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Splicing Variants of Estrogen Receptor in Breast Cancer

Progress reported for year 3 of this award includes: 1) completion of a thorough analysis of ER-α mRNA variants in MCF-7 cells, 2) the analysis of ER-α variants in an invasive ductal carcinoma specimen characterized by a discordant ER status, 3) continued characterization of the functional properties of six major ER-α splicing variants, and 4) the generation and preliminary characterization of cell lines that stably express ERÆ5. Our findings reconfirm that the estrogen-responsive MCF-7 breast epithelial cell line expresses a heterogeneous array of ER-α mRNA transcripts, less than half of which would encode a full-length, transcriptionally functional receptor. The most abundant variant present in MCF-7 cells is ERÆ7. Occasional breast tumors can display an even more extreme pattern of variant ER-α mRNA expression. There is diminishing evidence to support the contention that ERÆ5 represents a constitutively active variant. Indeed, ERÆ5 is essentially devoid of DNA-binding activity and transient and stable expression experiments indicate that it has little if any positive transcriptional activity. Rather, like ERÆ3, the ERÆ5 variant can act as a dominant inhibitory receptor when it is co-expressed with wt ER-α. Future work will address the potential of ER-α splicing variants to modulate the expression of estrogen responsive genes via non-classical (DNA-independent) pathways.
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PI - Signature / Date
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INTRODUCTION

Title: Splicing Variants of the Estrogen Receptor in Breast Cancer
ID No.: DAMD-1794-J-4372
PI: Richard J. Miksicek

Summary of Administrative Matters

This USAMRDC Breast Cancer Research Award was made to Richard J. Miksicek, who currently holds an appointment as Assistant Professor of Physiology and Member of the Cancer Center at Michigan State University (MSU). At the time this grant was awarded, Dr. Miksicek had been on the faculty at the State University of New York (SUNY) at Stony Brook. This report summarizes the third year of this award, through 09/30/97. During the first year of this award, the P.I. (Dr. Miksicek) accepted a new position (effective 07/01/95) in the Department of Physiology at MSU. At that time, permission was requested from the US Army MRMC through the awardee institution (The Research Foundation of SUNY) to effectively change the site of performance of this project from SUNY @ Stony Brook to MSU by establishing a research subcontract between these institutions. This research subcontract stipulated that Dr. Sandra Haslam (Professor of Physiology and Director of the MSU Breast Cancer Program) be named as Principal Investigator for the MSU subgrant with Dr. Miksicek continuing to serve as P.I. on the primary award to SUNY @ Stony Brook. In addition, Dr. Miksicek acted as Principal Co-Investigator at MSU with primary responsibility for the conduct of research and grant administration. This subcontract was accepted by both institutions on 03/28/96 to become effective retroactively to 07/01/95. Predictably, progress on this project was substantially delayed between 07/01/95 and 03/28/96 pending negotiation of the research subcontract and release of research funds at MSU. During 1996, the investigator's laboratory was re-established at MSU and the following research staff were recruited to resume work on this project:

Key Personnel:

<table>
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<th>Name</th>
<th>Degree</th>
<th>Role</th>
<th>Effort</th>
<th>Period of Service</th>
</tr>
</thead>
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<tr>
<td>MIKSICEK, Richard</td>
<td>Ph.D.</td>
<td>PI &amp; Co-PI</td>
<td>25%</td>
<td>09/94-present</td>
</tr>
<tr>
<td>BOLLIG, Aliccia</td>
<td>B.S.</td>
<td>Graduate Research Asst.</td>
<td>100%</td>
<td>10/95-present</td>
</tr>
<tr>
<td>MORRISON, Mary</td>
<td>M.S.</td>
<td>Research Asst.</td>
<td>15%</td>
<td>02/96-03/98</td>
</tr>
<tr>
<td>ANKRAPP, David</td>
<td>Ph.D.</td>
<td>Postdoctoral Research Fellow</td>
<td>100%</td>
<td>07/96-present</td>
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Dr. Miksicek received partial summer salary from this award from 6/97-8/97. Ms. Bollig and Dr. Ankapp currently draw full salary support from this award. Ms. Morrison was supported primarily from a non-overlapping award (DAMD1794-J-4411), but she provided significant technical support to this project in the form of laboratory management, cell culture, media preparation, antibody preparation, DNA sequencing, and technical assistance. In addition, this award provided supplemental salary for a part-time minority work study student, John Hicks.
Scope of the Project

Recent work from this and other laboratories have shown that human breast tumors and tumor-derived cell lines contain a mixed population of estrogen receptor-α (ER-α) messenger RNAs (mRNAs), representing both correctly and aberrantly processed transcripts (1-5). The aberrant ER-α mRNAs are predicted to give rise to structurally altered receptor molecules that are likely to interfere with the normal estrogen response pathway present in breast tumor cells. These variants may provide a molecular explanation for the loss of estrogen responsiveness or the acquisition of resistance to tamoxifen and other estrogen antagonists (6), thereby reducing the effectiveness of hormonal adjuvant therapy of breast cancer. We received this award to undertake a detailed analysis of splicing variants of ER-α mRNA in selected human breast tumor specimens and to characterize the functional activity of these variants in a model cell culture system. Additionally, we have been developing techniques and reagents (both molecular and immunological) to enable further investigation of the clinical significance of these ER-α splicing variants.

The approach being used in this project involves amplification of complementary DNAs (cDNAs) using the polymerase chain reaction (PCR), followed by sequence analysis of the resulting clones. In parallel, plasmid vectors have been developed to enable expression and functional characterization of selected ER-α splicing variants in a cell transfection system. The original statement of work for this project included the following specific tasks:

Task 1, Identification of ER mRNA variants in breast tissue (months 1-24):

- Preparation of oligonucleotide primers for the analysis of ER mRNA splicing variants and optimization of PCR amplification conditions.
- PCR amplification of ER cDNAs prepared from tamoxifen-resistant tumors.
- PCR amplification of Progesterone Receptor cDNAs prepared from tamoxifen-resistant tumors.
- PCR amplification of ER cDNAs prepared from normal human breast tissue.

Task 2, Functional analysis of ER mRNA splicing variants (months 18-48):

- Construction of ER expression plasmids harboring variant ER cDNAs.
- Analysis of the transcriptional stimulatory activity or inhibitory activity of ER splicing variants by transient transfection.
- Production and characterization of cell lines that stably express the “constitutive” ERAE5 variant.
EXPERIMENTAL METHODS

Synthetic oligonucleotides.

An extensive library of synthetic oligodeoxynucleotides corresponding to portions of ER-α cDNA sequence have been assembled. These oligonucleotides were designed to mimic the correct and aberrant splice junctions of processed ER-α mRNAs, but also include numerous upper and lower strand oligonucleotides that are contained entirely within individual exons of the ER-α transcript. New oligonucleotides added since the previous report are listed in Appendix A. Included within this set are 15 additional splice junction oligonucleotides synthesized for use as hybridization probes in a colony hybridization assay (see below) and three upper strand primers specific for the unique 5'-untranslated regions of ER-α transcripts derived from three discrete promoters that have been reported to drive transcription of the human ER-α gene (8). In addition to oligonucleotides specific for ER-α sequences, several PCR primers have been obtained corresponding to the human estrogen receptor-β (ER-β) cDNA, the human progesterone receptor (PR) cDNA, and a universal anchor primer for 5'-RACE analysis (see Appendix A).

Cell lines and cell culture.

MCF-7 cells (obtained from ATCC at passage 145) were routinely cultured in phenol red-free DMEM containing 10% fetal bovine serum supplemented with 0.6 μg/ml insulin, 5 mM HEPES (pH 7.4), 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. MDA MB231 (obtained from ATCC at passage 27), HeLa, and Cos7 cells were cultured as above except that calf serum was substituted for fetal bovine serum and supplemental insulin was omitted. Experiments involving RNA extraction and PCR analysis utilized cells that were subcultured fewer than 25 passages from receipt.

PCR analysis of ER-α mRNA splicing variants and preparation of ER-α cDNA libraries.

Standard conditions were employed for PCR amplification of cDNA sequences using AmpliTaq DNA polymerase (GIBCO/BRL) and a Stratagene thermocycler. Reactions typically involved 35-40 cycles of amplification, beginning with 30 seconds of denaturation at 94°C, followed by a 60 second annealing step (56-66°C) and 60 to 300 seconds of elongation at 74°C, depending on the length of the interval to be amplified. Annealing temperatures were carefully selected for each primer pair and were held as stringent as possible to optimize for the specificity and efficiency of amplification. RNA extractions were performed using Trisolve reagent (GIBCO/BRL) according to the manufacturer's instructions. Single stranded cDNAs were synthesized using MLV reverse transcriptase from 10 μg aliquots of total RNA, priming variably with oligo(dT)15 or a lower strand ER-α specific primer. In general, amplification reactions began with the cDNA equivalent of 1 μg of total RNA and no consistent differences in PCR yield or product profile were noticed comparing cDNAs made with oligo(dT) or with an ER-specific RT primer. Products of PCR reactions were routinely cloned using Invitrogen's pCR2-TOP kit according to manufacturer's instructions. When the goal was to perform a clonal analysis of the products of key PCR experiments, representative cDNA libraries were established by picking individual bacterial transformants into 96 well microtitre plates containing LB medium, incubating overnight at 37°C, and freezing at -70°C after the addition of glycerol to 10%.

Colony hybridization and DNA sequence analysis

Colony hybridization assays were performed using a battery of [32P] 5'-end labeled oligonucleotide probes corresponding to splice junction sequences from correctly and aberrantly spliced ER-α mRNAs (see Appendix A, section 1). Libraries of ER-α cDNAs were replica plated
using a Boekel replicator from 96 well microtitre dishes onto 20 X 20 cm nitrocellulose filters overlain on LB/agar plates, grown overnight at 37° C, and the filters were processed as described by Grunstein and Hogness (7). Hybridization reactions were performed using ~10^7 cpm of each oligonucleotide probe in 10 ml of 6X SSC, 5X Denhardt’s solution at 48-56° C for 5-6 hours, followed by successive room temperature washes in 6X SSC. Filters were exposed for autoradiography overnight at -70° C.

In some cases, transformants selected for further analysis were picked into 96 well submaster dishes and processed for colony hybridization and subsequent storage as above. To confirm the assignment of clones containing representative splice variants and ambiguous mutants, selected clones were subcultured on LB/ampicillin plates. Restriction reactions were performed on minilysate DNAs prepared using a STET boiling miniprep method. For DNA sequencing, we utilized double stranded miniprep DNA and the Sequenase V.2 sequencing kit from US Biochemical.

**Generation and functional analysis of cell lines that stably express the ERAE5 variant.**

As reviewed by ourselves and others (4,5,9,10), RNAs expressed from the ER-α gene in mammary epithelial tissue typically represent a mixed population of wild type and variant mRNAs, with the variants often representing 50% or more of the total ER-α transcript pool (see below). A wide variety of variant ER-α mRNAs have been described in the literature, but those most commonly observed harbor a precise deletion of one of the internal exons that contribute to the structure of the mature ER-α protein. We refer to these variants as ERAE2 through ERAE7, where the number indicates a missing exon. In our previous annual report, we summarized the construction and initial functional characterization of a series of ER-α expression plasmids derived from pCMV4 (11) that contain mutant ER-α cDNAs corresponding to ERAE2, ERAE3, ERAE4, ERAE5, ERAE6, and ERAE7. Since then, selectable versions of wt ER-α , ERAE3, ERAE4, ERAE5 have also been generated by subcloning these variant cDNAs into pcDNA3 or pCR3.1 (Invitrogen Corp., San Diego, CA). These plasmids represent CMV-based mammalian expression vectors that, in addition to the CMV-ER cDNA expression unit, contain an SV40 promoter-NeoR cassette that permits the production of stable cell lines by selecting for resistance to the aminoglycoside antibiotic, G418 (Geneticin). Stable cell lines harboring these Neo-containing expression plasmids were selected for their ability to grow in the presence of 0.4-0.8 mg/ml G418, following their transfection into Cos7, MB231, or MCF7 cells by treatment with calcium-phosphate DNA co-precipitates or by electroporation. In accordance with the priorities set within Task 2C, attention was focused on the stable expression of the ERAE5 variant; however, other plasmids were included in these experiments for control purposes.

Levels of receptor expression within G418-resistant cells lines were analyzed in whole cell extracts by western blotting, using an ERα-specific monoclonal antibody (Mab-17) that was previously developed in this laboratory (12). The DNA-binding activity of stably expressed receptors was measured using a gel mobility shift assay, as previously described (12). In some cases, transcriptional activity of stably expressed receptor was assessed by transient transfection of G418-resistant cells lines with the pERE-TK-CAT reporter plasmid, followed by the determination of CAT enzymatic activity, as described below.

**Transient transfection assays for characterizing ER-α splicing variants.**

Transient transfection assays were performed as previously described (1,12). Briefly, plasmids expressing wild type or variant ER-α cDNAs were transfected into recipient cells along with an estrogen-responsive reporter plasmid (pERE-TK-CAT). We have utilized both electroporation and the calcium phosphate co-precipitation technique for these experiments.
Transcriptional activity of receptor variants in the presence or absence of hormone was assessed by measuring the activity of chloramphenicol acetyltransferase (CAT) produced in cultures of transfected cells. A variation on this transfection strategy was also used to assess the transcriptional inhibitory potential of ERΔE2 through ERΔE7 on co-expressed wt ER-α. In this case, triple co-transfections were performed using the pERE-TK-CAT reporter, a constant low amount of pCMV-ERα, and increasing amounts of the variant ER-α expression vector. Total input DNA was normalized using empty pCMV-4 vector. Transfected cells were cultured for 48 hours in DMEM containing 5% charcoal-treated calf serum, in the absence and presence of 5 nM estradiol. CAT enzymatic activities were measured as described above.
RESULTS

Clonal analysis of variant ER-α transcripts in MCF-7 cells.

As previously described (year 2 annual technical report), we had been attempting to develop a competitive three-primer PCR assay to simultaneously amplify sequences from splicing variants in the presence of wild type (wt) ER-α mRNA transcripts. In principle, such an assay can be used to quantify the relative levels of two closely related mRNA species (13). In the present case, this strategy involves the use of splice junction primer pairs that include two upper strand primers (an exon-skipped primer and its corresponding wt primer) coupled with a common lower strand primer. Early during the third year of this project, a pilot study was undertaken to use this competitive three-primer PCR approach (employing incorporation of [32P]-labeled dNTPs and phosphorimage analysis) to quantify exon-skipped, relative to correctly spliced transcripts. MCF-7 cells were chosen for this analysis since they represent an established paradigm for estrogen-responsive breast epithelial cells and they have been one of the most common systems to be included in previously published studies on ER variants. Two significant technical limitations were discovered using the three-primer approach, which demands unusually stringent PCR conditions to insure correct priming of variant primers on exon-skipped, but not wt transcripts, and vice versa. First, while the required specificity could be achieved (using primer annealing temperatures in excess of 60° C) for some splice junctions, an unacceptable level of “cross-priming” was always observed for other splice junctions. Second, it became evident that the extensive heterogeneity of ER-α variants typically observed in MCF-7 cells and other ER-α expressing tissues includes numerous atypical variants that are missed or incorrectly assigned using this quantitative three-primer approach. Additionally, it appeared that the yield of wt and variant transcripts measured in MCF-7 RNA using this three-primer approach in some cases failed to accurately reflect their true relative abundance as determined using RNase protectional analysis or other independent techniques (3,5). For this reason, we chose to change our approach and base our quantification of variant ER-α transcripts on a clonal analysis strategy. This simpler approach entails reverse transcription (RT) of MCF-7 RNA, followed by a single PCR step using broadly spaced PCR primers intended to amplify as complete a population of transcripts as possible. Products of this RT/PCR reaction are then “shot-gun” cloned into a plasmid vector to generate a statistically representative library of ER-α cDNA transcripts. A similar strategy has also been adopted by Watson and coworkers in their studies on ER-α variants (14).

To assess the full variety of ER-α transcripts present in MCF-7 RNA, we selected priming sites within the first and last protein-coding exons of the ER-α gene, exons I and VIII, respectively (Fig. 1a). This E1Uwt/E8Lwt primer pair spans an interval 1,300 nt and is capable of scoring for deletions anywhere between positions 669 (residue Pro146) and 1969 (residue Leu579) of the ER-α mRNA, numbered according to Green et al (15). An ethidium bromide-stained agarose gel showing the products of this PCR experiment is presented in Fig. 1B (lane 2) and shows that the bulk of ER-α cDNAs present in MCF-7 cells are full-length, or within several hundred bp of this size. However, abundant PCR products less than this size were readily apparent and included a major band of approximately 1,100 bp and a heterogeneous smear of lower molecular weight products. As expected, a control reaction lacking a cDNA template failed to yield any detectable PCR products (lane 3). An aliquot from this MCF-7 PCR amplification reaction was ligated into the pCR2-TOP vector, transformed into E. Coli, and plated on LB/ampicillin plates containing IPTG and xgal. Approximately 800 white (insert-containing) colonies were picked into 96-well microtitre dishes to enable replica plating for hybridization analysis and permanent storage as glycerol cultures. As borne out by our subsequent hybridization analysis and DNA sequencing, this library was very highly enriched for ER-α cDNA sequences.
Fig. 1. RT/PCR amplification and clonal analysis of ERα mRNAs present in MCF-7 cells: A) design of the amplification experiment showing the locations of PCR primers, B) EtBr-stained agarose gel showing the size distribution of PCR products, and C) quantitative summary of the ERα cDNA clones characterized from this experiment by DNA sequencing.

Our initial intention was to score clones from this cDNA library for the presence of normal or aberrant splice junctions by simply performing a series of hybridization reactions with short (18-20 nt) oligonucleotide probes straddling ER-α splice junctions that were likely to be encountered among the clones (i.e., wt splice junctions and aberrant junctions from exon-skipped transcripts). Results from a representative filter (corresponding to DA-8, one of 9 master plates) hybridized with three splice junction probes (E2/3wt, E4/5wt, E6/7wt) are given in Fig. 2, along with the retrospective assignment of ERα cDNA clones present on this plate, based on DNA sequence analysis. With only a few exceptions, assignments based solely on oligonucleotide hybridization were subsequently confirmed by DNA sequencing. For example, wt ER-α cDNAs (e.g., DA8-C10, DA8-F9, etc.) gave clear hybridization signals with all three wt junction primers, while variant ER-α cDNAs such as ERAE7 (e.g., clones DA8-A4, DA8-D4, etc.) failed selectively to hybridize to junction probes (e.g., E6/7wt) that are disrupted by their deletions. The most common exceptions to this pattern involved the unexpected loss of some hybridization signals where signals were expected (e.g., wt clone DA8-A5 with E6/7wt). Such a loss of signal was most likely due to poor growth or incomplete lysis of some of the bacterial colonies on the nitrocellulose filters used for replica plating. What is also evident from a cursory examination of Fig 2 is that only about one fourth of the lac+ bacterial colonies that were picked into these microtitre dishes for further analysis actually contained ER-α cDNAs based on their ability to hybridize with the ER-α oligonucleotide probes. The remaining colonies probably included clones with very short inserts, “primer dimer” artifacts, inserts unrelated to ER-α cDNA, or no inserts at all.
Colonel hybridization analysis on a representative plate of MCF-7 PCR clones from the experiment presented in Fig. 1, hybridized against the wt ERα splice junction probes E2/3 wt, E4/5 wt, and E6/7 wt. The positions of ERα clones confirmed by DNA sequencing are indicated in the upper left panel.

It was decided that a careful analysis of the approximately 200 ER-α clones present in this library would provide a sampling large enough to include even rare ER-α cDNA variants present in MCF-7 cells. To this end, clones that afforded a strong hybridization signal with an exon 8 oligonucleotide (E8Lwt) were streaked out, and used for the isolation of plasmid DNA. A series of three restriction digests (EcoRI + XbaI, EcoRI + Bgl II, and Hind III + Apa I) were performed on each of these subclones to identify wt ER-α cDNAs among the clones and to establish the approximate location of deletions or insertions present among any variant cDNAs. The precise nature of the mutation(s) present in any PCR subclone with a restriction map differing from that of the wt ER-α cDNA was established by direct DNA sequencing. In addition to sequencing through all of the deletion junctions in ER-α variants, several wt ER-α cDNAs were also sequenced in their entirety. From this analysis of the RT/PCR products shown in Fig. 1, 179 clones were confirmed to represent ER-α cDNAs. Among these clones, 82 clones (46%) represented wt ER-α cDNAs, 92 (51%) harbored deletions varying from 3 bp to 918 bp in size, and 5 clones (3%) contained insertions. This population of clones was found to be exceedingly heterogeneous and included, in addition to wt ER-α, 38 different types of ER-α cDNA variants.
Among the clones analyzed, the only variants that were observed to occur as multiple isolates corresponded to previously reported "exon-skipped" transcripts (Fig. 3). By far the most common variant was ERΔE7 (49 clones, 27%), but each of the variants ERΔE3, ERΔE4, and ERΔE5 were also represented by multiple isolates (Fig. 3A). As reported by others (5), ER-α variants harboring deletions of more than one exon were also observed (12 isolates in total) and these variants included deletions of both contiguous as well as non-contiguous exons (Fig. 3B). Notably, exon 7 was almost invariably missing from clones that contained multiple exon deletions, consistent with its being the most common exon deleted individually.

A significant fraction of the ERα cDNA clones (12%) isolated from MCF-7 cells represented atypical deletion mutants, differing from those described above in that the deletion endpoints do not correspond to known exon boundaries. These variants constitute a remarkably heterogeneous group and are depicted in Fig. 4.

**Fig. 3.** Summary of ERα cDNAs in MCF-7 cells showing the loss of single (panel A) or multiple (panel B) exons.

**Fig. 4.** Structures of 21 atypical ERα deletion variants isolated from MCF-7 cells.
Five of the ER-\(\alpha\) cDNAs isolated from MCF-7 cells were observed to contain inserts of short DNA sequences (Fig. 5). Interestingly, three of these clones (4F4, 6F1, and 2A2) contained an identical 39 bp sequence (5'-GTGACTCAGGGATTCTTCTTGGCTGATCCATTGCTG-3') that was inserted precisely between the boundaries of exons 3 and 4. The consequence of this mutation would be to add 13 novel amino acids (AspSerGlyIleSerSerTrpLeuGlySerIleAlaGly) between Gly\(^{254}\) and Ile\(^{255}\) within the hinge region of ER-\(\alpha\), without otherwise disrupting the reading frame of the receptor. The functional properties of this unusual variant are currently under investigation.

![Fig. 5](image)

**Fig. 5.** Structures of ER\(\alpha\) cDNAs from MCF-7 cells that harbor DNA insertions.

**Analysis of the DNA-binding region of ER-\(\alpha\) mRNAs in MCF-7 cells by RT/PCR.**

An inadvertent design flaw in the PCR experiment described above was that the upper strand primer (E1Uwt) chosen for PCR amplification of MCF-7 mRNAs overlapped the exon I / exon II boundary by two nucleotides, thereby biasing the reaction against cDNAs (such as ERAE2) that lack a correct exon II splice junction. Not surprisingly, ERAE2 variants were entirely missing from the pool of RT/PCR clones analyzed above (see Fig. 3). To ask whether or not the splice variant heterogeneity observed in MCF-7 cells above for the distal two thirds of the ER-\(\alpha\) transcript is also seen in the proximal third of the transcript, we completed two additional PCR experiments, summarized below. Fig. 6 shows that when PCR amplification is anchored within exons I and III (with primers E1U-new and E3Lwt) this reaction does in fact reveal the presence of the ERAE2 variant (clone 27.14), as well as wt ER-\(\alpha\) clones and other atypical variants. Notably, two of these variants (clones 27.3 & 27.28) appear to utilize a cryptic splice donor site located 32 bp upstream of the nominal 3' end of exon I. This cryptic splice donor site appears to comply with the normal "GT" rule governing the selection of RNA cleavage sites at the 5' end of introns to be excised:
Just as with the ERΔE2 variant itself, use of this cryptic splice donor site in clones 27.3 & 27.28 would cause a shift in the translational reading frame of the ER protein. This is predicted to result in a severely truncated protein if these transcripts are in fact translated, \textit{in vivo}. Insertion mutants were also observed among the products of this amplification reaction, including one variant (clone 27.16) that contains an in-frame duplication of exon III. Finally, when a PCR experiment was designed to examine the integrity of exon I and the 5′-untranslated region of the ER-α mRNA in MCF-7 cells (Fig. 7), full length amplification products (such as that represented by clone M.3) were observed to co-occur with a variety of exon I deletion variants (e.g., clones M.1, M.6, and M.18). These variants harbor large deletions in the 5′-untranslated leader that cause loss of the initiating methionine residue. They would therefore be predicted to interfere with proper initiation of translation of the usual 67 kD ER-α protein.

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure6.png}
\caption{Fig. 6. Structures of ERα splicing variants in MCF-7 cells that involve the DNA-binding domain.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure7.png}
\caption{Fig. 7. Structures of ERα splicing variants in MCF-7 cells that contain deletions within exon I and the 5′-leader.}
\end{figure}

\textbf{RT/PCR analysis of ER-α mRNAs in a human breast tumor with discordant ER status.}

The reliability of immunological assays that are used clinically to evaluate the status of ER expression in human tumors depends on the assumption that the antibodies upon which these assays are based detect primarily full-length, transcriptionally competent receptor. Cases where tumors present with discordant laboratory findings represent a special challenge to physicians managing breast cancer cases. In collaboration with Jules Elias (Dept. of Pathology, SUNY at Stony Brook), we reported on a case of invasive ductal carcinoma in 1996 (16) which presented with a high proliferative index, 9/27 positive nodes, and bone involvement, but was paradoxically well differentiated (Grade I). While this tumor appeared to be ER- by ELISA (4 fmol/mg) as well as by independent immunocytochemical assays using the H222 anti-ER antibody, it displayed a discordant (positive) ER status when paraffin or frozen sections were stained with the 1D5 anti-ER antibody (>70% positive nuclei). Adding to the complexity of this case, this tumor was positive for pS2, but failed to express detectable PR (two independent markers that tend to correlate well
with ER expression). We reasoned that the discordant ER status of this tumor might reflect the expression of high levels of an unusual receptor variant that was predicted to retain the amino-terminal epitope recognized by the ER-1D5 antibody, but was missing a portion of the ligand binding domain that is recognized by H222. To pursue this hypothesis, total RNA was extracted from a 100 mg portion of this tumor that had been stored under liquid nitrogen, followed by RT/PCR analysis of ER-α transcripts using an approach analogous to that described above for MCF-7 cells. In this case, the PCR experiment utilized the primers E1U-new and E7L-wt, which are targeted to exons I and VII of the ER-α mRNA and are predicted to give rise to a 1,165 bp amplification product (Fig. 8). In contrast to the complex situation observed in MCF-7 cells, only two RT/PCR products were observed in the discordant tumor sample. The largest PCR product was estimated to be 380 bp in size and, when sequenced, was found to contain the aberrant joining of exon I to exon VI with complete loss of the intervening 783 bp comprising exons II through V. The shorter product suffered the additional loss of 32 bp of sequence at the very end of exon I as a result of the use of an apparent cryptic splice donor site just upstream of the exon I / exon II junction, just as described above for ER-α variants in MCF-7 cells (see Fig. 6). Thus, this tumor is highly unusual in that it appears to completely lack intact ER-α mRNA and to express a large deletion that removes the DNA-binding domain and the proximal half of the ligand-binding domain (including the epitope for the H222 antibody). From its structure, it is predicted to be entirely devoid of estradiol-binding, DNA-binding, and transcriptional activity.

**Fig. 8.** Unusual structure of a prominent pair of ER-α splicing variants expressed by an invasive ductal carcinoma presenting with a discordant ER status.
Characterization of the functional activity of ER splicing variants.

As noted in previous technical reports, HeLa cell co-transfection experiments designed to assess the transcriptional activity of individually expressed ER-α splicing variants have failed to demonstrate any significant ability of variant receptors to support gene activation through an estrogen response element (ERE), with the possible exception of the ERΔE5 variant which appears to display a low level of constitutive transcriptional activity on an ERE-reporter in some, but not all cell types examined. The presence or absence of 1 nM estradiol has no influence on the activity of ERΔE5 or the remaining ER-α splice variants (data not shown). These data agree with published studies (1,17) concluding that the exon-deleted ER variants ERΔE2, ERΔE3, and ERΔE7 fail to stimulate an estrogen-responsive reporter plasmid and add the ERΔE4 and ERΔE6 mutants to this list of transcriptionally impaired splicing variants. Our observations on the behavior of ERΔE4 represent the first functional characterization of this variant and are significant in light of the recently reported widespread occurrence of the ERΔE4 variant in human breast tumors (14). In contrast, the ERΔE6 variant remains a hypothetical curiosity in that the simple deletion of exon VI has never been observed to occur among ER-α transcripts in ER+ tissues and cells (see, for example, Fig. 3A), although rare deletions of exon VI are seen in conjunction with deletions of some of the other exons (see Fig. 3B).

While these experiments argue fairly conclusively that ER splicing variants other than ERΔE5 appear to be devoid of positive transcriptional activity on ERE-containing genes, they leave open the question of potential inhibitory transcriptional activity. Indeed, we previously reported that the ERΔE3 variant acts as a dominant negative mutant when it is co-expressed with wt ER-α (1). To clarify this point, we completed a series of experiments to assess the transcriptional inhibitory effects of the remaining exon skipped ER-α variants. When tested in a HeLa cell co-transfection assay in which the expression of pERE-TK-CAT was driven by wt ER-α, a 5-fold molar excess of any of the splice variants ERΔE2, ERΔE4, ERΔE6, and ERΔE7 failed to inhibit the estrogen-dependent induction of CAT expression by intact receptor (data not shown). In contrast, the ERΔE5 variant was found to share the dominant inhibitory activity of ERΔE3 (Fig. 9).

![Graph](image)

Fig. 9. Both ERΔE3 and ERΔE5 can repress the transcriptional stimulatory activity of co-transfected wt ERα in transiently transfected HeLa cells.
**Generation of cell lines that stably express the ERΔE5 splice variant.**

During the 1996-97 award period, significant time and effort was expended on the production of stable cell lines intended for use in functional studies on wt ER-α and its variants. In accordance with Task 2B of this project, priority was placed on stably expressing the ERΔE5 variant, due to published reports describing its constitutive activity (18) and speculation surrounding its ability to confer estrogen-independence and tamoxifen resistance (3). Early experiments, in which we attempted to generate stable clones by co-transfecting ER-α expression plasmids together with a neomycin resistance plasmid (pSV2-Neo) prior to selecting for G418 resistance failed to result in detectable levels of receptor co-expression among the resulting G418R colonies. To circumvent this problem and achieve better co-selection, cDNAs for wt ER-α and several variants where shuttled into pcDNA3 or pCR3.1, which represent cytomegalovirus (CMV) promoter driven expression vectors that incorporate the transgene of choice and the co-selected NeoR gene into a single plasmid. As a result of ongoing difficulties in achieving sustained expression of the ER-α transgenes within G418R cells, several additional parameters were tested, including:

1) varying the cell lines used for stable selection (HeLa, Cos7, MDA MB231, and MCF-7)
2) varying the concentration of G418 used for selection
3) performing step-wise selection with G418
4) performing selections in standard serum, in charcoal-treated serum, and in serum supplemented with estradiol
5) linearizing the transfection plasmids at a site distant from the ER-α transgene or the NeoR cassette.
6) comparing DNA transfection techniques (calcium phosphate vs. electroporation)
7) isolating both pools of G418-resistant cells, as well as individual colonies

Our repeated experience was that, while we could achieve excellent expression of the ER-α transgenes early during the selection period, expression rapidly diminished as the cells continued to grow in the presence of G418. This was true both of G418R pools, as well as individual colonies that were screened for ER-α expression. The apparent selection against sustained expression of ER was most pronounced for wt ER-α, but was also observed for the ERΔE5 variant. An example of this phenomenon is shown in Fig. 10 (below), which represents a western blot of whole cell extracts using the Mab-17 ER-α monoclonal antibody to follow the time course of ERΔE5 expression in MCF-7 cells transfected with pcDNA3-ERΔE5 after 2,5,7, and 27 days of G418 selection.

![Western Blot](image)

**Fig. 10.** Time course of ERΔE5 expression in MCF-7 cells during the course of G418 selection. MCF-7 cells were transfected with 5 μg of linearized pcDNA3-ERΔE5, followed by growth in the presence of 0.4 mg/ml G418 for 2, 5, 7, and 27 days. At the indicated times, cells were harvested and checked for ERΔE5 expression by western blot analysis using the Mab-17 ER-α-specific monoclonal antibody.

Since pools of G418-resistant cells invariably expressed unacceptably low levels of ERΔE5 in our experience, we focused our attention on screening individual colonies for receptor expression.
Eight clonal lines of MDA MB231 were isolated expressing various levels of ERΔE5, along with four additional clonal lines selected using Cos7 cells. In addition to these stable cell lines expressing ERΔE5, a smaller number of lines expressing ERΔE4 and a variety of engineered mutants of ER-α were also isolated. However, we were completely unsuccessful in constructing stable cell lines to express wt ER-α. Ampules corresponding to early passage cultures of all of our stable ER-positive cell lines were frozen for long-term storage in liquid nitrogen. A western blot showing ERΔE5 expression in several of these lines at approximately 5 weeks of G418 selection is shown in Fig. 11. Functional analysis of stably expressed ERΔE5 (see below) focused on MDA MB231 clones B.5, C.4, and D.1.

![Diagram showing levels of ERΔE5 expression in stable clones of pcDNA3-ERΔE5-transfected MDA MB231 and Cos7 cells after approximately 5 weeks of G418 selection.](image)

**Fig. 11.** Levels of ERΔE5 expression in stable clones of pcDNA3-ERΔE5-transfected MDA MB231 and Cos7 cells after approximately 5 weeks of G418 selection.

**Functional analysis of stably expressed ERΔE5.**

Previous experiments involving transient transfection of ERΔE5 into Cos7 cells have led us to conclude that the DNA-binding activity of this variant is severely impaired, due presumably to its loss of a strong subunit dimerization interface that has been localized within the carboxy-terminal end of the ligand-binding domain. To confirm if this is also the case when ERΔE5 is stably expressed in cells, we prepared whole cell extracts from MDA MB231 clones B.5, B.6, C.4, D.1 and tested them for their ability to bind to a double-stranded consensus estrogen response element (ERE) (5'-AGGTCACAGTGACCT-3') using a standard gel mobility shift assay. The results, which are given in Fig. 12 (below) and other experiments not shown, indicate a complete lack of DNA-binding by stably expressed ERΔE5, even when a bivalent ER-specific antibody (Mab-17) is added to the binding reactions to stabilize any transitory receptor / DNA complexes. Equivalent results were observed with our Cos7 clones that stably express ERΔE5. Under our conditions for DNA-binding, a modest "supershift" can be demonstrated with transiently expressed ERΔE5 (lane 5) but not stably expressed variant (lanes 11, 13, 15, & 17). The difference between the behavior of transiently vs stably expressed ERΔE5 is most likely due to the unusually high levels of expression that can be achieved in the Cos7 cell transient transfection system. The apparent inability of ERΔE5 to bind efficiently to a consensus ERE is consistent with the very low level of transcriptional activity that we have observed for this variant in a transient transfection assay.
Fig. 12. Lack of DNA binding by whole cell extracts from MDA MB231 clones that stably express the ERΔE5 protein. The DNA probe used for this experiment is a consensus (palindromic) 15 bp ERE. The control extracts shown in lanes 2-3 and lanes 4-5 are from Cos7 cells transiently transfected with wt ERα and ERΔE5, respectively. The arrow indicates the position of the retarded ER/DNA complex, while the asterix represents complexes supershifted by the addition of the Mab-17 (ER-specific) monoclonal antibody.

While stimulation of transcription by wt ER-α (and also ERΔE5) on an ERE is generally assumed to involve direct binding of receptor to DNA, other mechanisms involving an indirect effect of ERΔE5 on the transcription machinery are also conceivable. To examine such a possibility, we tried to assess the ability of our stable ERΔE5-expressing MDA MB231 cell lines to respond to the estrogen-responsive reporter pERE-TK-CAT. Under conditions where wt ER-α supports strong estradiol-dependent induction of the pERE-TK-CAT reporter in MDA MB231 cells, ERΔE5 produced a barely detectable increase in transcription relative to transfections lacking any receptor at all (see year 1 annual report). As expected, the low level of CAT activity seen with ERΔE5 was unchanged by the presence or absence of estradiol. Similarly, when we repeated this experiment in three independent MDA MB231 / ERΔE5-expressing stable cell lines (B.5, C.4, and D.1), no differences in CAT expression were observed comparing the pERE-TK-CAT reporter with pTK-CAT3, the corresponding control plasmid which lacks an ERE (Fig. 13). Additionally, we observed no differences in CAT expression when the stable MDA MB231 cell lines were treated with EGF or with high levels of insulin (intended to mimic the effects of IGF-1), two growth factors which might be considered potential candidates for ligand-independent activators of ERΔE5 function. These results reinforce our conclusions that, while ERΔE5 may display modest transcriptional activity in some special contexts, it is largely devoid of positive transcriptional effects that require it to bind directly to DNA. This leaves open the possibility, however, that ERΔE5 or some of the other ER-α splicing variants may display stimulatory effects on the growing list of estrogen responsive genes that lack a canonical ERE.
Fig. 13. Stably expressed ERΔE5 fails to support transcriptional stimulation through a consensus (palindromic) ERE in MDA MB231 cells.
DISCUSSION

The major accomplishments for this reporting period include: 1) completion of an intensive analysis of ER-α variants in MCF-7 cells (part of Task 1), 2) the analysis of ER-α variants in an invasive ductal carcinoma characterized by a discordant ER status, 3) continued characterization of the functional properties of six major ER-α splicing variants, particularly with respect to their transcriptional inhibitory behavior (Task 2.B), and 4) the generation and preliminary characterization of cell lines that stably express ERΔE5 (Task 2.C).

Several important revelations come from our analysis of ER-α transcripts present in MCF-7 cells. Significant is the finding that correctly processed mRNAs (i.e., wt ER-α) actually constitute a much smaller fraction (50% or less) of the total transcript pool than previously expected. In agreement with several previous reports, the ERΔE7 variant appears to be the only truly prevalent variant in MCF-7 cells, representing about one-fourth of total ER-α transcripts. The remaining transcripts are exceedingly heterogeneous in nature, including exon-skipped variants that suffer the loss of single or multiple exons, exon duplication variants, insertion mutants, and a wide variety of deletion mutants, many of which appear to be derived from the use of cryptic splice donor or acceptor sites. In their aggregate, these variants are quite prominent; however, no single variant (with the possible exception of ERΔE7) is ever likely to accumulate to sufficiently high levels to be easily detected at the protein level. A similar picture is likely to hold true in most human breast tumors, as evidenced by numerous published studies on ER-α variants (reviewed in 4,5,8). Unusual situations can also occur, however, as shown by our characterization of a discordant breast tumor that apparently lacks any wt ER-α and appears to exclusively express a pair of prominent ER-α mRNAs that lack exons II through V.

The other major accomplishments for this reporting period pertain to the functional analysis of ER-α splicing variants. While our work was in progress, two reports appeared describing the functional behavior of MCF-7 clones that stably express the ERΔE5 variant (6,19). This variant has attracted special interest due to an early report (18) suggesting that this receptor isoform may possess constitutive transcriptional activity that could possibly account for some aspects of tamoxifen resistance, a recurring problem in the clinical treatment of breast cancer. Our findings indicate that not only is the transcriptional stimulatory activity of ERΔE5 relatively weak and highly cell-type dependent, but that a more prominent activity of this receptor variant may actually be to interfere with activation of transcription by wt ER-α. We have formally demonstrated the dominant inhibitory activity of ERΔE5 in HeLa cell co-transfection experiments using a chimeric estrogen-responsive promoter (pERE-TK-CAT), but we suspect that the same behavior will be shared by naturally occurring ERE-containing genes in other cell types as well. Additionally, when we stably expressed ERΔE5 in the ERα-negative cell line MDA MB231, we failed to observe any evidence of DNA-binding or constitutive or growth factor-inducible transcriptional activity by ERΔE5 and we noticed no obvious change in the phenotypic behavior of the cells. Our assessment of the activity of ERΔE5 is in agreement with the findings of Rea and Parker (19) who reported that, when expressed stably in MCF-7 cells, ERΔE5 failed to alter the growth properties of these cells, failed to induce expression of the endogenous progesterone receptor or pS2 genes, and failed to confer tamoxifen resistance on these cells. Together with our data, this indicates that the common perception of ERΔE5 as an "outlaw" receptor needs to be reconsidered. These findings however contrast with those of Fuqua and Wolf who reported that a clonal line of MCF-7 cells harboring stably expressed ERΔE5 displayed elevated progesterone receptor expression, increased anchorage-independent colony formation in the absence of estrogen, and decreased sensitivity to the growth inhibitory effects of tamoxifen (6). Further work analyzing the behavior of ERΔE5 in stably and transiently transfected cell lines is in progress to clarify this issue.

An important question left open by our studies is whether ERΔE5 or any of the other ER-α
splicing variants that we and others observe may exert effects on gene expression using pathways distinct from the classical transcriptional effects mediated by direct DNA binding of receptor to palindromic estrogen response elements (EREs). Two obvious possibilities exist and they involve:

1) coactivator mediated effects of ER-α splice variants on genes that contain “non-canonical” estrogen response elements. Elements that have been reported to behave in this manner include AP-1 sites and GC boxes (SP-1 sites), but are likely to include others.
2) modulatory effects on signal transduction cascades mediated by direct interaction of ER-α splice variants with intracellular protein kinases (e.g., MAPK, pp60src, p90rsk-1, etc.)

Many of the genes that are regulated by estrogens apparently do so using “non-classical” mechanisms. Among these targets are numerous genes such as IGF-1, collagenase, cathepsin D, cyclins, and RARα that are believed to play important roles both during normal breast development and during tumor formation and progression. In the final year of this project, our work has been focusing on determining whether or not ER-α splice variants participate in non-classical pathways of gene regulation such as those described above.

Publications

1) year 3 publications related to this award (copies included as Appendix B)

2) manuscripts currently in preparation from this award
   b) Ankrapp, D.P., Morrison, M., Bollig, A.B., and Miksicek, R.J. MCF-7 breast cancer cells express a broad array of ERα mRNA size variants, in preparation.

3) previous publications related to this award

4) current publications unrelated to this award
REFERENCES


Appendix A: Sequences of Additional Oligodeoxynucleotides Obtained

**Human ER-α Splice Junction Oligos for Colony Hybridization Assays**

* positions of splice junctions are indented with a vertical slash (|)

1) 1/2 splice (wtER) 5'- CATTCTACAG|GCCAAATTCA -3'  20 mer
2) 1/3 splice (ERΔE2) 5'- CATTCTACAG|GACATAACGA -3'  20 mer
3) 1/4 splice (ERΔE2/3) 5'- CATTCTACAG|GGATACGAAA -3'  20 mer
4) 2/3 splice (wtER) 5'- AGTATTCAAG|GACATAACGA -3'  20 mer
5) 2/4 splice (ERΔE3) 5'- AGTATTCAAG|GGATACGAAA -3'  20 mer
6) 2/5 splice (ERΔE3/4) 5'- AGTATTCAAG|GCTTTGTGGA -3'  20 mer
7) 3/4 splice (wtER) 5'- ATGAAAGGTG|GGATACGAAA -3'  20 mer
8) 3/5 splice (ERΔE4) 5'- ATGAAAGGTG|GCTTTGTGGA -3'  20 mer
9) 4/5 splice (wtER) 5'- AGGATGCCCG|GCTTTGTGGA -3'  19 mer
10) 4/6 splice (ERΔE5) 5'- AGGATGCCCG|GAACCAGG -3'  18 mer
11) 5/6 splice (wtER) 5'- TCTTGGACAG|GAACCAGG -3'  19 mer
12) 5/7 splice (ERΔE6) 5'- TCTTGGACAG|GAGTGATAC -3'  20 mer
13) 6/7 splice (wtER) 5'- CTATGTTCTG|GAGTGATAC -3'  20 mer
14) 6/8 splice (ERΔE7) 5'- CTATGTTCTG|TAACAAAGGC -3'  20 mer
15) 7/8 splice (wtER) 5'- GCCACATGAG|TAACAAAGGC -3'  20 mer

**Exon 1 primer**

16) E1U-new (wt ER) 5'- CCTACTACCTGGAGAACGAG -3'  20 mer

**Oligos for RT/PCR Analysis of Human Estrogen Receptor-β Transcripts**

17) betaER(3'UT) 5'- CCACACAGCAGAAAGATGAAGCCCAG -3'  26 mer
18) betaER-1Uwt 5'- CCTGCTGTGATGAATTACAGC -3'  21 mer
19) betaER-5Lwt 5'- CCTTCTACGCATTTCCCCTC -3'  20 mer
20) betaER-8Lwt 5'- CCAGTTTCACCTCAGGGCCAG -3'  20 mer
### Promoter-Specific Oligos Priming in the 5'-Untranslated Regions of Human ER-α transcripts

21) Proximal (promoter A) specific oligo #1 for hER-α 5'-UT, upper strand:
   
   **Hero 18b**
   
   5' -GGCCAGAGCTCGCGTGTC-3'  
   18 mer

22) Proximal (promoter A) specific oligo #2 for hER-α 5'-UT, upper strand:
   
   **ER 5'UT, prox**
   
   5' -GGCGCAGCGCGTTCGTCCTG-3'  
   20 mer

23) Distal (promoter B) specific oligo for hER-α 5'-UT, upper strand:
   
   **ER 5'UT, distal**
   
   5' -CGAGCACATTCTCCTCCTCC-3'  
   20 mer

24) Liver (promoter C) specific oligo for hER-α 5'-UT, upper strand:
   
   **Promoter C primer**
   
   5' -CTCCAAAATCTGATACCAATCC-3'  
   22 mer

### Oligos for RT/PCR Analysis of Human Progesterone Resceptor Transcripts

25) **hPR(3'UT)**
   
   5' -TCTTTAAAAAGAAAAAGATGACATTCA-3'  
   22 mer

26) **hPR-lUwt**
   
   5' -CCCTATCTCAACTACCTGAG-3'  
   20 mer

27) **hPR-5Uwt**
   
   5' -GGTCATCAATATGTAAGTTTCG-3'  
   22 mer

28) **hPR-8Uwt**
   
   5' -AGTACAGATGAAGTTGTTTGAC-3'  
   22 mer

### Primer for 5'- RACE Analysis

29) **AUAP**
   
   5' -GGCCACGCGTCGACTAGTAC-3'  
   20 mer
Appendix B:

P3-420

FUNCTIONAL ANALYSIS OF ESTROGEN RECEPTOR SPlicing VARIANTS, A. Buliga, D. Ankrapp, and RJ Miksicek

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Analysis of messenger RNA (mRNA) prepared from a variety of estrogen-responsive cell lines (MCF7, T47D), breast tumor specimens, and normal breast tissue has established that estrogen receptor (ER) mRNA is typically expressed as a mixture of transcripts. Using PCR amplification, this heterogeneity has been shown to result largely from an incorrect pattern of mRNA splicing that gives rise to a family of correctly processed as well as exon-skipped ER transcripts. We have reconstructed ER cDNAs representing the exon-skipped variants ERAE2 through ERAE7 to enable their functional characterization in a well defined cell transfection system. All six of these ER splicing variants show efficient expression in Cos7 cells, and each displays a characteristic pattern of subcellular distribution that can be distinguished from that of intact receptor. Each of the variants also displays a dramatic reduction in DNA-binding activity, as measured using an in vitro gel mobility shift assay. While this DNA-binding defect appears to be complete for ERAE2, ERAE3, ERAE4, and ERAE6 weak residual DNA binding is discernible for ERAE5 and ERAE7 when DNA complex formation is stabilized by the addition of a bivalent antibody capable of recognizing the ER protein. We currently conclude that two factors contribute to the loss of efficient DNA-binding by these receptor variants: 1) disruption in the structure or accessibility of the "zinc-finger" DNA-binding domain caused by remnants of the hormone-binding domain still present in many of the variants or 2) absence of a critical dimerization element present in the carboxy-terminal end of the hormone-binding domain. The ERAE5 variant has attracted special interest due to a previous report suggesting that this isoform may possess constitutive transcriptional activity that could account for some forms of tamoxifen resistance in clinical breast cancer. Our findings indicate that the transcriptional activity of ERAE5 is relatively weak and is highly cell-type specific, indicating that the common perception of ERAE5 as an "outlaw" receptor needs to be re-evaluated.