-1-F	ATCCATCTTGCTCCAACACC
Luciferase-1-R	TTTTCCGTCATCGTCTTTCC
GAPDH-RT-F	GAAGGTGAAGGTCGGAGTCA
GAPDH-RT-R	GACAAGCTTCCCGTTCTCAG

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 200 M 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 ERβ 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).