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Development, Characterization, and Applications of a Novel Estrogen Receptor Beta Monoclonal Antibody

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Abstract

The role of estrogen receptor alpha (ER α) in breast cancer has been studied extensively, and its protein expression is prognostic and a primary determinant of endocrine sensitivity. However, much less is known about the role of ER β and its relevance remains unclear due to the publication of conflicting reports. Here, we provide evidence that much of this controversy may be explained by variability in antibody sensitivity and specificity and describe the development, characterization, and potential applications of a novel monoclonal antibody targeting full-length human ER β and its splice variant forms. Specifically, we demonstrate that a number of commercially available ER β antibodies are insensitive for ER β and exhibit significant cross-reaction with ER α . However, our newly developed MC10 ER β antibody is shown to be highly specific and sensitive for detection of full-length ER β and its variant forms. Strong and variable staining patterns for endogenous levels of ER β protein were detected in normal human tissues and breast tumors using the MC10 antibody. Importantly, ER β was shown to be expressed in a limited cohort of both ER α positive and ER α negative breast tumors. Taken together, these data demonstrate that the use of poorly validated ER β antibodies is likely to explain much of the controversy in the field with regard to the biological relevance of ER β in breast cancer. The use of the MC10 antibody, in combination with highly specific antibodies targeting only full-length ER α , is likely to provide additional discriminatory features in breast cancers that may be useful in predicting response to therapy.

Keywords

ESTROGEN RECEPTOR; ESTROGEN RECEPTOR BETA; BREAST CANCER; ANTIBODY

It is estimated that in 2011 over 230,000 women will be diagnosed with breast cancer in the United States alone [Siegel et al., 2011] with approximately 70% of these cases being classified as estrogen receptor (ER) positive breast tumors as defined by the expression of ER alpha (ER α) protein. For three decades, tamoxifen has been the most important therapeutic agent in the treatment of women with endocrine sensitive breast cancer since it

effectively inhibits the proliferation inducing effects of 17 α -estradiol (estrogen) in tumor cells. However, the use of ER α alone as an indicator of responsiveness to anti-estrogens is far from perfect as about 30% of ER α positive tumors do not respond to tamoxifen therapy [Osborne, 1998]. These observations have suggested that other estrogen receptors may be involved in mediating the responsiveness of endocrine sensitive tumors to hormonal agents. Following the discovery of a second estrogen receptor, ER α , in 1996 [Mosselman et al., 1996] many investigators began to explore the possible roles of this protein in mediating breast cancer development, progression, and response to therapy.

Like ER α , ER β is a member of the nuclear receptor superfamily of proteins which functions as a ligand-mediated transcription factor [Mosselman et al., 1996]. The human gene for ER β (ESR2) is comprised of eight exons which encode a 530-amino acid protein that is similar in structure to its closely related family member, ER α , as well as that of other nuclear hormone receptors. As with ER α , it contains five distinct protein domains designated as A/B, C, D, E, and F (Fig. 1). The A/B domain, located at the N-terminal end of the protein, contains an activation function (AF1) which has been shown to exhibit ligand independent activity [Tora et al., 1989]. The C domain contains a highly conserved DNA binding domain and is also involved with receptor dimerization. The D domain functions as a hinge region and is thought to contain a nuclear localization signal [Picard et al., 1990]. The ligand-binding domain lies within the E domain and contains another activation function referred to as AF2 [Tora et al., 1989]. At present, the functions of the F domain, located at the C-terminus, are not known.

In addition to this “full-length” receptor (ER α 1), the ER β gene also encodes an additional four variants designated as ER α 2, ER α 3, ER α 4, and ER α 5 (Fig. 1). These variants are identical to that of ER α 1 from amino acids 1–469. Amino acids 470–530, encoding the C-terminal portion of the E domain and the entire F domain of ER α 1, are deleted in ER α 2–5. However, each variant contains a unique C-terminal amino acid sequence which varies in length and results from alternative splicing of exon 8 [Moore et al., 1998; Lewandowski et al., 2002; Poola et al., 2005a] (Fig. 1).

Since the discovery of ER β [Mosselman et al., 1996], its role in the development, progression, and treatment of breast cancer has been hotly debated, and to date, no real consensus regarding its clinical utility has been established. Potential explanations include the lack of standardized methodologies for detecting expression of ER α , the use of poorly validated antibodies, the presence of highly conserved variants whose functions remain unresolved and/or the inconsistent interpretation of what constitutes ER β positivity. The first studies aimed at addressing the role of ER β in breast cancer were conducted using mRNA-based assays [Leygue et al., 1998; Leygue et al., 1999; Speirs et al., 1999a; Speirs et al., 1999b; Iwao et al., 2000; Shaw et al., 2002; Park et al., 2003; Zhao et al., 2003]. However, it has been reported that mRNA levels of ER β do not correlate with its protein levels in human tumors [Shaw et al., 2002; Balfe et al., 2004; O’Neill et al., 2004]. More recently, a significant number of immunohistochemical based studies have been performed using paraffin embedded breast tumor samples [Jarvinen et al., 2000; Jensen et al., 2001; Mann et al., 2001; Miyoshi et al., 2001; Omoto et al., 2001; Roger et al., 2001; Skliris et al., 2001, 2003, 2006; Murphy et al., 2002; Saunders et al., 2002; Fuqua et al., 2003; Iwase et al., 2003; Shaaban et al., 2003, 2008; Esslimani-Sahla et al., 2004; Fleming et al., 2004; Hopp et al., 2004; Myers et al., 2004; Nakopoulou et al., 2004; O’Neill et al., 2004; Poola et al., 2005b; Miller et al., 2006; Umekita et al., 2006; Gruvberger-Saal et al., 2007; Sugiura et al., 2007; Honma et al., 2008; Novelli et al., 2008; Motomura et al., 2010]. Unfortunately, the techniques for tissue preparation and processing, the antibodies employed, and the scoring systems used to determine ER β positivity are highly variable making it extremely difficult to compare the results and draw specific conclusions as to the relevance of this protein in

breast cancer. Complicating the matter even more is the fact that these studies differ significantly with regard to their patient populations, the number of samples utilized, menopausal status, ethnicity, types of therapies, and consideration of other important biomarkers such as ER α , progesterone receptor, and Her2 as well as patient follow-up times.

These realities highlight the need to develop more reliable and consistent strategies to detect ER β expression which will ultimately enable scientists to further define the relevance of this protein in breast cancer progression and treatment. As a start, we have utilized multiple highly controlled cell model systems and technical approaches to confirm that a number of the commercially available ER β antibodies are non-specific and insensitive for detection of this protein. Even more troubling is the fact that some of them actually cross-react with ER α and therefore lead to erroneous conclusions during data analysis. To address these issues, we have now developed and characterized a novel, highly specific and sensitive, monoclonal ER β antibody (MC10) which detects all forms of ER α . We have also identified a highly specific commercially available antibody which recognizes only ER α 1 (PPG5/10). We hypothesize that the use of both of these antibodies in parallel will allow for a more accurate and complete characterization of the expression of ER β in human breast cancer biopsies and will further our ability to elucidate the potential roles of this protein, and its variants, in breast cancer. Identification of highly specific ER β antibodies, and thorough characterization of such antibodies, is an essential first step in our quest to eventually use ER β as a predictive and/or prognostic biomarker in breast cancer.

MATERIALS AND METHODS

CELL CULTURE

293T cells and parental U2OS osteosarcoma cells were purchased from American Type Culture Collection and grown in phenol red-free Dulbecco's modified Eagle's medium/F12 medium (DMEM/F12) (Sigma-Aldrich, St. Louis, MO) containing 10% Fetal Bovine Serum (FBS) (ISC Bioexpress, Kaysville, UT) and 1% antibiotic/antimycotic (AA) (Invitrogen, Carlsbad, CA). Doxycycline inducible U2OS-ER α , and -ER β cell lines were originally developed in our laboratory as described previously [Monroe et al., 2003]. U2OS-ER α and ER β cells were routinely maintained in phenol red-free DMEM/F12 containing 10% FBS, 1% AA, 5 mg/L blasticidin S (Roche Applied Science, Indianapolis, IN), and 500 mg/L zeocin (Invitrogen).

EXPRESSION AND PURIFICATION OF THE ER β FUSION PROTEIN FOR ANTIBODY GENERATION

The 5'-region of the human ER β gene encoding amino acids 1–140 was PCR amplified and cloned into the pGEX-5X-3 vector (GE Healthcare, Piscataway, NJ) using BamH1 and XhoI restriction enzyme sites. The ER α -GST construct was sequenced to ensure nucleotide integrity and the fusion protein was expressed in DH5 α Escherichia coli cells and purified using a glutathione affinity column (Qiagen, Valencia, CA) as specified by the manufacturer. Purified ER α -GST fusion protein was dialyzed three times in 1 \times PBS and subsequently used for antibody development.

DEVELOPMENT OF ER β MONOCLONAL ANTIBODIES

Balb/c mice were immunized with the purified ER β -GST fusion protein (1–140 aa) and spleen cells were subsequently isolated and fused to HAT sensitive myeloma F/O cells to form hybridomas. Immunizations, cell fusions, and production of individual hybridoma clones were performed by the Mayo Clinic Antibody Core Facility. Fused hybridoma cells were plated as a single cell suspension into 96 well plates for further propagation and

characterization. Individual clones were first screened for GST or ER β antibody production by Enzyme-Linked Immunosorbent Assay (ELISA) analysis using both GST and ER β protein as antigen. ER β positive and GST negative clones were further screened by western blotting for detection of ER β protein. All ELISA- and western-positive clones were finally confirmed for ER β antibody production via immunofluorescence staining using U2OS-ER β expressing cells. ER β positive clones were then again sub-cloned and rescreened as above. This screening method led to the identification of two highly positive clones which were designated as Mayo Clinic (MC) 9 and 10. For large scale production of the ER β monoclonal antibody, MC10 hybridoma cells were sent to Cocalico Biologicals Inc (Reamstown, PA), injected into the peritoneal cavity of Balb/C mice and allowed to proliferate at which time ascites fluid was collected. Following collection, the ascites fluid was centrifuged and the supernatant was used as the source of the MC10 ER β monoclonal antibody described in this manuscript.

WESTERN BLOT AND IMMUNOPRECIPITATION ANALYSIS

293T cells were transfected with Flag-tagged ER α or ER β pcDNA4/TO expression constructs (Invitrogen). Cells were harvested following two days of transfection and lysed in NETN buffer (150 mM NaCl, 1 mM EDTA, 20 mM Tris [pH 8.0], 0.5% Nonidet P-40). Cell lysates were centrifuged and supernatants were immunoprecipitated using 1 μ g of either Flag (M2) (Sigma-Aldrich), ER α (H-20) (Santa Cruz, Santa Cruz, CA), ER β (MC9 and MC10), or mouse IgG (Beckman Coulter, Brea, CA) antibodies. Whole cell lysates and immunocomplexes were separated by SDS-PAGE, transferred to PVDF membranes, probed with primary [Flag, ER α , and ER β (MC9 and MC10)] and secondary antibodies and visualized using enhanced chemiluminescence (GE Healthcare).

PREPARATION OF CELL PELLETS FOR IMMUNOHISTOCHEMISTRY AND IMMUNOFLUORESCENCE

U2OS cells stably expressing Flag-tagged ER α or ER β , under the control of a doxycycline promoter, were expanded in a total of 10 T150 tissue culture flasks. All U2OS cell lines were cultured in both the presence and absence of doxycycline to generate ER $^{+}$ and ER $^{-}$ preparations respectively. At approximately 80% confluence, growth medium was removed, cell monolayers were washed twice with 1 \times PBS and fixed in 10% neutral buffered formalin for 5 min. Following fixation, cells were scraped from each flask, pooled and incubated in formalin at 4 $^{\circ}$ C for a minimum of 12 h. Pooled cells were pelleted, processed, and paraffin embedded prior to use for immunohistochemistry and immunofluorescent staining. The resulting paraffin blocks were sectioned directly and also incorporated into tissue microarrays (TMAs) for use as positive and negative controls on a single slide for testing the specificity of antibodies for immunofluorescence and immunohistochemistry.

IMMUNOHISTOCHEMICAL AND IMMUNOFLUORESCENCE STAINING

Numerous staining protocols were tested using the following ER β antibodies on sections of the formalin-fixed, paraffin embedded, cell pellets described above: Calbiochem GR40, Santa Cruz sc-6820, Chemicon AB1410, Thermo MA1-81281 (PPG5/10), and Mayo Clinic MC10. EDTA pH8.1 and Borg pH 9.5 antigen retrievals were tested on all antibodies. In addition, a citrate-based retrieval solution, low-pH Target Retrieval Solution (Dako, Carpinteria, CA) was used for the Chemicon, MC10, and PPG5/10 antibodies. Antibodies were tested in dilutions ranging from 1:50 to 1:400, with most commercial antibodies providing the best staining results at 1:100. For those antibodies with weak staining at the standard incubation time of 1 h, incubation times were increased to overnight. Envision Dual + Link HRP (Dako) was the secondary label used for all antibodies in the initial evaluations. Staining was visualized by both immunofluorescence and immunohistochemistry for all antibodies. For immunohistochemistry, secondary antibodies were used as described in more

detail below. Slides were digitized using an Olympus NDP brightfield system. For immunofluorescence staining, Alexa Fluor 568 secondary antibodies (Invitrogen) were used to visualize the primary antibody staining. Hoechst 33342 was used to stain nuclear DNA. Slides were viewed and images captured using a Zeiss 510 confocal microscope. In addition to immunofluorescence and immunohistochemistry for ER β , sections were also stained with antibodies against Flag (M2, Sigma-Aldrich) and ER-alpha (clone ID5, M7047, Dako) as controls.

Antibodies which showed specificity for ER β , i.e., exhibited staining in ER β expressing cells with no staining in ER α expressing cells or in cells expressing neither ER α or ER β , were selected for further refinement of staining protocols. The protocols developed in these studies were designed specifically for detection of ER β in formalin-fixed, paraffin embedded human tissues used for standard clinical diagnostic purposes. Ultimately, protocols were finalized for only the PPG5/10 and MC10 ER β antibodies since these were the only two ER β antibodies which were highly specific and sensitive for detection of this protein. Sections obtained from formalin-fixed, paraffin embedded human tissue were deparaffinized in xylene, washed in decreasing concentrations of ethanol, and rehydrated in distilled water. Antigen retrieval was performed by placing slides in a preheated citrate-based solution (low-pH Target Retrieval Solution (Dako)) in a streamer at 98°C for 40 min. The staining procedure was carried out in a Dako Autostainer Plus as follows. Tissue sections were treated with Peroxidase Blocking Reagent (Dako) for 5 min, washed with 1 \times Wash Buffer (Dako), and treated with Background Sniper (Biocare Medical, Concord, CA) for 10 min. The primary antibodies for ER β were diluted in Background Reducing Antibody Diluent (Dako) and incubated for 60 min at room temperature. The PPG5/10 antibody was used at 1:100 and MC10 was used at 1:300. After washing once in Wash Buffer, sections were incubated in a mouse MACH3-HRP two-step system (Biocare Medical) for 20 min each. Slides were washed twice with 1 \times Wash Buffer between steps and before applying Betazoid DAB (Biocare Medical) for 5 min for colorimetric visualization. Counterstaining with hematoxylin and eosin, followed by dehydration in increasing concentrations of ethyl alcohol and xylene, was performed prior to cover slipping. These studies were approved by the Mayo Clinic Institutional Review Board and were conducted under the following protocol: IRB #08-008233.

SCORING OF ER β PROTEIN EXPRESSION IN HUMAN TISSUES

Expression of ER β was scored manually by a dedicated breast pathologist (CR) for proportion and intensity of nuclear staining and the intensity of cytoplasmic staining. The proportion score represents the estimated percentage of positive cells with nuclear staining. No staining, or less than 1%, was considered negative. Intensity staining was assigned none, weak, moderate, or strong for both nuclear and cytoplasmic staining.

CONSTRUCTION OF ER β VARIANT EXPRESSION VECTORS

Our laboratory previously cloned Flag-tagged ER β 1 and ER β 2 into the pcDNA4/TO expression vector (Invitrogen) as described [Monroe et al., 2003; Secreto et al., 2007]. Flag-tagged ER β variants 3–5 were generated using a PCR based approach and primers containing the unique C-terminal sequences for each variant. PCR amplified fragments were sub-cloned into the pcDNA4/TO expression vector. DNA sequencing was performed to ensure proper orientation and sequence integrity.

TRANSIENT TRANSFECTION AND CONFOCAL MICROSCOPY

For detection of ER β 1 and its variants by western blotting, 293T cells were plated in 10 cm dishes, grown to approximately 70% confluence and transiently transfected with 10 μ g of individual ER β expression constructs using FuGENE[®]6 (Roche Diagnostics, Indianapolis,

IN). Twenty-four hours post-transfection, cells were lysed in NETN buffer and western blot analysis was performed as described above. For determination of the sub-cellular localization of ER β variants, parental U2OS cells were grown on cover slips to approximately 30% confluence and subsequently transfected with 250 ng of individual ER β expression constructs using FuGENE[®]6 (Roche Diagnostics) for 24 h. Cells were subsequently fixed with methanol for 1 h and permeabilized with 0.1% Triton X-100 for 5 min on ice. Slides were pre-incubated in 5% goat serum for 1 h to block non-specific binding sites and subsequently incubated with a 1:50 dilution of either Flag, MC10, or PPG5/10 antibodies for another hour. Slides were washed with 1 \times PBS and incubated with a FITC-conjugated anti-mouse secondary antibody (Jackson ImmunoResearch, United Kingdom) for 30 min at room temperature. DAPI was used as a counter-stain for nuclei (Invitrogen). Immunofluorescent detection was conducted using a Zeiss Laser Scanning Microscope 510.

RESULTS

CHARACTERIZATION OF NEWLY DEVELOPED MONOCLONAL ER β ANTIBODIES

In order to determine the specificity of our newly developed monoclonal antibodies for ER β , 293T cells were transiently transfected with human Flag-tagged ER α or ER β expression constructs. Twenty-four hours post-transfection, cells were lysed and immunoprecipitations were performed using an ER α specific antibody, our MC9 and MC10 ER β antibodies, a Flag antibody or an IgG control. Western blotting was then performed using these same antibodies. As shown in Figure 2A, the MC9 and MC10 antibodies did not immunoprecipitate ER α protein, nor did they detect ER α expression in whole cell extracts. However, these two antibodies were able to specifically immunoprecipitate and detect significant levels of ER β protein in whole cell extracts of cells expressing ER β similar to that of the Flag antibody (Fig. 2B). While both MC9 and MC10 were equally sensitive for detection of ER β via immunoprecipitation and western blotting, the MC10 antibody was chosen for use in all of the experiments described below. These data demonstrate that the newly developed ER β monoclonal antibodies are highly specific for detection of ER β without cross-reaction to ER α or other non-specific proteins.

COMPARISON OF MC10 TO COMMERCIALY AVAILABLE ER β ANTIBODIES VIA IMMUNOHISTOCHEMISTRY

We hypothesized that much of the controversy with regard to the role of ER β in mediating breast cancer progression and therapeutic responses was largely due to the lack of highly specific antibodies to detect the protein levels of this receptor in human tissues. Therefore, we proceeded to compare the ability of the MC10 antibody to specifically detect ER β protein via immunohistochemistry, with that of other commercially available antibodies targeting ER β . In order to accomplish this goal, it was essential to first utilize highly controlled cell model systems which were known to be completely ER negative or to specifically express ER α or ER β . Therefore, we utilized our U2OS cell models which express Flag-tagged versions of these receptors under the control of a doxycycline inducible promoter allowing us to turn on or turn off their expression [Monroe et al., 2003]. U2OS cells were cultured in the presence or absence of doxycycline, collected, paraffin embedded, and processed for immunohistochemistry. As a positive control, sections of these cell pellets were first stained with a Flag-specific antibody. As shown in Figure 3A, some non-nuclear background staining was observed in non-doxycycline induced U2OS cells. However, intense nuclear staining was observed in the doxycycline induced U2OS-ER α and -ER β cell lines confirming the expression of the ERs in these model systems (Fig. 3A). We next examined the staining patterns in sections of these cell pellets using the ER α specific antibody purchased from Dako (ID5) which is utilized at the Mayo Clinic for determination

of hormone sensitivity of breast cancer patients. As expected, this antibody was highly specific for detection of ER α with no cross-reaction to ER β (Fig. 3B). Our newly developed MC10 monoclonal antibody exhibited strong nuclear staining only in cells expressing ER β with no cross-reaction to ER α and no background staining in the absence of doxycycline (Fig. 3C). In contrast, the ER β specific antibody purchased from Chemicon (AB1410) exhibited extremely high levels of nuclear and cytoplasmic staining in all cell pellets (Fig. 3D). The ER β antibody purchased from Santa Cruz (sc-6820) revealed strong reactivity in the ER β expressing cells, however, appreciable levels of background staining, both cytoplasmic and nuclear, were also observed in ER α expressing cells and non-doxycycline induced U2OS cells (Fig. 3E). The antibody purchased from Calbiochem (GR40) was unable to detect expression of ER β (Fig. 3F). In contrast, the ER β specific monoclonal antibody (PPG5/10), which can be purchased from Thermo Scientific, Serotec, GeneTex and Dako, exhibited strong nuclear staining in ER β expressing cells with slight background staining observed in the non-doxycycline induced and ER α expressing cell pellets (Fig. 3G). These studies further demonstrate that our newly developed MC10 antibody, unlike that of many of the commercially available antibodies, is able to specifically detect ER β protein via immunohistochemistry without cross-reaction to ER α or other non-specific proteins.

COMPARISON OF MC10 TO COMMERCIALY AVAILABLE ER β ANTIBODIES VIA IMMUNOFLUORESCENCE

The specificity of the MC10 and commercially available antibodies for their targeted proteins were further examined using immunofluorescence detection in the U2OS cell models. As expected, nuclear staining was observed in ER α and ER β expressing U2OS cells using the Flag antibody with little to no background in the absence of doxycycline (Fig. 4A). As with the results observed in Figure 3B, the ER α specific antibody (Dako-ID5) was highly selective for expression of this protein (Fig. 4B). The MC10 antibody exhibited strong nuclear staining only in ER β expressing cells and exhibited no cross-reactivity with ER α or other non-specific proteins (Fig. 4C). The antibody purchased from Chemicon (AB1410) exhibited strong cytoplasmic staining in ER α and ER β expressing cells as well as in the non-doxycycline induced U2OS cells (Fig. 4D). Unlike that observed using immunohistochemistry in Figure 3E, the Santa Cruz antibody (sc-6820) did not result in any appreciable levels of staining as detected by immunofluorescence (Fig. 4E). The antibody purchased from Calbiochem (GR40) was unable to detect ER β expression, but did result in weak cytoplasmic staining in ER α expressing cells (Fig. 4F). The PPG5/10 antibody again detected ER β protein expression with no cross-reaction to ER α (Fig. 4G). These studies further confirm the sensitivity and selectivity of the MC10 antibody for the detection of ER β using immunofluorescence.

DETECTION OF ER β PROTEIN IN HUMAN TISSUE SAMPLES USING MC10 AND PPG5/10 ANTIBODIES

The above studies revealed that the MC10 and PPG5/10 ER β antibodies were specific for detection of ER β protein. However, the cell model systems utilized in these experiments over-expressed ER β 1 and were therefore not necessarily representative of endogenous receptor levels in human tissues. Additionally, these cell model systems do not account for the potential presence of other ER β variants. To address this issue, we next examined the ability of these two antibodies to detect ER β via immunohistochemistry in human tissues known to express this protein. As shown in Figure 5, substantial staining was observed using both antibodies in normal human breast, prostate, and testis tissue. However, the staining patterns were not identical between these two antibodies. More specifically, substantial cytoplasmic staining was observed using the MC10 antibody while the large majority of staining using the PPG5/10 antibody was localized to the nuclei (Fig. 5). The pathological

reports regarding the staining of these tissues for both the PPG5/10 and MC10 antibodies are summarized in Table I.

DETECTION OF ER β VARIANTS USING THE MC10 ANTIBODY

In order to confirm that the MC10 antibody cross-reacts with all variant forms of ER β , pcDNA4/TO expression vectors for ER β 1-5 were transiently expressed individually in 293T cells. Twenty-four hours post transfection, whole cell lysates were prepared and western blotting was performed using the MC10 and PPG5/10 antibodies. As expected, the MC10 antibody was shown to cross-react with all ER β variants while the PPG5/10 antibody was shown to be specific for detection of only ER β 1 (Fig. 6).

SUB-CELLULAR LOCALIZATION OF ER β VARIANTS

Since appreciable levels of cytoplasmic staining were observed in human tissue samples stained with the MC10 antibody (Fig. 5), and since this antibody cross-reacts with all ER β variants, we proceeded to determine and compare the sub-cellular localization of ER β 1-5. Along these lines, parental (ER negative) U2OS cells were plated on cover slips and transiently transfected with Flag-tagged ER β 1-5. Localization of these proteins was first analyzed by immunofluorescent confocal microscopy using a Flag-specific antibody. As shown in Figure 7A, ER β 1 was completely localized to the nucleus. Similar staining patterns were also observed for ER β 2 (Fig. 7A). However, in addition to nuclear staining, appreciable levels of ER β 3-5 were detected in the cytoplasm (Fig. 7A). Identical staining patterns were detected with the MC10 antibody (Fig. 7A). As with the western blotting results (Fig. 6), the PPG5/10 antibody was only able to detect full-length ER β 1 (Fig. 7A). Interestingly, a significant number of cells transfected with either ER β 4 or ER β 5 exhibited a completely different staining pattern characterized as primarily peri-nuclear with some cytoplasmic staining but no nuclear staining (Fig. 7B). This pattern was identical when using the both the Flag and MC10 antibodies (Fig. 7B). As with Figure 7A, no such staining patterns were detected by the PPG5/10 antibody (data not shown). To our knowledge, these studies are the first to have comprehensively characterized the sub-cellular localization of all ER β variants and suggest that the cytoplasmic and/or peri-nuclear staining observed in human tissues with the MC10 antibody (Fig. 5) are likely explained by expression of ER β 3, ER β 4, or ER β 5, or any combination of the three.

DETECTION OF ER β PROTEIN IN HUMAN BREAST TUMORS

The original goals of this study were to produce and/or identify an antibody that was specific for the detection of ER β protein, without cross-reaction to ER α or other unrelated proteins, which would ultimately enhance our ability to further characterize the role of ER β in human breast cancer. Along these lines, we next screened normal breast tissue and a subset of human breast tumors for ER α and ER β protein expression using an ER α specific antibody (Dako-ID5), as well as the MC10 and PPG5/10 ER β specific antibodies. Normal breast ducts were shown to be highly positive for ER β with primarily nuclear staining detected with the PPG5/10 antibody and strong cytoplasmic staining detected with the MC10 antibody (Fig. 8A). Interestingly, of the six breast tumors examined, five exhibited some degree of ER β positivity with both the PPG5/10 and MC10 antibodies (Fig. 8B-F). Of these, one ER α + tumor exhibited nuclear and cytoplasmic staining with both antibodies (Fig. 8B). However, there was significantly more cytoplasmic staining observed with the MC10 antibody (Fig. 8B). Another ER α + tumor exhibited strong cytoplasmic staining using both antibodies with little to no nuclear staining for ER β (Fig. 8C). Of the three ER α -tumors, all exhibited ER β positivity with both antibodies to varying degrees (Fig. 8D-F). Finally, the weakly ER α + breast tumor shown in Figure 8G was essentially negative for ER β using both antibodies. The pathological reports regarding the staining of these specimens for ER β as determined by both the PPG5/10 and MC10 antibodies are summarized in Table II.

DISCUSSION

In the present study, we describe the development and characterization of a novel monoclonal ER β antibody, MC10, which is highly specific and sensitive for detection of full-length ER β 1 as well as all four of its variant forms (ER β 2-5). We have also analyzed the specificity and sensitivity of other commercially available ER β antibodies and have shown that, with the exception of PPG5/10, they are either insensitive or non-specific for detection of this protein. This study is also the first to analyze and compare the sub-cellular localization of all of the ER β variants and has demonstrated that some of these variants exhibit substantial cytoplasmic and peri-nuclear localization patterns. Immunohistochemical staining of normal human tissues and breast cancer biopsies using the PPG5/10 and MC10 antibodies have revealed distinct staining patterns including tumors which exhibit primarily nuclear staining, and others which exhibit primarily cytoplasmic staining, both in the presence and absence of ER α . While significant conclusions regarding the expression of ER β in breast cancer cannot be drawn from this limited cohort of breast tumors, it is apparent that ER β is expressed in a proportion of ER α -tumors and that the sub-cellular localization of ER β as detected by the PPG5/10 and MC10 antibodies is variable.

The differences in nuclear staining between the two antibodies could be explained by the fact that the PPG5/10 antibody recognizes the C-terminal end of ER β 1 while the MC10 antibody was designed against the N-terminal region. It is possible, and potentially likely, that the N-terminal region of ER β is much less accessible than the C-terminal domain due to tertiary protein structure and/or interaction with co-factors in the nucleus which are not disrupted by the antigen retrieval methods. While little is known about the functional role of the N-terminal domain(s) of ER β , we have previously demonstrated that this region, particularly the AF1 domain, confers specificity for at least two known ER β target genes, TIEG1 and RBBP1, and is essential for their regulation in response to estrogen [Monroe et al., 2006; Hawse et al., 2008]. This domain was also shown to be necessary for interaction with the classic co-regulators, SRC1 and SRC2, as they pertain to regulation of these two target genes [Monroe et al., 2006; Hawse et al., 2008]. Interaction with, and epitope masking by, such co-factors in the nucleus could lead to less nuclear staining for ER β when using the MC10 antibody compared to the PPG5/10 antibody. Increased levels of cytoplasmic staining as detected by the MC10 antibody are likely explained by the presence of ER β variants 3-5 since the MC10 antibody was produced using a N-terminal domain which is 100% conserved between all ER β variants while the PPG5/10 antibody was designed to recognize the C-terminal region of only ER β 1. Furthermore, we have demonstrated that ER β 3-5 at least partially localize to cytoplasmic regions. Since these antibodies result in distinct staining patterns in human tissues, it is our opinion that their combined use in large cohorts of well-annotated samples is likely to provide a more comprehensive and accurate analysis regarding the biological significance of total ER β protein expression in breast cancer. Additionally, the use of both antibodies will allow for further stratification of ER β + tumors on the basis of nuclear versus cytoplasmic staining and as a function of ER β 1 positivity in the presence or absence of ER β variants.

A number of past in vitro studies have shown that re-expression of ER β 1 in ER negative breast cancer cells results in the reduction of both basal and estrogen induced proliferation rates [Lazennec et al., 2001; Secreto et al., 2007; Thomas et al., 2011]. Expression of ER β 1 in ER α positive breast cancer cells also results in suppression of proliferation following estrogen exposure [Paruthiyil et al., 2004; Strom et al., 2004; Sotoca et al., 2008]. Furthermore, ER β expression has been shown to increase the effectiveness of selective estrogen receptor modulators (SERMs) such as endoxifen, 4-hydroxytamoxifen, raloxifene, and fulvestrant in cell culture model systems [Strom et al., 2004; Hodges-Gallagher et al.,

2008; Wu et al., 2011]. The summation of these studies suggests that ER β could serve as a prognostic and/or predictive marker in breast tumors.

Unfortunately, the value of ER β protein expression in the clinical setting has not been realized. Indeed, significant discrepancies have been reported for both the rate of ER β expression in breast cancer, as well as its predictive and prognostic value with regard to patient outcomes and responses to targeted therapies. Our findings strongly suggest that some of these discrepancies are related to the use of highly variable and non-specific antibodies. Many studies, including the present report, have demonstrated that ER β is expressed in normal breast epithelial cells [Jarvinen et al., 2000; Roger et al., 2001; Shaaban et al., 2003; Skliris et al., 2001, 2003] and several studies have indicated that ER β expression levels are suppressed or lost in many breast cancers [Leygue et al., 1998, 1999; Iwao et al., 2000; Miyoshi et al., 2001; Roger et al., 2001; Shaw et al., 2002; Park et al., 2003; Skliris et al., 2003; Zhao et al., 2003; Bardin et al., 2004]. However, other studies analyzing the frequency of ER β expression in breast tumors have reported extremely variable results which range from 17–100% [Jarvinen et al., 2000; Jensen et al., 2001; Mann et al., 2001; Miyoshi et al., 2001; Omoto et al., 2001; Skliris et al., 2001, 2006; Saunders et al., 2002; Fuqua et al., 2003; Myers et al., 2004; Nakopoulou et al., 2004; O'Neill et al., 2004; Poola et al., 2005b; Miller et al., 2006; Umekita et al., 2006; Sugiura et al., 2007]. This wide range of ER β positivity is in stark contrast to the well accepted and reported rate of ER α expression which is observed in approximately 70% of all breast cancer cases. Additionally, the frequency of ER β expression in ER α negative tumors which has been reported in the literature ranges from 13–83% [Jensen et al., 2001; Mann et al., 2001; Saunders et al., 2002; Shaaban et al., 2003; O'Neill et al., 2004; Honma et al., 2008]. With regard to the potential functions of ER β in breast cancer, a number of more recent studies have suggested that the presence of this receptor correlates with improved rates of recurrence, disease-free survival, and overall survival [Mann et al., 2001; Omoto et al., 2001; Murphy et al., 2002; Iwase et al., 2003; Esslimani-Sahla et al., 2004; Fleming et al., 2004; Hopp et al., 2004; Myers et al., 2004; Nakopoulou et al., 2004; Sugiura et al., 2007]. However, others indicate little to no correlation [O'Neill et al., 2004; Miller et al., 2006] or even worse prognosis [Speirs et al., 1999a; Speirs et al., 1999b; Jensen et al., 2001]. Lastly, several studies have reported that the presence of ER β in breast tumors increases the effectiveness of tamoxifen therapy [Poola et al., 2005b; Honma et al., 2008; Novelli et al., 2008; Shaaban et al., 2008] or aromatase inhibitor therapy [Motomura et al., 2010].

These matters become further complicated when considering the fact that the majority of studies have utilized a single ER β antibody, which either detects only ER β 1 or cross-reacts with all forms of ER β , making it impossible to analyze the individual contributions of the full-length receptor or its variants. While the functions of ER β 2–5 remain poorly understood, a few publications have suggested that their presence in breast tumors is associated with increased overall survival and increased sensitivity to tamoxifen therapy [Mann et al., 2001; Murphy et al., 2002; Esslimani-Sahla et al., 2004; Hopp et al., 2004; Honma et al., 2008]. It should be of note that these studies have employed antibodies which were designed to cross react with all forms of ER β (ER β 1–5) and therefore make it impossible to attribute these favorable responses to either the full-length receptor or one or more of its variants. A couple of reports using an ER β 2 specific antibody have shown that its presence in breast tumors correlates with increased overall survival [Sugiura et al., 2007; Shaaban et al., 2008] and one report stated the same for ER β 5 [Shaaban et al., 2008]. Another factor to be considered is the sub-cellular localization of these receptors since we have now shown that ER β 3–5 can localize to cytoplasmic and peri-nuclear regions of the cell in addition to the nucleus. A number of past studies have noted the appearance of cytoplasmic staining for ER β , but the majority have ignored the potential significance of this observation with the exception of one manuscript which reported that cytoplasmic ER β 2

positivity was correlated with decreased overall survival [Shaaban et al., 2008]. Of note, our studies suggest that ER β 2 is localized within the nuclei of cells and again call into question the specificity of this particular antibody.

In summary, we describe the development and characterization of a novel ER β monoclonal antibody, MC10, which is highly specific for the detection of ER β 1 and its four variant forms via western blotting, immunoprecipitation, immunohistochemistry, and immunofluorescence. We also provide evidence that some commercially available ER β antibodies are either non-specific or insensitive for the detection of ER β with the exception of the ER β 1 specific PPG5/10 antibody. The use of these, and other, non-specific ER β antibodies in clinical samples has likely created much of the controversy regarding the functions of this protein in breast cancer. Additionally, our results raise significant concerns relating to the interpretation of data presented in previous manuscripts which utilized either commercially obtained, or independently developed, ER β antibodies in the absence of thorough characterization with regard to their sensitivity and specificity. The present data also reveal that ER β variants 3–5 exhibit some extent of cytoplasmic and peri-nuclear sub-cellular localization and further research is needed to determine whether such staining patterns in breast tumors could be of predictive and/or prognostic value. The summation of our studies highlight the need to further analyze the role of ER β in breast cancer using highly specific and validated antibodies. Additionally, the use of ER β antibodies which do not detect all ER β variants, or the use of a single ER β antibody which does not discriminate between ER β 1 and its variants, is unlikely to reveal the complete biological significance of total ER β expression in breast cancer and may in part explain the conflicting studies which have been reported for ER β in the literature. The use of the newly developed MC10 antibody, in combination with other highly specific ER β antibodies such as PPG5/10, may provide additional discriminatory features of value in predicting the response of breast cancer patients to endocrine therapies and/or their association with other clinicopathological factors. Lastly, the ability to accurately identify ER β expression in ER α negative breast tumors could provide a basis to treat a large number of women with safe and effective hormonal based therapies which at present are not considered as an option.

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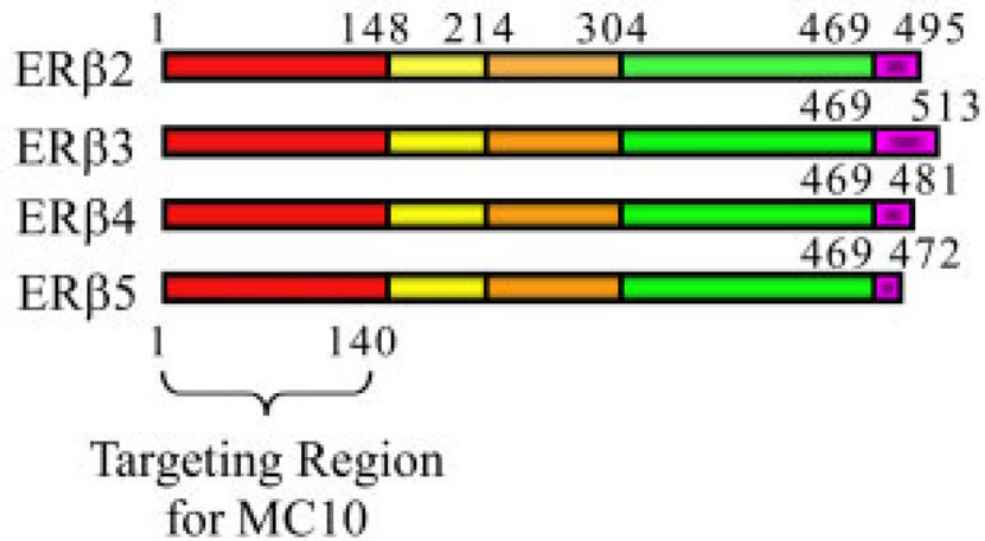
ER β 1 Protein Domains:**Variants:**

Fig. 1. Diagram depicting the domain structures of human full-length ER α 1 and its variant forms (ER α 2–5) as well as the targeting region for the MC10 monoclonal ER β antibody.

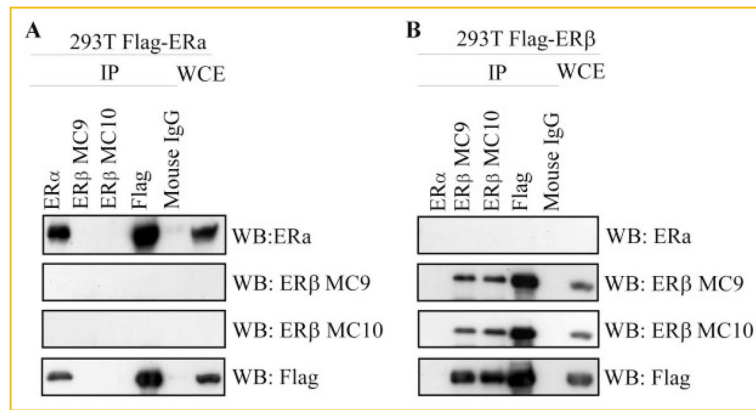


Fig. 2. Specificity of MC9 and MC10 monoclonal antibodies for ER β protein as determined by immunoprecipitation and western blotting. 293T cells were transfected with either a Flag-tagged ER α (A) or Flag-tagged ER β (B) expression vector for 24 h and equal amounts of cell lysates were immunoprecipitated with indicated antibodies. Immunoprecipitated proteins (IP) were separated by SDS-PAGE and western blotting (WB) was performed using indicated antibodies. Detection of non-immunoprecipitated ER α or ER β protein levels were also determined by western blotting in whole cell extracts (WCE).

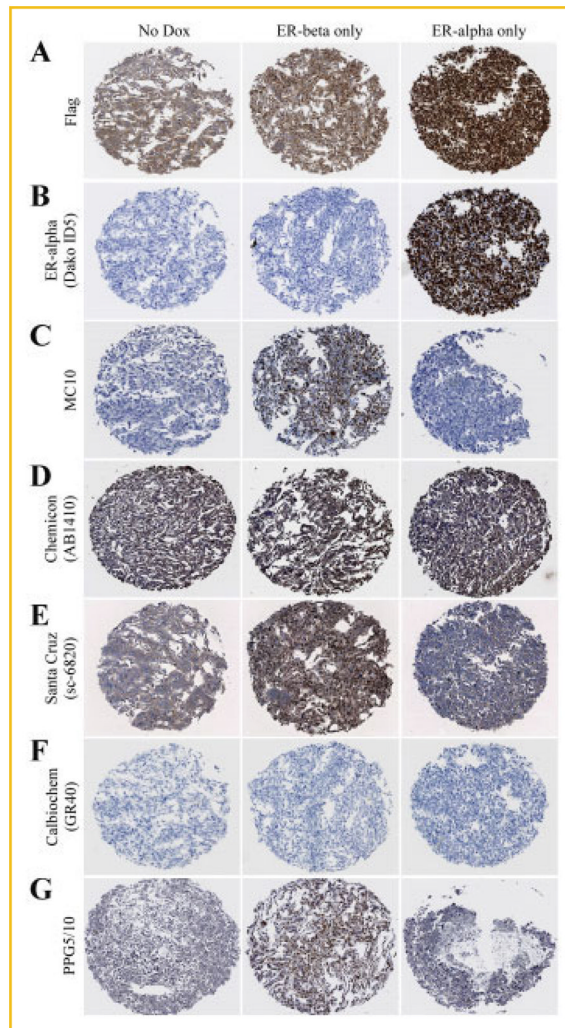


Fig. 3. Specificity and comparison of the MC10 monoclonal antibody for ER β protein to that of other commercial antibodies as determined by immunohistochemistry. U2OS cells expressing either Flag-tagged ER β or ER α under the control of a doxycycline inducible promoter were pelleted, paraffin embedded, sectioned, and processed for immunohistochemistry. Sections of un-induced cells, ER β expressing cells and ER α expressing cells were stained with a monoclonal Flag antibody (1:100) (A), an ER α antibody (Dako ID5, 1: 50) (B), or the following ER β antibodies: MC10 (1:300) (C), Chemicon (AB1410, 1:400) (D), Santa Cruz (sc-6820, 1:100) (E), Calbiochem (GR40, 1:100) (F), and PPG5/10 (1:100) (G).

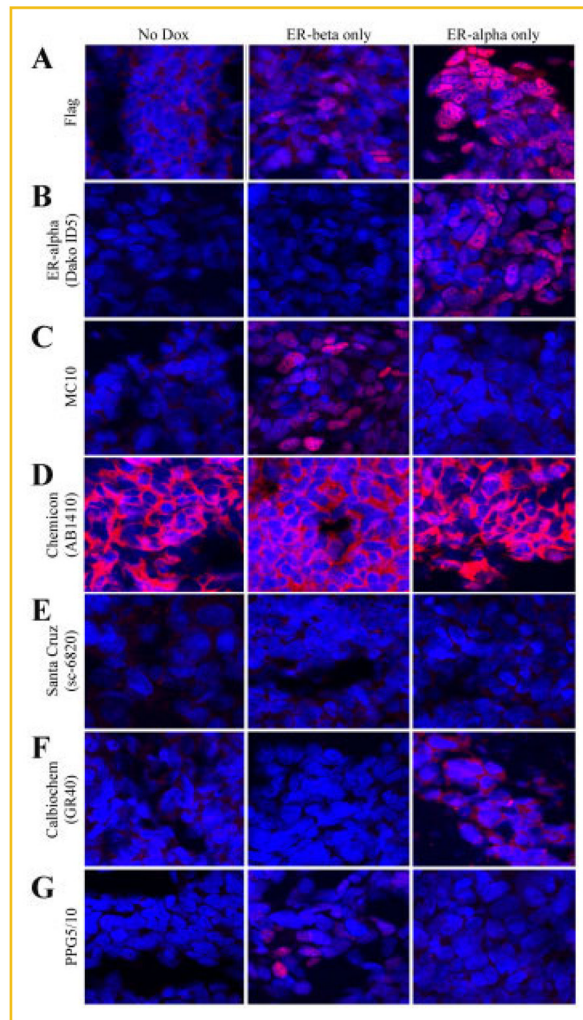


Fig. 4. Specificity and comparison of the MC10 monoclonal antibody for ER β protein to that of other commercial antibodies as determined by immunofluorescence. U2OS cells expressing either Flag-tagged ER β or ER α under the control of a doxycycline inducible promoter were pelleted, paraffin embedded, sectioned, and processed for immunofluorescence. Sections of un-induced cells, ER β expressing cells and ER α expressing cells were stained with a monoclonal Flag antibody (1:100) (A), an ER α antibody (Dako ID5, 1: 50) (B), or the following ER β antibodies: MC10 (1:300) (C), Chemicon (AB1410, 1:400) (D), Santa Cruz (sc-6820, 1:100) (E), Calbiochem (GR40, 1:100) (F), and PPG5/10 (1:100) (G).

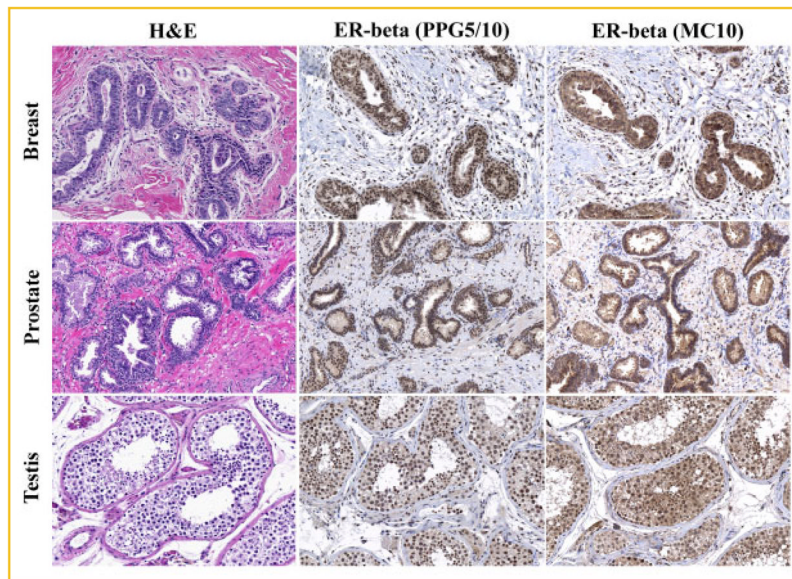


Fig. 5. Detection of ER β protein in normal human tissues using the MC10 and PPG5/10 monoclonal antibodies. Serial sections of normal human breast, prostate, and testis tissue were processed for immunohistochemistry and stained with the PPG5/10 (1:100) and MC10 (1:300) antibodies. One section was also stained with hematoxylin and eosin (H&E) for histological purposes.

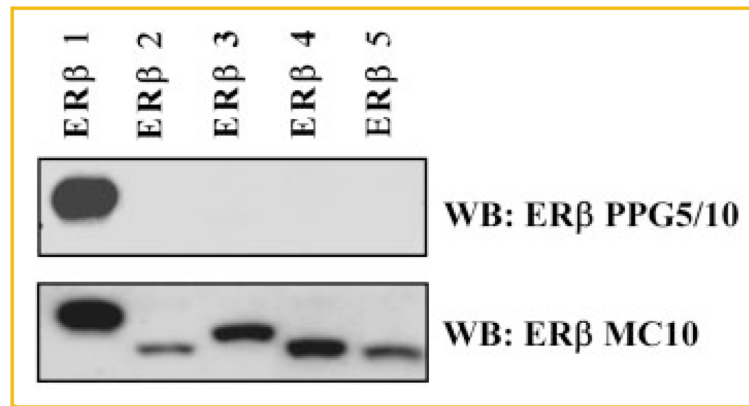


Fig. 6. Detection of full-length ERβ1 and its variants (ERβ2-5) by the MC10 and PPG5/10 monoclonal antibodies. 293T cells were transiently transfected with expression vectors for ERβ1-5 for 24 h. Equal amounts of whole cell lysates were separated by SDS-PAGE and western blotting (WB) was performed using the PPG5/10 and MC10 antibodies. PPG5/10 was shown to only detect ERβ1 while the MC10 antibody was shown to cross-react with ERβ1-5.

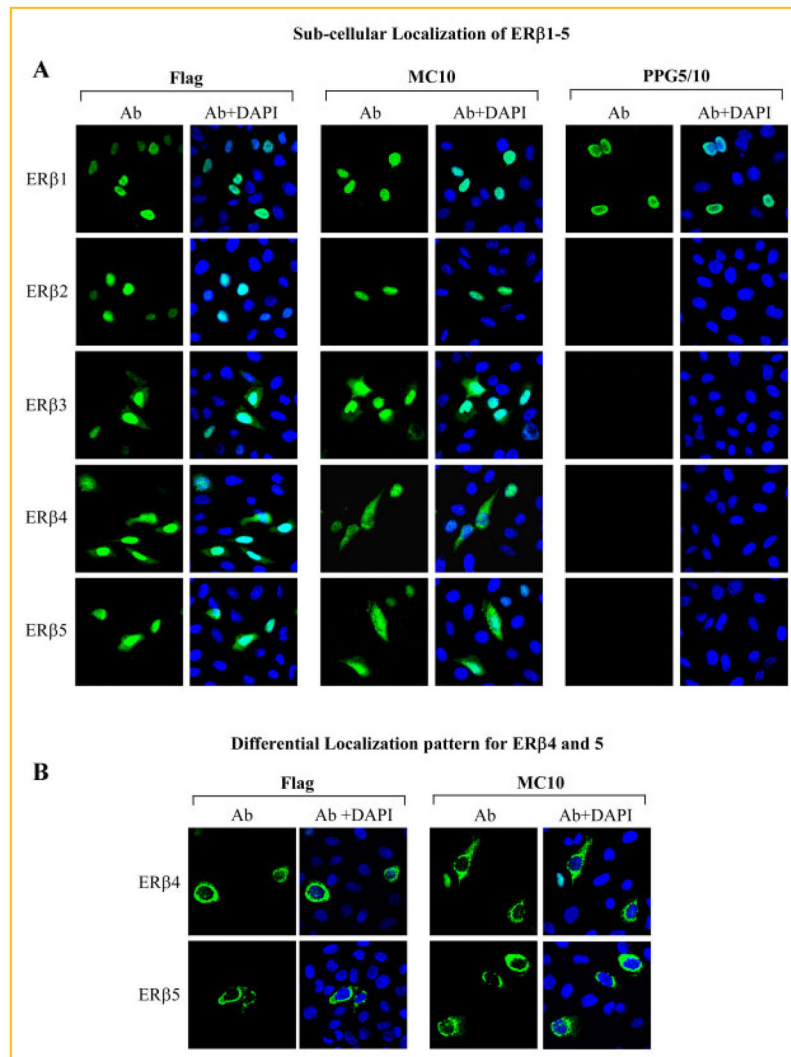


Fig. 7. Sub-cellular localization of ERβ1–5. U2OS cells were plated on cover slips and transiently transfected with Flag-tagged expression vectors for ERβ1–5 for 24 h. Cells were subsequently fixed, permeabilized, and incubated with either the monoclonal Flag (1:50), MC10 (1:50), or PPG5/10 (1:50) antibodies. Localization of ERβ1–5 was determined using a FITC-labeled anti-mouse secondary antibody and confocal microscopy. A: The PPG5/10 antibody was only able to detect ERβ1 expression while the MC10 antibody was able to detect all forms of ERβ. ERβ1 and ERβ2 were completely localized within the cell nucleus while appreciable levels of cytoplasmic localization were also observed for ERβ3–5 using both the Flag and MC10 antibodies. B: A significant number of cells transfected with ERβ4 and ERβ5 expression vectors also exhibited a differential sub-cellular localization pattern characterized by strong peri-nuclear and punctate cytoplasmic staining in the absence of nuclear staining with both the Flag and MC10 antibodies.

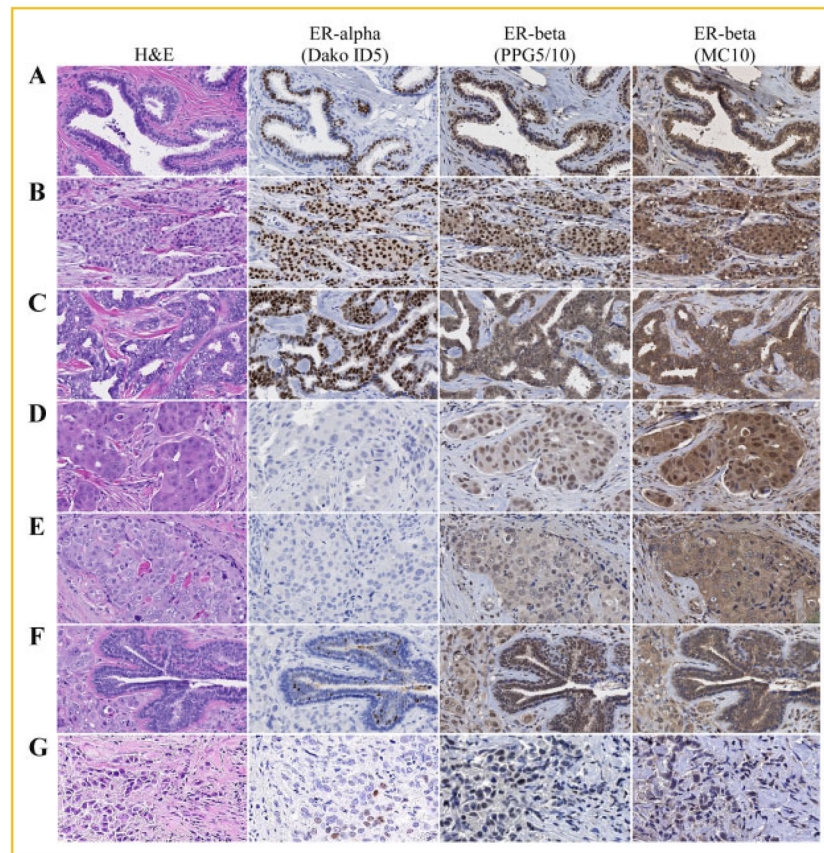


Fig. 8. Detection of ER β protein in normal and cancerous human breast tissue using the MC10 and PPG5/10 monoclonal antibodies. Serial sections of a normal human breast, as well as six randomly selected breast tumors were processed for immunohistochemistry and stained with the ER α specific Dako ID5 antibody (1:50) and the ER β specific PPG5/10 (1:100) and MC10 (1:300) antibodies as indicated. One section was also stained with hematoxylin and eosin (H&E) for histological purposes. A: Normal human breast tissue. B,C: ER α + human breast tumors. D–F: ER α -human breast tumors. G: A weakly ER α + and ER β -human breast tumor. Note the different staining patterns for ER β as detected by the PPG5/10 and MC10 antibodies for many of these specimens.

TABLE I

Pathological Scoring for ER β in Normal Human Breast, Prostate, and Testis Tissue Sections as Detected by the PPG5/10 and MC10 ER β Monoclonal Antibodies.

Tissue type	PPG5/10		MC10	
	Nuclear	Cytoplasmic	Nuclear	Cytoplasmic
Breast	>95%; Strong	Weak	<1%	Moderate
Prostate	60–75%; Strong	Weak	<1%	Weak to Moderate
Testis	80–90%; Strong	Weak	50–60%; Strong	Moderate

TABLE II

Pathological Scoring for ER β in Normal Human Breast and Breast Tumor Sections as Detected by the PPG5/10 and MC10 ER β Monoclonal Antibodies.

Tissue	PPG5/10		MC10	
	Nuclear	Cytoplasmic	Nuclear	Cytoplasmic
Normal Breast	>90%; Strong	Weak	<1%	Moderate
Tumor B	80%; Strong	Weak	<1%	Strong
Tumor C	<1%	Moderate	<1%	Strong
Tumor D	70%; Moderate	Weak	30–40%; Moderate	Moderate to Strong
Tumor E	<1%	Weak	<1%	Moderate
Tumor F	10–20%; Strong	Weak	<1%	Moderate
Tumor G	<1%	None	<1%	Weak