



Estrogen regulates a tissue-specific calpain in the anterior pituitary[☆]

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Received 28 May 2002

Abstract

A PCR-based cDNA subtraction hybridization was performed to identify the genes stimulated by estrogen in the pituitary. A novel tissue-specific calpain (nCL-2'), previously shown to be expressed mainly in the stomach, was markedly induced in the pituitary after estrogen treatment. The 5'-flanking region of the calpain nCL-2' gene was analyzed to assess the molecular mechanism of estrogen regulation. Sequence analysis of the nCL-2' promoter (1.9 kb) revealed a perfectly palindromic putative estrogen-response element (ERE), GGTCATGCTGACC. In transient transfection studies, the nCL-2' promoter was highly responsive to estrogen in the presence of estrogen receptor (ER). Transcriptional activation by estrogen was prevented by an ERE mutation as well as by mutations in the ER DNA-binding domain. An ER antagonist, ICI 182780, blocked estrogen inducibility of the nCL-2' promoter. We conclude that the nCL-2' form of calpain is expressed in the pituitary and upregulated by estrogen at the transcription level. © 2002 Elsevier Science (USA). All rights reserved.

Keywords: Calpain; Estrogen; Pituitary; Estrogen-response element

Estrogen is a key regulator of reproduction, acting in part to control hypothalamic–pituitary axis. In particular, estrogen feedback modulates the synthesis and secretion of the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), and prolactin (PRL) [1]. Targeted disruption of the mouse estrogen receptor- α revealed elevated gonadotropin mRNA levels and marked reduction in PRL mRNA and lactotrope cell number [2], consistent with findings of direct and major actions of estrogen on these hormones in the anterior pituitary. Although the physiological effects of estrogen on the pituitary are well known, molecular mechanisms and molecules that mediate the effects of estrogen remain largely unknown.

Calpains, intracellular calcium-dependent cysteine proteases, represent a superfamily of structurally related proteins that are widely distributed in various tissues. A large number of calpain-like molecules have been identified and cloned. These include ubiquitously expressed and tissue-specific isoforms. Although the physiological

function of calpains remains obscure, they have been implicated in the regulation of a variety of cellular functions, including intracellular signaling, proliferation, differentiation, and apoptosis [3–5]. Calpain-mediated proteolysis is involved in hormone maturation and secretion as well as cell signaling in the pituitary [6–8]. Recently, a possible link between estrogen levels and calpain activity was described in post-exercise skeletal muscle [9]. Calpain activity has also been associated with estrogen effects on cell proliferation in breast cancer cell lines [10,11].

In this study, we used a cDNA subtraction technique to identify new genes regulated by estrogen in the anterior pituitary. We demonstrate that a novel tissue-specific calpain (nCL-2') previously described to be expressed predominantly in the stomach [12] is upregulated by estrogen in the pituitary.

Materials and methods

Animal models. All surgical and experimental procedures were conducted in accordance with the policies of Northwestern University's Animal Care and Use Committee. Sprague–Dawley rats (Charles River Laboratories, Wilmington, MA) (200–250 g) were housed in a controlled environment. Female rats were ovariectomized bilaterally

[☆]The DNA sequence has been submitted to GenBank and the accession number is AF514419.

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for 14 days and received 17 β -estradiol-filled SILASTIC implants (Dow Corning, Midland, MI; id, 0.062 in.; od, 0.125 in.; 5 mm in length) subcutaneously under light anesthesia with metofane for 10 days. Rats were killed by decapitation. Anterior pituitary glands were rapidly removed and frozen. Blood was collected for measurement of estrogen to verify the effectiveness of estrogen implantation.

RNA preparation, PCR-based subtraction, and cloning. Total RNA was isolated from the anterior pituitaries of ovariectomized and estradiol-treated rats as described before [13]. The PCR-based cDNA subtraction was performed by using a PCR-Select cDNA Subtraction Kit (CLONTECH, Palo Alto, CA) according to manufacturer's instructions. "Tester" (estrogen-treated) and "driver" (ovariectomized) double-stranded cDNAs were synthesized from total RNA using the SMART PCR cDNA Synthesis Kit (CLONTECH). Double-stranded cDNAs were digested with *RsaI* and the digested tester cDNA was ligated with adapters 1 and 2 provided in the kit in separate reactions. Each of the adapter-ligated cDNAs was heat-denatured and annealed to excess heat-denatured driver cDNA (first hybridization). The two samples from the first hybridization were combined and a fresh portion of heat-denatured excess driver cDNA was added (second hybridization). PCR amplification was performed twice for the subtracted cDNA. All of the primers (PCR primer 1 and nested PCR primers 1 and 2R) for the PCR were provided in the kit. The products of the second PCR were inserted into the pCR2.1 TOPO vector using a T/A cloning kit (Invitrogen, Carlsbad, CA).

Differential screening the subtracted cDNA library, DNA sequencing, and alignments. Dot blot hybridization was performed with PCR-Select Differential Screening Kit (CLONTECH). A total of 500 clones were selected and grown. Bacterial cultures were used to amplify cDNA inserts by PCR. The amplified cDNA was blotted onto duplicated Zeta-Probe Blotting nylon membranes (BIO-RAD, Hercules, CA). Membranes were hybridized with ³²P-labeled double-stranded cDNA pools of equal specific activity derived from subtracted or un-subtracted tester mRNA in Rapid-hyb buffer (Amersham Pharmacia, Piscataway, NJ). Membranes were washed in 2 \times SSC, 0.1% SDS and exposed to X-ray films. The signals of corresponding clones from two hybridizations were compared and the positive clones were selected. Sequences of the inserts for clones differentially expressed were determined by using M13 reverse and forward primers and a dRhodamine terminator cycle sequencing kit. Sequences were analyzed using an ABI377 automated DNA sequencer (PE Applied Biosystems, Foster City, CA). A nucleic acid homology search was performed using the BLAST program (National Institutes of Health, Bethesda, MD).

Northern blot analysis. Equal amounts (10 μ g) of total RNA isolated from a variety of tissues of random cycling female rats and the testis of male rats were separated on a formaldehyde agarose gel and transferred onto nylon membranes with a TurboBlotter (Schleicher and Schuell, Keene, NH). An nCL-2'-specific probe was generated by PCR amplification of the nCL-2' insert. The primers used were 5'-ATTGCCGTAATGCTACATTC-3' and 5'-CTGAGGAGAGGCTGAATAAGT-3'. Membranes were hybridized with the probes labeled with [³²P]dCTP in Rapid-hyb buffer overnight, washed, and exposed to films.

Isolation and cloning of the nCL-2' 5'-flanking sequence. Fragments of the 5'-flanking region of the rat gene encoding nCL-2' were amplified by using rat GenomeWalker kits (CLONTECH). Primers were designed within the coding and 5'-untranslated regions (Fig. 2A and B) according to the published rat nCL-2' cDNA sequence [12]. The primary PCR was performed with the outer adapter primer (AP1) provided in the kit and an nCL-2' gene-specific antisense primer (GSP1, 5'-ACGTTGCTGGATACTCCCCTGCCAGAGC-3'). The primary PCR products were diluted and used as a template for a secondary PCR with the nested adapter primer (AP2) and a nested nCL-2 gene-specific antisense primer (GSP2, 5'-CAGAGCTGCCATGGCCTGCCTGTGAGTCCT-3'). The PCR products were subcloned into pCR2.1 TOPO vector (Invitrogen), sequenced with T7 and M13 reverse primers, and analyzed as described above. Based on the sequence obtained, PCR primers were designed to amplify the promoter region (-1937 to +50). For subcloning into a re-

porter gene, a *KpnI* site and an *NcoI* site (CCATGG) corresponding to the initiation codon of the calpain gene were introduced into the sense and antisense primers, respectively. PCR was performed using advantage genomic polymerase mix (CLONTECH) and rat genomic DNA as a template. After restriction digestion with *KpnI* and *NcoI*, the PCR product was subcloned into the pGL3 basic luciferase reporter vector (Promega, Madison, WI). The DNA sequences of several independent clones were directly determined and the promoter sequence was also confirmed by direct sequencing of the PCR products from genomic DNA. A mutation was introduced to the estrogen-response element (ERE) identified in the nCL-2' promoter using an overlapping PCR method.

Cell culture, transfection, and luciferase assays. TSA-201 cells, derived from human embryonic kidney 293 cells, were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and transfected using the calcium phosphate method. Before transfection, TSA-201 cells were estrogen-depleted for four days in charcoal-stripped phenol red-free media and then seeded onto 12-well plates in estrogen-depleted medium. Twenty-four hours later, cells were co-transfected with mouse wild type estrogen receptor (ER) or mutated ER expression vector (5 ng) and luciferase reporters containing either wild type or an ERE mutated calpain promoter (0.5 μ g). Estradiol (1 nM) was added to treatment media and ethanol was added to control wells to produce the same final solvent concentrations (0.1%). Twenty-four hours later, cells were assayed for luciferase activity.

Results

Identification of nCL-2' expression in the rat pituitary induced by estrogen

To identify genes upregulated by estrogen in the pituitary, cDNA subtraction was performed with mRNA populations obtained from the pituitaries of either ovariectomized or estradiol-treated rats. Five hundred colonies containing inserts verified by PCR were screened by dot blot hybridization and 239 colonies showing strong positive signals with the subtracted cDNA probe were sequenced. Partial sequences of these 239 clones were compared with the GenBank database. Among them, 72 clones were identified as PRL and seven clones were galanin, both of which are known to be upregulated by estrogen. An additional 56 positive clones were found to share homology with known genes.

One of the genes (nCL-2') verified by Northern blot analysis was a tissue-specific calpain that contains only the cysteine protease domain but lacks the calcium-binding domain [12]. The cDNA isolated from subtracted pituitary cDNA library was 554 bp, corresponding to the 3'-untranslated region of nCL-2' that shares no similarity with other isoforms [12]. A probe that specifically recognizes nCL-2' was generated using the nCL-2' insert as a template. The same pool of total RNA used in the subtraction hybridization was hybridized with the nCL-2' probe. As shown in Fig. 1, the nCL-2' gene was highly responsive to estrogen. Pituitary calpain nCL-2' mRNA increased markedly after 10 days of estrogen treatment, whereas it was undetectable in ovariectomized rats. In addition to two major

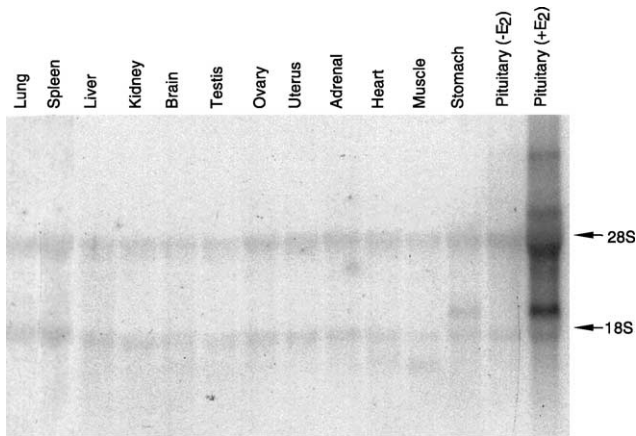


Fig. 1. Induction of nCL-2' mRNA by estrogen in the pituitary. Total RNA was obtained from a variety of tissues of random cycling female rats, the testis of male rats, and ovariectomized rats treated with or without estrogen for 10 days. Equal amounts of RNA (10µg) were subjected to Northern blot analysis with a ³²P-dCTP-labeled nCL-2'-specific probe. The blot was exposed to films for 5 days. Positions of 28S and 18S are indicated by arrows.

transcripts that have been previously reported [12], two weak transcripts were also detected at lower mobility than 28S RNA. A faint band corresponding to the smallest transcript found in the estrogen-treated pituitary was observed in the stomach after 5 days exposure (Fig. 1). Additionally, a very weak band below 18S was visible in skeletal muscle and heart after more than 10 days of exposure to a film. The nCL-2' mRNA was not detected in other tissues examined.

Identification of a functional ERE in the promoter region of rat nCL-2' gene

To define the mechanism of transcription regulation of the nCL-2' gene by estrogen, the promoter region was cloned using a PCR-based GenomeWalking method (Fig. 2A). PCR-amplification from a *StuI* digested rat genomic DNA library generated a 2.0-kb DNA. Fig. 2B shows the nucleotide sequence of the rat nCL-2' promoter region. A search for the putative transcription factor-binding sites using the TRANSFAC database [14] revealed a perfectly palindromic ERE (GGTCATGCTGACC) between position -1139 and -1152 of the nCL-2 promoter.

We constructed luciferase reporter plasmids driven by 1.9-kb fragment of the nCL-2' promoter to analyze the function of the putative ERE. Promoter constructs were co-transfected into ER-deficient cells with an ER expression vector. Transfected cells were treated with or without estrogen for 24h and assayed for luciferase activity. As shown in Fig. 3B, in the absence of transfected ER, there was no induction of reporter gene activity, confirming the absence of endogenous ER. Estrogen stimulates significantly the expression of

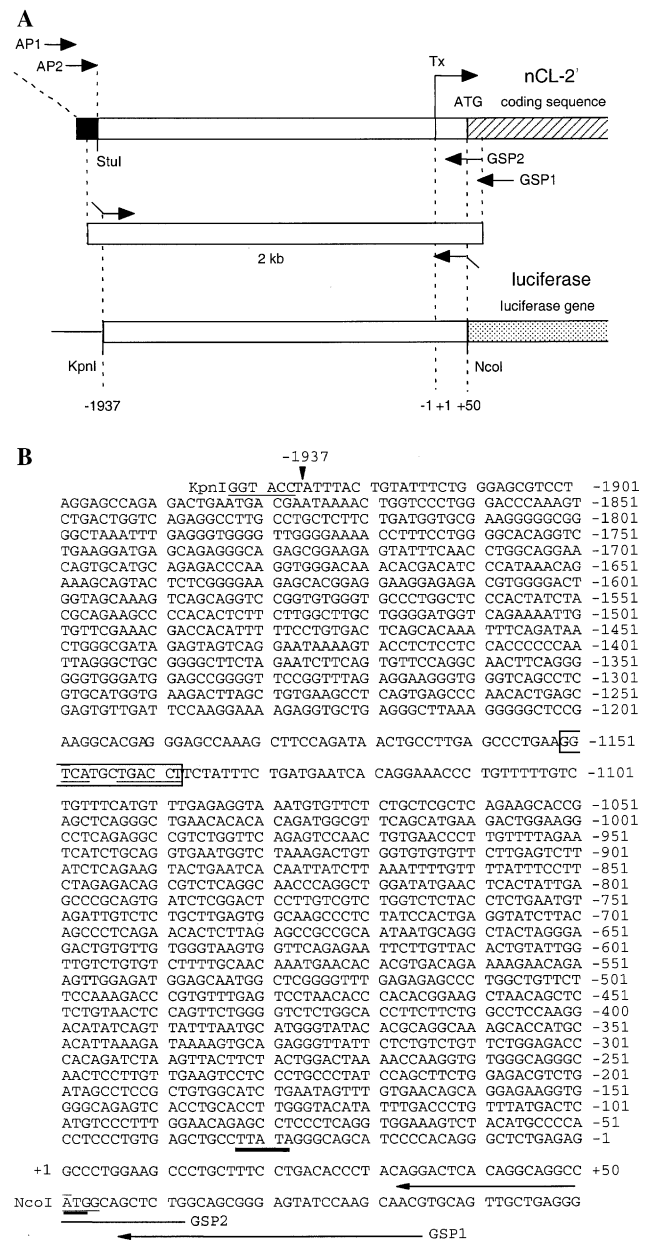


Fig. 2. Cloning of the nCL-2' promoter. (A) Schematic representation of the cloning strategy and construction of a reporter gene. The nCL-2' promoter was cloned from a rat genomic DNA library using a PCR-based GenomeWalking method and it was subcloned into the pGL3 basic luciferase reporter plasmid. (B) Nucleotide sequence of the promoter. DNA sequences for the gene-specific primers (GSP1 and GSP2) and restriction sites (*KpnI* and *NcoI*) are underlined. Sequence for the ERE is framed. The initiation codon and TATA box are shown by bold underlines.

reporter gene when the ER is also present. Co-transfected ER alone, in the absence of estrogen, slightly increases the nCL-2' promoter activity. The reporter gene activation by both ER and estradiol can be entirely blocked by an estrogen antagonist, ICI 182780 (ICI).

A mutation was introduced to the ERE to establish the specificity of ERE-mediated activation of the

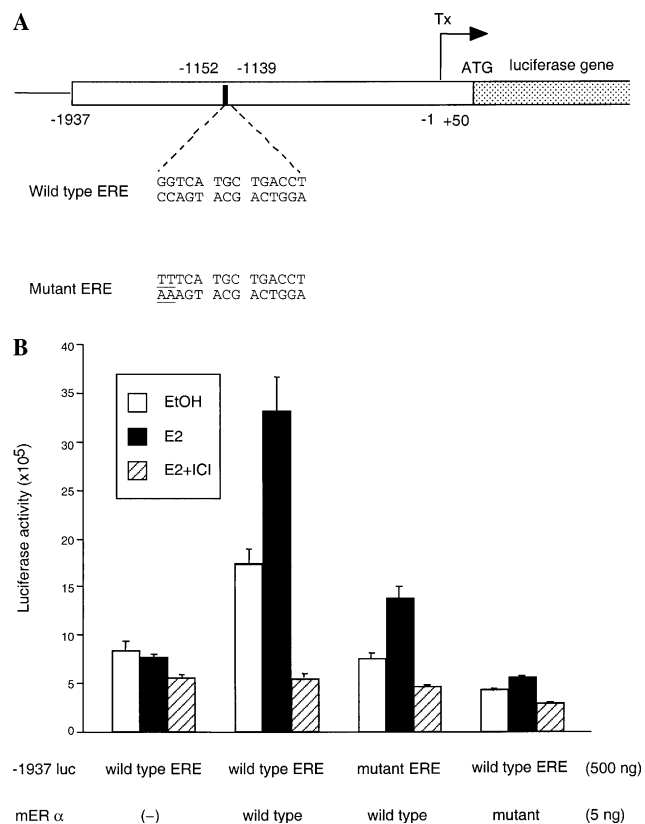


Fig. 3. Activation of the nCL-2' promoter by estrogen in the presence of estrogen receptor. (A) Schematic of the nCL-2' luciferase reporter (-1937 to +50) containing a wild type or mutant ERE (-1152 to -1139). A mutation was introduced into the ERE to disrupt DNA binding of ER. (B) TSA-201 cells maintained in estrogen-depleted medium for 4 days were co-transfected with a wild type or mutant mouse ER expression vector (5 ng) and a reporter gene containing a wild type or mutant ERE (0.5 μ g). Estradiol (1 nM) was added to treatment media after transfection. Twenty-four hours later, cells were assayed for luciferase activity. Results are means \pm SEM of triplicate transfection. Experiments were repeated at least three times and similar data were obtained (mER α , mouse estrogen receptor alpha; ERE, estrogen response element).

nCL-2' promoter and to further confirm the function of this ERE. The two 5'-guanosine residues were replaced with thymidines (Fig. 3A). This mutation has previously been shown to prevent receptor binding as well as transcriptional activation by estrogen [15]. There was no induction of reporter gene activity when this mutated construct was transfected (Fig. 3B) in the absence of estrogen. The stimulatory effect of estrogen was blunted by the mutation (less than 2-fold of induction remained). ICI completely eliminated the remaining activity. In addition to the ERE mutation, a DNA-binding domain mutant of ER that has double mutations at the P-box (E207G/G208S) was also tested in the transfection [16,17]. Co-transfection of the ER mutant completely prevented estrogen induction of the reporter gene activity (Fig. 3B).

Discussion

We have identified a number of genes upregulated by estrogen in the pituitary using a subtraction hybridization technique [18]. Among the positive clones sequenced, 30% was recognized as PRL and 3% was galanin, indicating that the subtraction hybridization was effective. One of the estrogen stimulated clones proved to be a tissue-specific calpain. This calpain was initially isolated from a rat stomach cDNA library and found to be expressed predominantly in the stomach [12]. Analysis of this tissue-specific cDNA revealed two forms, nCL-2 and nCL-2', that share identity at 5'-region but no similarity at 3'-end [12]. The amino acid sequence of nCL-2 shows a high homology to μ -(μ CL) and m-calpain (mCL) and contains four domains including the cysteine protease domain and the calcium-binding domain. Conversely, nCL-2' possesses only the cysteine protease domain but lacks most of the third domain and the calcium-binding domain [12]. The gene for nCL-2 and nCL-2' is located at the same locus and generated by alternative splicing [12]. It is likely that nCL-2' and nCL-2 are both regulated similarly by estrogen as they share the 5'-flanking region. The sizes of the mRNAs for both nCL-2' and nCL-2 are similar and they are expressed equally in the stomach [12]. Two sizes (3 and 6 kb) of mRNA for nCL-2' were described in the rat stomach [12]. However, in our study, only the smaller mRNA band was observed in the stomach. On the other hand, two dominant mRNA transcripts were detected in the pituitary after estrogen stimulation. Consistent with an earlier report [12], trace expression of nCL-2' was also observed in skeletal muscle and heart. Though the nCL-2' mRNA was not detected in most tissues examined, it remains to be determined whether nCL-2' or other calpain isoforms are induced by estrogen in other estrogen target tissues.

Since estrogen responsiveness often involves transcriptional regulation by ER interactions with promoter regulatory elements, the nCL-2' promoter region was isolated and analyzed for potential EREs. Sequence analysis of the promoter revealed a consensus palindromic ERE similar to those found in the promoters of other estrogen-responsive genes [19–21]. It has been shown previously that this ERE binds the estrogen receptor and it is sufficient to mediate hormone-dependent transcription [19,20]. To test the function of the calpain promoter ERE, a 1.9-kb promoter region was linked to a reporter gene and transfected into ER-deficient cells. The calpain promoter is highly responsive to estrogen only when estrogen receptor is present. In addition, the observed induction of the nCL-2' promoter activity was abolished by the ER antagonist, ICI, supporting estrogen action via the ER. Estrogen receptor alone, in the absence of estrogen, slightly increases expression of the reporter gene. This is probably due to a low amount of

estrogen still present in the charcoal-treated serum as ICI entirely reversed this effect.

The importance of the two guanosine residues of the ERE for estrogen responsiveness and ER binding has been demonstrated previously [15]. Mutations in the half site of the c-fos-ERE (5'-GGTCA-3' to 5'-ttTCA-3') destroyed both the transcriptional response to estrogen and ER binding [15]. Introduction of the same mutation into the ERE of the nCL-2' promoter markedly impaired estrogen inducibility. A mutation was also introduced into the DNA-binding domain of the mouse ER to examine whether DNA binding to the ERE is necessary for nCL-2' responsiveness. As we anticipated, a selective DNA-binding domain mutation that abolished ER binding to the ERE [17] completely eliminated estrogen responsiveness of the nCL-2' promoter. Our data demonstrate that the transcriptional effect of estrogen on the calpain promoter is mediated through an interaction of the estrogen receptor and the ERE, consistent with the classical model of ER action.

In this study, we described a tissue-specific calpain, nCL-2', expressed in the pituitary in response to estrogen. Nevertheless, it is uncertain which cell types produce nCL-2'. Given the fact that nCL-2' is stimulated by estrogen, lactotropes, and gonadotropes are the most likely candidates for nCL-2' expression. Despite no direct evidence suggesting that calpains participate in pituitary hormone processing and secretion, calpain-mediated proteolysis has been shown to be important in cytokine control of gene expression [22] and activation of Gs α in pituitary cells [7]. Moreover, calpain activity is inducible after activation of the pituitary thyrotropin-releasing hormone receptor [8]. Therefore, calpains may be involved in mediating effects of stimulating hormones from the hypothalamus. Unlike calpain-5 and 6 with a novel C-terminal domain [23], nCL-2' exhibits a distinctive feature. It is a truncated form of nCL-2 with a unique 3'-untranslated region. The absence of domain IV suggests that nCL-2' is independent of calcium for its activity and it may be unable to interact with the endogenous inhibitor calpastatin, as domain IV is involved in calcium binding and interactions with calpastatin [24]. Instead, the activity of nCL-2' may be regulated primarily by estrogen. nCL-2' may have diverse cellular functions and may play a role in estrogen actions to control pituitary hormone gene expression, secretion or cell proliferation.

The association of calpain activity with an estrogen effect on the cell proliferation has been observed in breast cancer cell lines [10,11]. A calpain inhibitor repressed cell growth of ER positive breast cancer cells in the presence of estrogen. In contrast, the inhibitor did not impair the cell growth of ER negative cells [10]. Calpain activity was significantly higher in ER-positive tumors than in ER-negative ones [11]. Our finding that estrogen directly stimulates calpain expression supports

the idea that calpain may be involved in the regulation of ER function in breast cancer tissues. It would be interesting in the future to investigate whether the promoters of other calpain family members contain ERE's estrogen responsiveness.

Acknowledgments

We thank Malcolm Parker for providing mER cDNA, Monika Jakacka for mutant ER, and Tom Kotlar and Leah Sabacan for assistance with DNA sequencing. This work was conducted as part of the National Cooperative Program on Infertility Research and supported by NIH Grant U54-HD-29164. Rachel Duan is a recipient of NIH Fellowship Award HD-08311.

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