Differential Expression in Estrogen Receptor alpha (ESRα) and beta (ESRβ) in breast (MCF-7) and prostate (PC3) cell lines

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Abstract

The importance of estrogen in both men and women for cell growth and development is widely documented. Estrogen initiates alteration of cell behavior within target tissues by binding to Estrogen Receptor (ESR). The two documented estrogen receptor isoforms, alpha (ESRα) and beta (ESRβ) subsequently trigger gene activation. Because ESR's mediate all estrogen effects, they are important in oncology and specifically prostate and breast cancer. This study aimed to identify expression level differences between ESRα and ESRβ in breast (MCF-7) and prostate (PC-3) cell lines. RT-PCR was used to amplify the ESR's from purified mRNA. The PCR product was fractionated on a 1% agarose gel, purified, sequenced and aligned with the ESR mRNA sequences from the GenBank database. ESRα was expressed in the breast (MCF-7) but not in prostate (PC-3) cell lines, whereas, ESRβ was expressed in both cell lines. Amplification of ESRβ yielded more PCR product in PC-3 compared to MCF-7 in 4/5 replicates (80%). The growing literature pertaining to ESR expression in specific tissues can aid in the development of new selective estrogen receptor modulators (SERMs) for disease treatment. This experiment adds to the documented literature and provides additional replication of results found in previous studies.

Introduction

Estrogen is important for cell differentiation, growth and development in both men and women. A non-exhaustive list of specific roles includes the following: differentiation and development of reproductive tissues including mammary glands in women and testis, epididymus, and prostate in men; protection against osteoporosis via maintenance of bone density; reduction of lipid and cholesterol levels in blood acting as a cardioprotective hormone; regulation of reproductive behavior, homeostasis and general mood (Couse et al. 1997, Kurebayashi et al. 2000, Osborne et al. 2000, Signoretti and Loda 2001, Matthews and Gustafsson 2003, *Public communication*: http://newscenter.cancer.gov/sciencebehind/,accessed 5/3/06).

In addition to its normal physiological role, the prevalence and progression of cancerous cells within the body has been linked to prolonged stimulation by higher than normal levels of estrogen (Osborne et al. 2000). In fact, anti-estrogens are used in the treatment of certain breast cancers and some prostate cancers.

Estrogens and estrogen-like molecules act on target tissues and alter cell activity and behavior by binding to estrogen receptors (ESR's). The estrogen receptor is a ligand-activated transcription factor that belongs to the nuclear receptor superfamily and mediates all biological activity of estrogen in target tissues (Matthews and Gustafsson 2003). Currently, two ESR's, alpha (ESRα) and beta (ESRβ), are documented in the literature. A thorough description of the promotor region and exons found within ESRα is provided by Kos et al. (2001). Since the cloning of ESRα in 1986 (Green et al. 1986a, Green et al. 1986b), it was believed for over a decade that ESRα was the only ESR. Then in 1996, ESRβ was first cloned from rat prostate by Kuiper et al. (1996) and then from humans by Mosselman et al. (1996). The full-length human ESRβ was isolated by Ogawa et al. (1998). Interesting to note, a novel isoform called ESR gamma may have been discovered another decade later in 2006,

although the work is not yet published *(Personal Communication*; Brian Fox, Fordham University).

ESRβ is smaller than ESRα but both possess distinct functional domains, termed A through F, characteristic of the nuclear receptor superfamily (Couse et al. 1997). Specifically, ESRβ is homologous to ESRα in the DNA and ligand binding domains (Mosselman et al. 1996). ESRβ also binds estradiol, a prevalent form of estrogen, with similar affinity as ESR α (Kurebayashi et al. 2000).

Despite sharing high sequence homology, ESRα and ESRβ differ in many facets including tissue location, cellular function and expression level. (Krege et al. 1998, Matthews and Gustafsson 2003). Furthermore, each has several isoform variants possibly due to exon splicing, exon duplication and/or multiple start codons. These differences will be briefly discussed below with an emphasis on breast and prostate cells.

ESRα is expressed primarily in the uterus, liver, kidney and heart, whereas, ESRβ is expressed primarily in the ovary, prostate, lung, bladder and central nervous system. Both are co-expressed in a number of tissues including but not limited to mammary glands, thyroid and parts of the brain (Matthews and Gustaffson 2003).

Overall, ESR expression usually varies among tissues. Adding to the complexity, even if both ESR's are expressed in the same tissues, they may not be expressed in the same cells (Matthews and Gustafsson 2003). For example, expression of ESRβ in epithelial normal prostate cells is reproducible; however, ESRβ can exhibit both high expression (Lau et al. 2000, Horvath et al. 2001) and decreased expression (Latil et al. 2001) in prostate cancer epithelial cells. Further, Saji et al. (2000) determined that although both ESR's are expressed in rat mammary glands, their presence and cellular distribution is unique.

Adding to the complexity, both ESR's can posses multiple variant forms as well. For example, there is a 46

kDa isoform of ESRα that lacks the N-terminal 173 amino acids found in the normal 66 kDa isoform (Flouriot et al. 2000). This isoform variant is documented in MCF-7 (Flouriot et al.2000). Several ESRβ isoform variants have also been identified. Fuqua et al. (1999) was the first to characterize ESRβ expression and variant ESRβ isoforms at the protein level in breast cancer cells. Even splice variants within the ESRβ family like ERβ1 and ERβ2 are known to vary in expression and functional roles (Saunders et al. 2002). Additionally, ESRα and ESRβ can form heterodimers when both are co-expressed, however, the biological roles of these heterodimers remains unknown.

With such variation, it is not surprising that the definitive roles of estrogen receptor in the initiation, suppression or progression of prostate and breast cancer have not yet been fully defined. This gap in understanding has direct implications for effectiveness of treatments. Selective estrogen receptor modulators, (SERMs), selectively stimulate or inhibit the estrogen receptors of different target tissues. Unfortunately, even the most common SERM, Tamoxifen, is not fully effective. In fact, Tamoxifen has many of the same antagonistic effects documented for estrogen. Information on development and future goals of SERM's is found in Osborne et al. (2000).

Obviously more research is needed on the role and expression of ESR to improve upon or to develop new drugs. There is a need to document expression level differences among tissues and within variant tissue cell lines from normal to cancerous. This study was designed to provide new information on ESR expression. Specifically, the aim of this study is to characterize expression level differences between ESRα and ESRβ in two cancer cell lines, MCF-7 (breast) and PC-3 (prostate).

Methods

Cell Lines

The MCF-7 breast cancer and PC-3 prostate cancer cell lines were grown at Fordham University under the following conditions: cultured in DMEM (Gibco) supplemented with 2 mM L-Glutamine (Gibco), 1 mM sodium pyruvate (Gibco), 5 ml/L PenStrep 50 units/ml penicillin, and 50 µg/ml streptomycin (Gibco), and 10% fetal bovine serum (Hyclone).

RNA Purification

RNA from MCF-7 cells and PC3 cells was purified by Brian Fox, Fordham University and Paul Bechtel, respectively. RNA was isolated from 10cm tissue culture dishes using TRI reagent (Molecular Research Center) as outlined in Fox and Kandpal (2004). The RNA was visualized by running on a 1% agarose gel with ethidium bromide and the presence of ribosomal RNA bands (18S and 28S), as detected using UV light, indicated that the RNA was not degraded (Figure 1). The RNA concentration was read in a spectrophotometer and then diluted to 20 ng/µl.
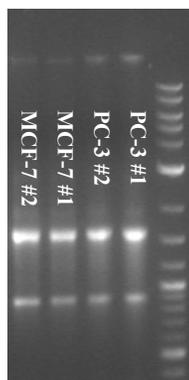

Figure 1. Purified RNA from MCF-7 and PC-3 cell lines.

Primer Design

Multiple intron-spanning primer sets were designed by hand or using the Primer 3 Output Program (http://www.genome.wi.mit.edu/cgi-bin/primer/primer 3.cgi) from published gene sequences within the NCBI GenBank database for each target gene. Specifically, *Homo sapiens* mRNA Accession #'s NM_000125 and NM_001437 were used, respectively. GAPDH was the housekeeping control gene and was amplified in parallel. The GAPDH primers were designed by Jinsong Qiu to amplify a 214 base pair segment. Primers were developed in different exons to avoid amplification of contaminating genomic DNA. Primer sequences for ESRα (Set A, Set B) and ESRβ (Set A, Set B) are given in Table 1. The primers were ordered from Invitrogen Inc.

Furthermore, primer sets were initially screened on HeLA cell lines using RT-PCR and 1% agarose gel fractionation. Primer sets were deemed successful if the presence of a single band at the expected size was visualized. Primer sets B for both alpha and beta were chosen as the main primer sets for use in all RT-PCR amplifications. Primer set B for ESRα amplified a 180 bp segment that spanned exon 5 and exon 6 with a 49,197 base intron between. Primer set B for ESRβ amplified a 201 bp segment that spanned exon 7 and exon 8 with a 14,405 base intron between. Primer set A for ESRα was used in conjunction with a DNA vector to confirm that the ESRα amplified was the normal rather than truncated form as described in Flouriot et al. (2000). The forward primer in this set is located in exon 1 and would be unable to anneal if the truncated form was present because exon 1 would be missing. Primer set A for ESRβ was used sporadically in an attempt to replicate an intriguing banding pattern that was obtained on 4/12/06 (Figure 2).
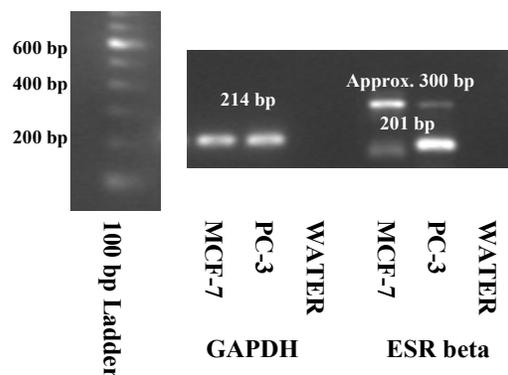

Figure 2: Multi-band expression of ESR beta in breast (MCF-7) and prostate (PC-3) cell lines. This result was replicated for PC-3 but not MCF-7 during this study. No sequencing was completed.

RT-PCR Setup

RT-PCR and subsequent agarose gel fractionation was completed on 4/12/06, 4/19/06, 4/24/06 and 4/27/06. RT-PCR was performed on the RNA's (20ng/µl) prepared from the MCF-7 and PC-3 cell lines using the QIAGEN OneStep™ RT-PCR kit. Specifically, the 15 µl RT-PCR samples contained 7.8 depc water, 3µl 5×RT buffer, 0.6µl enzyme mix 0.6µl 10mM dNTPs, 0.75µl 10pmol/µl forward primer, 0.75µl 10pmol/µl reverse primer and 1.5µl 20ng/µl RNA. Negative controls included substitution of RNA with 1.5µl depc water.

One-step RT-PCR for ESR alpha and ESR beta was carried out as follows: one cycle of 50 °C X 30 min and 95°C X 15 min, followed by 50 cycles of denaturation at 94 °C X 20s, annealing at 59 °C X 30s, extension at 72 °C X 30s and then a final extension of 72 °C X 2 min. The same RT-PCR protocol was carried out for GAPDH however the samples were removed from the thermal cycler after 25 cycles. PCR products were analyzed by gel electrophoresis through a 1% agarose gel at 139 volts and visualized by ethidium bromide staining under ultraviolet (UV) illumination.

PCR product Purification

PCR products were purified by using a Rapid PCR Purification System (Marligen BioSciences Inc., Ljamsville MD, Catalog No. 11458-023, Lot # 0786) with slight alterations to the manufacturer's protocol. Rather than eluting with 50µl of warm TE Buffer (TE), we used 35µl of warm depc water.

Sequencing

Both ESR alpha and ESR beta were first identified on a 1% agarose gel on 4/24/06. Any PCR products that produced a single band in the expected size range were then pooled together and sequenced on 4/26/06. DNA sequencing was performed using a modified version of the Sanger's dideoxy method and using a AmpliCycle™ Sequencing Kit (Perkin Elmer, Foster City CA, Part No. N808-0175) following the manufacture's protocol. Fifty fmoles of purified PCR products were mixed with 4µl of 10X cycling buffer, 0.5µl of ^{33}P-dATP, 13.5 µl depc water, 10µl of DNA and 2 µl of primer for a total volume of 30µl. 6µl from this master mix was then added to four tubes, each containing 2µl of either ddGTP, ddATP,

Table 1: Primer sequences used, location and expected size of amplicons

Gene	Primer Name	Sequence	Exon Location (nucleotide position)	Product Size
Estrogen Receptor alpha	**Set A** **ESR1AF** **ESR1AR**	5' ACCGTCCGCAGCTCAAGAT 3' 5' TCCTTGGCAGATTCCATAGC 3'	**Exon 1 (442-460)** **Exon 2 (5552-5571)**	**463 bp**
	Set B **ESR1BF** **ESR1BR**	5' GGATTTGACCCTCCATGATC 3' 5' AGCATGTCGAAGATCTCCAC 3'	**Exon 5 (1466-1485)** **Exon 6 (4811-4830)**	**180 bp**
Estrogen Receptor beta	**Set A** **ESR2AF** **ESR2AR**	5' CCGGTGTGTTTATCTGCAAG 3' 5' GTATGTATATGGAGCCGTGC 3'	**Exon 1 (309-328)** **Exon 2 (1496-1515)**	**207 bp**
	Set B **ESR2BF** **ESR2BR**	5' TGACATGCTCCTGGCAACTA 3' 5' CTCTTGGCAATCACCCAAAC 3'	**Exon 7 (1549-1568)** **Exon 8 (262-281)**	**201 bp**
(GAPDH) Glyceraldehyde-3 phosphate dehydrogenase: Primer set produced a 214 bp amplicon. Nucleotide location for the forward and reverse primers were (100-119) and (327-208), respectively.				

ddTTP, or ddCTP. Next, a drop of mineral oil was added to each tube in order to prevent evaporation during the subsequent sequencing reaction. The sequencing reactions were conducted for 35 cycles by denaturing at 94°C for 30 seconds, annealing at 58°C for 30 seconds, and elongating at 72°C for 1 minute.

Following the sequencing reactions, 4μl of stop solution was added to each tube and they were then heated to 94°C for 3 minutes to denature the products before running them on a sequencing gel (72ml Sequa Gel 6, 18 ml Sequa Gel Complete, 720μl 10% APS). The gel was then dried for two hours and exposed overnight to x-ray film. Sequence read from the film was compared to the NCBI-Genbank database using the Blast function and aligned via ClustalW in MacVector.

Results

PCR products and Primers:

Firstly, the RNA was not degraded (Figure 1). All four of the designed primers, listed in Table 1, produced PCR product. Primer set B for ESRα and ESRβ amplified the expected band at 180 and 201 bp, respectively (Figure 3). Primer Set A for ESRα produced a band of the expected 463 bp as shown in Figure 4. This primer set requires presence of exon 1 for amplification of ESRα, thus the regular 66 kDa isoform rather than the
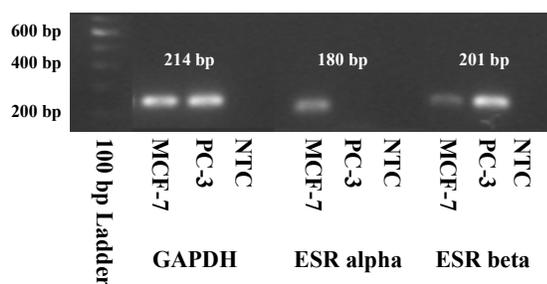

Figure 3: Expression of Estrogen Receptor alpha (ESRά) and beta (ESRβ) in breast (MCF-7) and prostate (PC-3) cell lines. GAPDH was run as a control and water as a negative control. The RT-PCR products were run on a 1% agarose gel.

46 kDa truncated isoform was present in the MCF-7 cell lines. Primer set A for ESRβ exhibited multiple banding (Figure 2).

ESR alpha was expressed in MCF-7 but not in PC-3, whereas, ESR beta was expressed in both MCF-7 and PC-3 (Figures 3-4). These results were observed 100% of the time after gel fractionation. See Figure 4. Further, ESR beta showed slightly more PCR product indicating more mRNA was present in 4/5 gel fractionations or 80%. GAPDH was consistently expressed throughout the study and the non-template control (NTC) was negative as expected.

The PCR products were aligned to the documented ESR mRNA sequences listed in GenBank (NCBI) for *Homo sapien* and exhibited near 100% homology.

Discussion

Expression level differences between the ESR subtypes are identified in the literature. Likewise, differences were observed in this study. ESRα was expressed in MCF-7 but not in PC-3, whereas, ESRβ was expressed in both MCF-7 and PC-3.
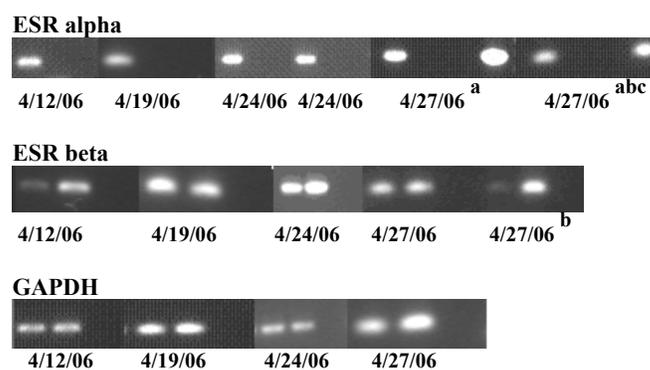

Figure 4: Replicated expression of ESR alpha and beta in MCF-7 and PC-3 cell lines. The order of the gel is as follows; MCF-7, PC-3, Water (non-template negative control) and [a] DNA vector (positive control, only for ESR alpha 4/27/06). [b] Amplified with Set A alpha, beta primers, respectively. [c] The size of the amplicon is 463 bp, whereas, all other ESR alpha amplicons are 180 bp.

Observing expression of ESRα in MCF-7 was not surprising. In fact, research like Speirs et al (2000) even used cDNA from MCF-7 cell lines as a positive control for ESRα.

The absence of ESRα expression in PC-3 both supports and contrasts previous research. Lau et al. (2000) documented ESRα presence in PC-3 but not in other prostate cell lines (DU145 or LNCaP) indicating variation among cell lines. Carruba et al. (1994) also found ESRα expression in prostate cancer cells, however, Hobisch et al. (1998) did not. Possible explanations exist for the conflicting results, mainly variable distribution within cells. ESRα was detected exclusively in normal

Formatted Alignments

A

Formatted Alignments

B

Figure 2: A) Formatted alignments of purified PCR product sequence (ESR1{alpha}PCR) versus *Homo sapien* ESR1, mRNA (Accession # NM_000125). B) Formatted alignments of sequenced purified PCR (ESR2{beta}PCR) versus *Homo sapien* ESR2, mRNA (Accession # NM_001437.1). PCR product was purified via the Rapid PCR Purification System (Marligen BioSciences Inc., Ljamsville MD), sequenced via the Sanger's dideoxy method AmpliCycle™ Sequencing Kit (Perkin Elmer, Foster City CA), compared to the NCBI-Genbank database and aligned via ClustalW in MacVector.

and malignant human prostate stromal but not epithilial cells (Couse et al. 1997, Speirs et al. 1999, Lau et al. 2000, Leav et al 2001). Perhaps, ESRα is lost during cancer progression or was not detected because this study did not use stromal cells.

ESR beta is expressed in both human breast tumors (Dotzlaw et al. 1997) and prostate cancer (Couse et al. 1997, Speirs et al. 1999, Lau et al. 2000, Leav et al 2001). This study parallels these findings.

Expression levels for ESR can change depending on several factors: normal versus cancerous cell lines, the type and severity of cancer within tissues and the interaction effects (inhibitory, neutral or synergistic) between the ESR subtypes.

The complexity further increases when the ESR's are dually studied. Hall and McDonnell (1999) determined that ESRβ functions as a trans-dominant inhibitor of ESRα transcriptional activity when estrogen is at normal levels and also decreases overall cellular sensitivity to estradiol. Leav et al. (2001) and Horvath et al. (2001) reported a down-regulation or progressive loss of ESRβ expression during prostatic carcinogenesis from hyperplasia to invasive cancer. Also, ESRβ was found to be predominant over ESRα in rat prostate (Kuiper et al 1996, Enmark et al. 1997) and significantly lower in prostate tumors than in normal prostate tissues (Latil et al. 2001).

Significant positive and negative correlations are documented between ESRα and ESRβ (Latil et al. 2001, Kurebayashi et al. 2000). Speirs et al. (1999) studied co-expression of ESRα and ESRβ on sixty breast tumor samples and 23 normal breast samples. Beta was expressed alone (without alpha and no co-expression) in 22% of the samples. Further, most breast tumor cells expressed ESRα alone or co-expressed with ESRβ, whereas, ESRβ was not observed alone in breast cancer. This highlights the variability and problematic nature of

elucidating definitive scenarios from tissue to tissue and person to person.

Deciphering the result of interaction between ESRα and ESRβ is crucial; however, the complexity of teasing this apart has confounded scientists to date. Alpha/beta heterodimers (Ogawa et al. 1998, Cowley et al. 1997, Pettersson et al. 1997), alternative splicing (Carruba et al. 1994, Speirs et al. 2000, Lau et al. 2000), truncated isoforms and multiple start codons (Cowley et al. 1997) are documented. Multiple variants of ESR beta (β1, β2, and β5) were observed in MCF-7 by Leygue et al. (1999) and may partly explain the multiple banding pattern in Figure 2. This multi-band pattern is intriguing and future research should revisit this result and properly sequence the bands. This may add a valuable addition to the literature on Estrogen Receptors.

The incomplete research on ESR and the inherent "unknowns" can explain the seemingly contradictory results abound within the literature. Couse et al. (1997) reported that normal adult female mammary glands exhibited only ESRα and not ESRβ, however, Speirs et al. (2000) detected both ESR's were present. Kurebayashi et al. (2000) identified a possible down-regulation of ESRα during the progression of breast cancer, whereas, Fuqua et al (1999) observed ESRα was the predominating ESR in cancerous cells.

Understanding and identifying differences in expression and ligand-binding specificity can provide the basis for development of novel Selective Estrogen Receptor Modulators (SERM's) (Barkhem et al, 1998). For example, Latil et al. (2001) suggest decreased ESRβ could influence cancer cell growth and may normally protect against abnormal growth when fully expressed. Also, ESRβ may mediate estrogen action and even suppress cell proliferation in prostate tissue (Bonkhoff et al. 1999). Estrogen and anti-estrogen are effective for

therapy in PC-3 cells because it is documented that both subtypes are present in within the cell (Lau et al. 2000).

ESR may even represent a potential bioindicator because the progression of normal to cancerous may be visualized via the expression shift of ESRα and ESRβ. Leygue et al. (1998) suggested that the observed alteration in ESRα:ESRβ ratio during carcinogenesis implies definitive roles for each estrogen receptor. Running normal cell lines in parallel to the cancerous cell lines would have helped identify any ESR ratio shifts.

In summary, ESRα was expressed in the breast cell line MCF-7; however, it was not expressed in the prostate cell line PC-3. ESR β was expressed in both the breast cell line MCF-7 and the prostate cell line PC-3. This study represents another part of the continuing investigations into the expression level differences of ESRα and ESRβ among various tissues of the body. Compiling reproducible results are a precursor to the development of an effective treatment. As of yet a complete guide describing which ESR subtypes are present in which tissues during which stage of cancer does not exist. The function and role of each variant isoform remains unsettled as well.

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