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Comprehensive assessment of estrogen receptor beta antibodies in cancer cell line models and tissue reveals critical limitations in reagent specificity

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ABSTRACT

Estrogen Receptor- β (ER β) has been implicated in many cancers. In prostate and breast cancer its function is controversial, but genetic studies implicate a role in cancer progression. Much of the confusion around ER β stems from antibodies that are inadequately validated, yet have become standard tools for deciphering its role. Using an ER β -inducible cell system we assessed commonly utilized ER β antibodies and show that one of the most commonly used antibodies, NCL-ER-BETA, is non-specific for ER β . Other antibodies have limited ER β specificity or are only specific in one experimental modality. ER β is commonly studied in MCF-7 (breast) and LNCaP (prostate) cancer cell lines, but we found no ER β expression in either, using validated antibodies and independent mass spectrometry-based approaches. Our findings question conclusions made about ER β using the NCL-ER-BETA antibody, or LNCaP and MCF-7 cell lines. We describe robust reagents, which detect ER β across multiple experimental approaches and in clinical samples.

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1. Introduction

Estrogen receptor beta (ER β) was first discovered in the rat prostate (Kuiper et al., 1996). Since then, there has been considerable interest in understanding its role in both breast and prostate cancer. Despite a large body of literature, the function of ER β in these two cancers remains unclear (Haldosen et al., 2014; Nelson et al., 2014). Most authors agree that ER β has a predominantly antiproliferative, pro-apoptotic and tumor-suppressive role (Attia and Ederveen, 2012; Bottner et al., 2014; Chang and Prins, 1999; Ellem and Risbridger, 2007; Horvath et al., 2001; Madak-Erdogan

et al., 2013; McPherson et al., 2010; Muthusamy et al., 2011; Nakajima et al., 2011; Rizza et al., 2014; Ruddy et al., 2014; Zhu et al., 2004), however ER β has also been implicated as an oncogene. This is particularly in the context of Castrate Resistant Prostate Cancer (CRPC) where it has been proposed as a driver of androgen receptor (AR)-dependent gene transcription (Yang et al., 2012, 2015), along with a potential role in mediating the transition from hormone-sensitive to CRPC (Zellweger et al., 2013). In breast cancer, it has been suggested that ER β may have a 'bi-faceted role' and should not simply be considered a tumor-suppressor (Jonsson et al., 2014). ER β has been reported to 'cross-talk' with androgen receptor-positive breast cancer (Rizza et al., 2014) and may be an important factor in ER α -negative breast cancer (Gruvberger-Saal et al., 2007; Smart et al., 2013).

Inconsistencies in the reported expression of ER β in breast and

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prostate cancers as determined by immunohistochemistry (IHC) have contributed to this uncertainty. In prostate, most data support the conclusion that ER β is highly expressed in benign epithelial cells, with expression declining in cancer development and inversely correlating with increasing Gleason grade (Asgari and Morakabati, 2011; Attia and Ederveen, 2012; Dey et al., 2014; Horvath et al., 2001; Leav et al., 2001; Risbridger et al., 2007). However, it has also been reported that ER β expression is high in bone and lymph node metastases (Bouchal et al., 2011; Zhu et al., 2004) and that high ER β expression correlates with poor clinical prognosis (Horvath et al., 2001; Zellweger et al., 2013). In breast cancer, high ER β expression has been described both as a poor (Guo et al., 2014) and favorable (Esslimani-Sahla et al., 2004; Gruvberger-Saal et al., 2007; Hieken et al., 2015; Leygue and Murphy, 2013; Myers et al., 2004; Omoto et al., 2002; Roger et al., 2001) prognostic marker, with others finding no association between clinico-pathological parameters and ER β expression (Umekita et al., 2006).

It is recognized that there is wide variability in the sensitivity and specificity of ER β antibodies, which may contribute to the uncertainties surrounding its molecular action and tissue expression (Choi et al., 2001; Hartman et al., 2012; Skliris et al., 2002; Weitsman et al., 2006; Wu et al., 2012). Previous ER β antibody validation studies have been published (Carder et al., 2005; Choi et al., 2001; Skliris et al., 2002; Weitsman et al., 2006; Wu et al., 2012), however some of them are limited by reliance on two key assumptions. Firstly, that when assessing an antibody by Western blotting in a cell line model, the factor of interest is expressed and secondly, when assessing an antibody's specificity by IHC in tissue, the tissue expression of the factor has been well characterized. In the case of ER β , these assumptions are problematic, as its expression in commonly used cell line models and in tissues is not universally accepted (Al-Bader et al., 2011; Asgari and Morakabati, 2011; Attia and Ederveen, 2012; Bouchal et al., 2011; Dey et al., 2014; Gruvberger-Saal et al., 2007; Guo et al., 2014; Hieken et al., 2015; Holbeck et al., 2010; Horvath et al., 2001; Leav et al., 2001; Nakajima et al., 2011; Omoto et al., 2002; Risbridger et al., 2007; Shaaban et al., 2003; Skliris et al., 2002; Umekita et al., 2006; Zellweger et al., 2013; Zhou et al., 2012; Zhu et al., 2004).

In light of this, we sought to test and validate six commonly used, commercially available ER β antibodies and two non-commercially available ER β antibodies (Choi et al., 2001; Wu et al., 2012) in a systematic manner that addresses these assumptions. To achieve this, we employed a number of assays for antibody validation, including a novel proteomic-based pull down method called Rapid Immunoprecipitation Mass spectrometry of Endogenous protein (RIME) (Mohammed et al., 2013). We then applied successfully validated antibodies to cell line models of breast and prostate cancer commonly used for studies of ER β to assess them for ER β expression. ER β expression in the cell lines was validated by a non-antibody dependent, targeted proteomics method known as Parallel Reaction Monitoring (PRM) (Gallien et al., 2012). Finally, benign and malignant prostate and breast tissues were stained with the validated ER β antibody to assess tissue expression of ER β by IHC.

2. Materials and methods

2.1. Cell culture

The cancer cell line MDA-MB-231 with doxycycline-inducible ER β expression (MDA-MB-231-ER β) (Reese et al., 2014) was cultured in Dulbeccos Modified Eagle Medium with F12 supplement (DMEM/F12) with 10% heat-inactivated tetracycline-free fetal bovine serum (FBS) (Fisher-Scientific), 2 mM L-glutamine, 50 U/ml

penicillin, 50 μ g/ml streptomycin, 5 μ g/ml blasticidin S (Invivogen) to select for the tetracycline repressor and 500 μ g/ml zeocin (Invitrogen) to select for the ER β expression vector. To induce ER β expression in MDA-MB-231-ER β cells, 15 cm² plates were seeded with 5×10^6 cells and doxycycline added at either 0.1 μ g/ml (for Western blot, real-time polymerase chain reaction (qRT-PCR) and PRM) or 0.5 μ g/ml (for RIME) for 24 h. The MCF-7 breast cancer cell line was cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% heat-inactivated FBS (Fisher-Scientific), 2 mM L-glutamine, 50 U/ml penicillin and 50 μ g/ml streptomycin. The LNCaP prostate cancer cell line was cultured in RPMI 1640 with 10% heat-inactivated FBS (Fisher-Scientific), 2 mM L-glutamine, 50 U/ml penicillin and 50 μ g/ml streptomycin. All cells were incubated at 37 °C with 5% CO₂ and cultured to 80–90% confluence. LNCaP and MCF-7 cell lines were obtained from ATCC (Middlesex, UK) and validated by STR genotyping.

2.2. Preparation of mRNA and qRT-PCR

MDA-MB-231-ER β +, MDA-MB-231-ER β –, MCF-7 and LNCaP cells were harvested for collection of mRNA using the RNeasy Mini Kit (Qiagen, California USA). On-column DNase digestion was performed to remove contaminating genomic DNA. RNA was quantified with the NanoDrop 8000 (Thermo Scientific, Delaware USA). Samples containing 250 ng random primers, 1 μ g RNA, 1 μ l 10 mM dNTP mix and water to a total volume of 13 μ l were heated to 65 °C for 5 min, followed by 1 min incubation on ice. To each sample 4 μ l 5X First-strand buffer, 1 μ l 0.1 M DTT, 1 μ l RNaseOUT and 1 μ l SuperScript III reverse transcriptase (RT) (ThermoFisher Scientific, Leicestershire, UK) were added and incubated at 25 °C for 5 min then 50 °C for 60 min followed by heating at 70 °C for 15 min. qRT-PCR primers for wild type ER β (Table 1) were designed based on published sequence of ESR2 (available from USCS genome browser at <http://genome.ucsc.edu/>) using the Primer3 software package (Koressaar and Remm, 2007; Untergasser et al., 2012) available at <http://bioinfo.ut.ee/primer3-0.4.0/primer3/>. UBC primers (SY121212648) were obtained from Sigma-Aldrich (Dorset, UK). Each qRT-PCR reaction contained 7.5 μ l Power SYBR Green PCR Master Mix (Applied Biosystems, California USA), 0.5 μ l of 10 μ M primer mix, 2 μ l of a 1:5 dilution of cDNA and nuclease-free water to a final volume of 15 μ l. Reactions were performed with the Stratagene Mx3005P RealTime machine in triplicate. Hot-start Taq polymerase was heat-activated at 95 °C for 10 min followed by 40 cycles of 15 s at 95 °C and 30 s at 60 °C. Fluorescence was read in each cycle and a melting curve constructed as the temperature was increased from 65 °C to 95 °C with continuous fluorescence readings. UBC was used as a control gene to normalize between the samples and relative expression determined using the delta-delta Ct method (Livak and Schmittgen, 2001).

2.3. Western blotting

MDA-MB-231-ER β +, MDA-MB-231-ER β –, MCF-7 and LNCaP cells were harvested for nuclear extract using the Ne-Per nuclear extraction kit (Thermo Scientific Pierce, Rockford IL USA) according to the manufacturer's instructions. Extracted protein was quantified using the Direct Detect system (Merrick Millipore, Massachusetts USA). Nuclear extracts were prepared with 4X protein sample loading buffer (LI-COR Biosciences, USA), 10X NuPage sample reducing agent (ThermoFisher Scientific, Leicestershire, UK) and water, and 15 μ g protein per lane loaded into Bolt 4–12% Bis-Tris gels (ThermoFisher Scientific, Leicestershire, UK). Gels were run with MOPS running buffer for 30 min at 60 V followed by 30 min at 120 V. Western transfer was performed using the iBlot system (Invitrogen, Paisley, UK) according to the manufacturer's

instructions. Odyssey blocking buffer (LI-COR Biosciences, USA) was added to membranes for one hour at room temperature. Primary antibodies (Table 2 and Supplementary Fig. 1) were added at the following dilutions and incubated overnight at 4 °C: Novocastra-ER-beta (EMR02-NCL-ER-BETA) (Leica Biosystems, Newcastle, UK) 1:100, ERβ1 PPG5/10 (MAI-81281) (Thermo Scientific Pierce, Rockford IL USA) 1:100, ERβ-antibody H150 (sc8974) (Santa Cruz Biotechnology, Dallas TX, USA) 1:200, CWK-F12, USA) (Choi et al., 2001) 1:200, MC10 (Wu et al., 2012) 1:300, GeneTex ERβ 70182 (Irvine, CA, USA) 1:200, ERβ 06-629 (Merck Millipore, Watford, UK), 1:500, Abcam 288 [14C8] (Cambridge, UK) 1:500. The following were used as loading controls: rabbit anti-beta actin (ab8227) (Abcam, Cambridge, UK) 1:5000 or mouse anti-beta actin [AC-15](ab6276) 1:1000 according to the species of the ERβ antibody. The membranes were washed three times with PBS/0.1% tween and incubated with secondary antibodies for one hour at room temperature: Goat anti-mouse (green) 1:5000 with Goat anti-rabbit (red) 1:20000 or Goat anti-rabbit (green) 1:5000 with Goat anti-mouse (red) 1:20000 according to the species of the ERβ antibody. Membranes were imaged using the Li-Cor Odyssey fluorescent imaging system (LI-COR Biosciences, USA).

2.4. Immunohistochemistry (IHC)

Formalin-fixed, paraffin-embedded MDA-MB-231-ERβ− and MDA-MB-231-ERβ+ cell pellets were generated, with $\sim 2 \times 10^7$ cells per pellet. ERβ expression was induced with 0.5 µg/ml doxycycline for 24 h. Antigen retrieval was achieved by incubating in citrate-based retrieval solution for 20 min. Sections were stained using CWK-F12 ERβ antibody, diluted 1:250 in standard Bond diluent using Leica's Polymer Refine Kit (Catalogue No: DS9800) on the automated Bond platform (Leica Biosystems Newcastle Ltd, Newcastle UK). Images were captured using Aperio® software (Leica Biosystems Newcastle Ltd, Newcastle UK).

A prostate tissue microarray (TMA) was created from a random selection of prostate cancers, including a range of different tumor grades, and benign prostatic tissue (10 cancer, 5 benign in total) (ethical approval: ProMPT study MREC/01/4/061). The areas to be sampled from the formalin-fixed and paraffin embedded tissue blocks were marked on the corresponding Haematoxylin and Eosin stained paraffin sections. Each block was assessed to ensure that there was an adequate amount of tissue for sampling, and cores of tissue punched from the selected area of the block using 5 mm skin biopsy punches. Each core was re-embedded into a new recipient paraffin block and its position in the block recorded on a TMA map. Cores of pig kidney were used as orientation markers.

The breast TMA was constructed using the Chemicon Advanced Tissue Arrayer (Merck Millipore, Germany) according to the manufacturer's instructions. This contained 30 benign samples, 56 grade I, 55 grade II and 57 grade III ER alpha positive tumors. An additional TMA was constructed from 10 invasive carcinomas and 10 non-malignant tissues for optimisation of antibody staining. To ensure adequate representation of the tissue, core size of 1 mm was selected and cores arranged in duplicate with liver and spleen as orientation cores. The study protocol for tissue collection was approved by the University of Adelaide Human Research Ethics

Committee (#s H-2005-065).

For the prostate IHC, 3.5 µm sections were cut and mounted onto charged slides, dried and sealed with paraffin. The CWK-F12 ERβ antibody was further optimized to the clinical samples and diluted at 1:200 in diluent consisting of 1% donkey serum, 0.05% Tween20 in 300 mM TBS to reduce background staining. Antigen retrieval was achieved by incubating in Tris EDTA for 20 min at 100 °C. Images were captured at 250 × magnification using Image Pro-Insight (Media Cybernetics, Rockville, MD, USA).

For the breast IHC, 4 µm sections were cut and adhered to Superfrost UltraPLUS slides (Thermo-Fisher Scientific #1014356190). Slides were dewaxed in xylene followed by 100% EtOH and then PBS. Endogenous peroxidase was quenched with 0.3% hydrogen peroxide (Ajax Finchem #7722-84-1). Antigen retrieval was performed in 10 mM Citric acid buffer (pH 6.0) within a decloaking chamber (Biocare Medical #DC2012), for 5 min at 120 °C. Slides were blocked in 5% normal goat serum (Sigma-Aldrich #G9023) in PBS for 30 min at room temperature. CWK-F12 antibody was added at a dilution of 1:100 and incubated overnight at 4 °C. A second section of TMA tissue that received buffer in the absence of primary antibody served as a negative control. Secondary antibody (biotinylated anti-mouse antibody (Dako #E0433) diluted in PBS with 5% normal goat serum was added and incubated for 60 min at room temperature. Sections were washed twice in PBS followed by addition of HRP-conjugated streptavidin (Dako #P0397). Tissue was counterstained with haematoxylin and mounted under DPX mountant (Sigma #06522). Slides were scanned on a Nanozoomer slide scanner (Hamamatsu #C9600).

2.5. Rapid immunoprecipitation and Mass Spectrometry of Endogenous Protein (RIME)

RIME experiments were conducted as previously described (Mohammed et al., 2013). Briefly, MDA-MB-231-ERβ−, MDA-MB-231-ERβ+ (2×10^7 cells per condition for antibody evaluation), LNCaP and MCF-7 cells (4×10^7 cells per condition for cell line characterization) were grown in 15 cm² plates to 90% confluency. Cells were crosslinked with media containing 1% EM grade formaldehyde (TEBU biosciences, Peterborough UK) for 8 min and the formaldehyde quenched with 0.1 M glycine. Cells were washed, harvested and pelleted in cold PBS. To enrich the nuclear fraction the cell pellet was suspended in 10 ml of lysis buffer 1 (50 mM HEPES-KOH [pH 7.5], 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP-40 or Igepal CA-630, and 0.25% Triton X-100) for 10 min at 4 °C. Cells were pelleted and resuspended in lysis buffer 2 (10 mM Tris-HCl [pH 8.0], 200 mM NaCl, 1 mM EDTA, and 0.5 mM EGTA) for five minutes at 4 °C. Cells were pelleted and resuspended in 300 µl of lysis buffer 3 (10 mM Tris-HCl [pH 8], 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.1% Na-deoxycholate, and 0.5% N-lauroylsarcosine) and sonicated (Diagenode bioruptor, Diagenode, Seraing Belgium) for 45 min 30 µl of 10% Triton-X was added and the sonicated lysate centrifuged at 17,000G for 10 min to remove cell debris. The supernatant was incubated with 100 µl of magnetic beads (Dyna-beads®, Thermo Fisher Scientific, Waltham MA USA) pre-bound with antibody.

For evaluation of the 8 ERβ antibodies, immunoprecipitations (IP) were set up each for MDA-MB-231-ERβ− and MDA-MB-231-ERβ+ cells using 10 µg of antibody (NCL-ER-BETA, GeneTex 70182, Millipore 06-629, Abcam 288 [14C8], MC10, CWK-F12, sc8974 and PPG5/10). For characterization of LNCaP and MCF-7 cells, 20 µg of MC10 ERβ antibody was used in each IP. In all cases, 10 µg of E2F1-C20 IP was used as a positive control (Sc-193, Santa Cruz Biotechnology, Dallas TX, USA) and species-specific IgG used to detect non-specific pull-down (Mouse sc2025 or Rabbit sc2027, Santa Cruz Biotechnology, Dallas TX, USA). Samples were incubated

Table 1

Sequence of ERβ mRNA primers used in qRT-PCR validation of the MDA-MB-231-ERβ cell line. These primer sequences flank a region spanning exons 2 and 3, which is common to wild type ERβ and ERβ isoforms.

Primer	Sequence
ERβ – fwd	5' AAAACCGCGCAAGACGCTG 3'
ERβ – rev	3' TGCTCGTCGGCACTTCTCTG 5'

Table 2

Details of ER β antibodies validated. Application details are as recommended by the manufacturer. IHC, immunohistochemistry; WB, western blot; IF, immunofluorescence; ChIP, chromatin immunoprecipitation; ELISA, enzyme-linked immunosorbent assay; Flow cyt, flow cytometry; ICC, immunocytochemistry; IP, immunoprecipitation; Wt, wild type; NTD, N terminal domain; LBD, ligand binding domain.

Antibody	Immunogen	Host species	Class	Binding region	Application
NCL-ER-BETA	Recombinant protein. Wt ER β . C terminus	Mouse	Monoclonal	C terminus	IHC, WB
PPG5/10	Synthetic peptide C terminus of wt ER β	Mouse	Monoclonal	C terminus	IF, IHC, WB
GeneTex 70182	Amino acids 1–153 of human ER β expressed in E.coli	Mouse	Monoclonal	N terminus	IP, WB, ChIP
Millipore 06-629	Amino acids 46–63 of human ER β	Rabbit	Polyclonal	NTD	WB, IHC
Santa cruz sc8974	Amino acids 1–150 of human ER β	Rabbit	Polyclonal	N terminus	WB, ChIP, IF, ELISA
Abcam 288 [14C8]	Recombinant fusion protein. Amino acids 1–153 of human ER β in E.coli	Mouse	Monoclonal	N terminus	WB, Flow cyt, IHC, ICC, ChIP
CWK-F12	Recombinant protein. Amino acids 272–285 of human wt ER β	Mouse	Monoclonal	LBD	WB, IP, IHC
MC10	Fusion protein. Amino acids 1–140 of human ER β in E.coli	Mouse	Monoclonal	N terminus	IHC, IP, WB, IF

overnight at 4 °C. Beads were washed 10 times in 1 ml RIPA buffer (50 mM HEPES pH 7.6, 1 mM EDTA, 0.7% Na deoxycholate, 1% NP-40, 0.5 M LiCl) and twice in 100 mM ammonium hydrogen carbonate (AMBIC) solution. Dry, frozen beads were submitted for tryptic digestion of bead-bound protein, and peptides pulled down by IP identified by mass-spectrometry (LTQ Velos-Orbitrap MS, Thermo Fisher Scientific, Waltham MA USA). Raw MS data files were processed using Proteome Discoverer v.1.3 (Thermo Scientific). Processed files were searched against the SwissProt human database using the Mascot search engine version 2.3.0 with a false discovery rate (FDR) of less than 1%. For each ER β antibody tested, the resulting list of purified peptides identified was filtered against the corresponding IgG control to remove non-specific proteins pulled down. Mean percentage ER β peptide coverage, and mean number of unique ER β peptides identified in biological duplicate experiments were calculated.

2.6. Parallel Reaction monitoring (PRM)

Nuclear pellets of MDA-MB-231-ER β +, MDA-MB-231-ER β –, LNCaP and MCF-7 cells were prepared using the Panomics nuclear extraction kit (Affymetrix, CA USA) as per the manufacturer's provided instructions. Nuclear pellets were lysed in 8 M Urea, 0.1% SDS in 50 mM TEAB by sonication twice, each for 5 min. After protein estimation 20 μ g of protein was taken for tryptic digestion. 50 mM of TEAB (pH = 8) was added up to a total volume of 100 μ l. Cysteines were reduced in 0.1 mM DTT for 1 h at room temperature and alkylated in 0.1 mM IAA for 30 min at room temperature in the dark. Alkylation was quenched by adding 0.1 mM DTT for 15 min. Trypsin (Promega trypsin (V5111)) was added in a 1:100 trypsin:protein ratio for 1 h at room temperature. Another batch of trypsin (1:100 ratio) was added to have a final ratio of 1:50 for incubation overnight. Samples were acidified to a final concentration of 1% formic acid (FA) and cleaned over C18 spin columns (Harvard apparatus C18 Micro SpinColumn™). After elution from the columns samples were lyophilized in a speedvac and resolubilized in 0.1% FA, 5% ACN to a final peptide concentration of 1 μ g/ μ l. Samples were subjected to liquid chromatography-electrospray ionization in an Orbitrap nano-ESI Q-Exactive mass spectrometer (Thermo Scientific), coupled to a nanoLC (Dionex Ultimate 3000 UHPLC). Samples were trapped on a 100 μ m $\times$ 2 cm, C18, 5 μ m, 100 trapping column (Acclaim PepMap 100) in μ l-pickup injection mode at 4 μ l/min flow rate for 10 min. Samples were loaded on a Rapid Separation Liquid Chromatography, 75 μ m $\times$ 25 cm nanoViper C18 3 μ m 100 column (Acclaim, PepMap) retrofitted to an EASY-Spray source with a flow rate of 300 nL/min (buffer A, HPLC H₂O, 0.1% FA; buffer B, 100% ACN, 0.1% FA; 60-min gradient; 0–5 min: 5% buffer B, 5–45 min: 5 to >56% buffer B, 45.1–50 min: 56% to >95% buffer B, 50.1–60 min, 5% buffer B). Peptides were transferred to the gaseous phase with positive ion electrospray ionization at 1.8 kV. Precursors were targeted in a 2Th selection window around the m/z of interest.

Precursors were fragmented in high-energy collisional dissociation mode with normalized collision energy dependent on the target peptide. The first mass analysis was performed at a 70,000 resolution, an automatic gain control target of 3×10^6 , and a maximum C-trap fill time of 200 ms; MS/MS was performed at 35,000 resolution, an AGC target of 5×10^4 , and a maximum C-trap fill time of 100 ms. Spectra were analyzed using Skyline with manual validation.

2.7. Statistics

Differences in ER β mRNA levels observed in MDA-MB-231-ER β – and MDA-MB-231-ER β – conditions were analyzed using unpaired t-tests. Differences were considered statistically significant at $p \leq 0.05$. Data presented are mean of technical triplicate experiments $\pm$ standard deviation. Analysis was performed in GraphPad Prism version 6.

3. Results

3.1. ER β antibody validation

Given the confusion in the ER β field and the concern associated with variable and potentially non-specific reagents, we sought to extensively validate commonly used ER β antibodies in a systematic manner that does not rely upon *a priori* assumptions regarding ER β expression in cell line models or in tissues. As a control, we employed a cell line system with doxycycline-inducible expression of the ER β protein, allowing us to assess antibodies in ER β negative and matched ER β positive conditions (Fig. 1A). One hundred-fold induction of ER β mRNA in MDA-MB-231-ER β cells treated with doxycycline 0.1 μ g/ml for 24 h ($p = 0.01$) was confirmed by qRT-PCR (Fig. 1B).

Western blots of MDA-MB-231-ER β – and MDA-MB-231-ER β – cell lysates with 8 different ER β antibodies were performed (Fig. 1C). Six commonly used antibodies in the literature were included; PPG5/10 (Asgari and Morakabati, 2011; Carder et al., 2005; Ciucci et al., 2014; Shaaban et al., 2003; Wimberly et al., 2014), NCL-ER-BETA (Ellem et al., 2014; Hussain et al., 2012; McPherson et al., 2007; 2010; Morais-Santos et al., 2015; Oliveira et al., 2007; Umekita et al., 2006; Yang et al., 2015; Zellweger et al., 2013), Genetex 70182 (Celhay et al., 2010; Madak-Erdogan et al., 2013; Mak et al., 2013, Mak et al., 2015, ; Nakajima et al., 2011), Millipore 06-629 (Bouchal et al., 2011; Chen et al., 2009; Grubisha et al., 2012), Abcam 288 [14C8] (Abd Elmaged et al., 2013; Carder et al., 2005; Colciago et al., 2014; Cotrim et al., 2013; Dey et al., 2012, 2014; Setlur et al., 2008; Shaaban et al., 2003; Vivar et al., 2010; Yang et al., 2012) and Santa Cruz 8974 (Al-Bader et al., 2011; Foryst-Ludwig et al., 2008; Han et al., 2015; Rossi et al., 2011; Zhou et al., 2012) antibodies. The PPG5/10 antibody detected a protein band of 77 kDa with no difference between

ER β + or ER β – conditions, suggesting it is recognizing a non-specific protein. Similarly, the NCL-ER-BETA antibody detected a band of ~59 kDa, which is the correct size for ER β however, there was no difference between ER β + or ER β – conditions implying that this band was not ER β . The GeneTex 70182 antibody detected a band of 59 kDa with differential signal between ER β + and ER β – conditions, and a non-specific band was present at around 65 kDa. The Millipore 06-629 antibody detected a band of 59 kDa in both ER β + and ER β – conditions, however the band was stronger in the ER β + condition, suggesting that the antibody could be cross-reacting with another protein of 59 kDa in addition to detecting ER β . MC10, CWK-F12, Abcam 288 [14C8] and sc8974 ER β antibodies all detected protein bands of 59 kDa with differential signal between ER β + and ER β – conditions, confirming their specificity for ER β by Western blotting. Further confirmation of the specificity of CWK-F12 to ER β was demonstrated by IHC of MDA-MB-231-ER β + and MDA-MB-231-ER β – cell pellets (Fig. 2), showing differential nuclear staining between the two conditions. The 8 ER β antibodies were then assessed by an independent method called RIME, which uses an antibody-based purification followed by mass spectrometry (MS) to identify enriched peptides. We conducted RIME in MDA-MB-231-ER β – and MDA-MB-231-ER β + cells using all 8 antibodies. E2F1 antibody was included in parallel as a positive control since E2F1 is a ubiquitous protein (Fig. 3A) and an IgG was used as a negative control (Fig. 3C). In MDA-MB-231-ER β – cells, no ER β peptides were purified by any of the ER β antibodies, confirming the ER β negative status of the uninduced MDA-MB-231-ER β cell line (Fig. 3C). Following ER β induction, RIME revealed diverse coverage of the ER β protein by the different antibodies. The percent coverage of the ER β protein following purification with each of the ER β antibodies, and the location of the peptide fragments identified by MS are shown in Fig. 3B. To provide an indication of the specificity of each antibody, we ranked all the proteins purified by the IP and identified by MS according to the number of unique peptides (confirmed with a false discovery rate (FDR) of <1%). We hypothesized that the higher the ranking of ER β , the greater the specificity of the antibody. Hence, if ER β has the greatest number of unique peptides relative to all other proteins, it is ranked 1st.

NCL-ER-BETA did not purify any ER β peptides (Fig. 3B), which is consistent with the lack of specificity identified from the Western blot result (Fig. 1C). The Millipore 06-629 antibody positively pulled down ER β in the test condition, although coverage and ranking were not as favorable as compared with some of the other antibodies. Interestingly, LACTB, a 60 kDa protein was purified by Millipore 06-629 in both ER β + and ER β – conditions (data not shown), which may explain the ~60 kDa band identified from Western blotting. Whilst the PPG5/10 did not detect ER β by Western blotting, by RIME it detected ER β with 25% coverage, with ER β ranking 3rd in the list of identified peptides, suggesting differences in the specificity of this antibody from one experimental assay to another. PPG5/10 has been previously validated for IHC in a doxycycline-inducible U2OS-ER β cell line, developed using the same plasmids as the MDA-MB-231-ER β cell line (Wu et al., 2012). The Abcam 288 [14C8] antibody is a very commonly used ER β antibody (Abd Elmageed et al., 2013; Colciago et al., 2014; Cotrim et al., 2013; Dey et al., 2012, 2014; Setlur et al., 2008; Shaaban et al., 2003; Vivar et al., 2010; Yang et al., 2012), which performed well by Western blotting, and also had the best antibody coverage by RIME (31.9%). However ER β ranked 20th in the list of identified peptides when using Abcam 288 [14C8], suggesting that this antibody might also be purifying additional non-specific proteins. The MC10 antibody had the second-greatest coverage (28.2%) with ER β ranking 1st in the list of identified peptides. In view of this finding, along with the positive Western blot result (Fig. 1), the MC10 antibody was carried forward into the RIME experiments for the

cell line characterization. The CWK-F12 antibody had 17.7% coverage, with ER β ranking 2nd in the list of purified peptides. As the CWK-F12 antibody produced very clean results by Western blotting, IHC and ranked ER β second in the list of purified proteins, it was used for Western blotting in the cell line characterization and directly compared against the non-specific NCL-ER-BETA antibody. The goal was to use independent validated ER β antibodies and additional independent methods to assess whether the most commonly studied breast and prostate cancer cell line models express ER β .

3.2. Characterization of LNCaP and MCF-7 cell lines for ER β expression

Given the wealth of publications assessing ER β in breast (MCF-7) and prostate (LNCaP) cancer cell lines (Abd Elmageed et al., 2013; Al-Bader et al., 2011; Bouchal et al., 2011; Chen et al., 2009; Dey et al., 2014; Ellem et al., 2014; Fuqua et al., 1999; Hinsche et al., 2015; Kim et al., 2002; Lau et al., 2000; Mak et al., 2013; Shaaban et al., 2003; Skliris et al., 2002; Weng et al., 2013; Yang et al., 2012, 2015; Zhou et al., 2012), we sought to investigate the expression of ER β in these models, using the newly validated ER β antibodies. Protein lysate and RNA was collected from LNCaP and MCF-7 cells. Using primers validated in the inducible MDA-MB-231-ER β cell line, which binds to sequence common to wild type (wt) ER β and its isoforms (Fig. 1B), LNCaP and MCF-7 were shown to express no detectable levels of ER β mRNA (Fig. 4A). Using the validated CWK-F12 ER β antibody, ER β protein was undetectable by Western blotting in these cells. By way of contrast, using the NCL-ER-BETA antibody on the same cell lysates, we detected a protein band of approximately 59 kDa in all conditions tested, including the MDA-MB-231-ER β – cell line, confirming the non-specificity of this antibody to ER β (Fig. 4B). Importantly, this demonstrates that the NCL-ER-BETA antibody is not detecting ER β in either LNCaP or MCF-7 cancer cell line models and is instead identifying a non-specific protein of similar molecular weight.

Furthermore, RIME analysis of LNCaP and MCF-7 cells using the validated MC10 ER β antibody did not purify any ER β peptides by MS (Fig. 4C). This result was confirmed by an antibody-independent approach known as Parallel Reaction Monitoring (PRM), which demonstrated that no ER β peptides were present in either of these cell lines (Fig. 4D). As such, our early passage LNCaP and MCF-7 cell line models are ER β negative and these cancer models should not be used for the analysis of this protein.

3.3. ER β expression in prostate and breast tissue

Importantly, whilst the LNCaP and MCF-7 cell-line models do not express ER β , application of the validated CWK-F12 ER β antibody to prostate and breast cancer TMAs demonstrated variable ER β expression in differing cancer grades. In prostate tissue, previous reports have described an inverse correlation between ER β expression and increasing Gleason grade of prostate cancer (Asgari and Morakabati, 2011; Attia and Ederveen, 2012; Dey et al., 2014; Horvath et al., 2001; Leav et al., 2001; Risbridger et al., 2007), whereas others have reported an association between increased ER β expression and higher Gleason grade (Zellweger et al., 2013) or increased expression of ER β in bone and lymph node metastases (Bouchal et al., 2011; Zhu et al., 2004). In our prostate TMA (Fig. 5A–D) we observed high expression of ER β in the basal epithelium of benign glands, with no expression in Gleason grade 3 cancer. Gleason grade 4 cancer showed weak nuclear staining of ER β and in areas of Gleason grade 5 cancer, ER β nuclear expression was of moderate intensity. In breast tissue, previous studies have shown greatest ER β expression in benign tissue, with a gradual

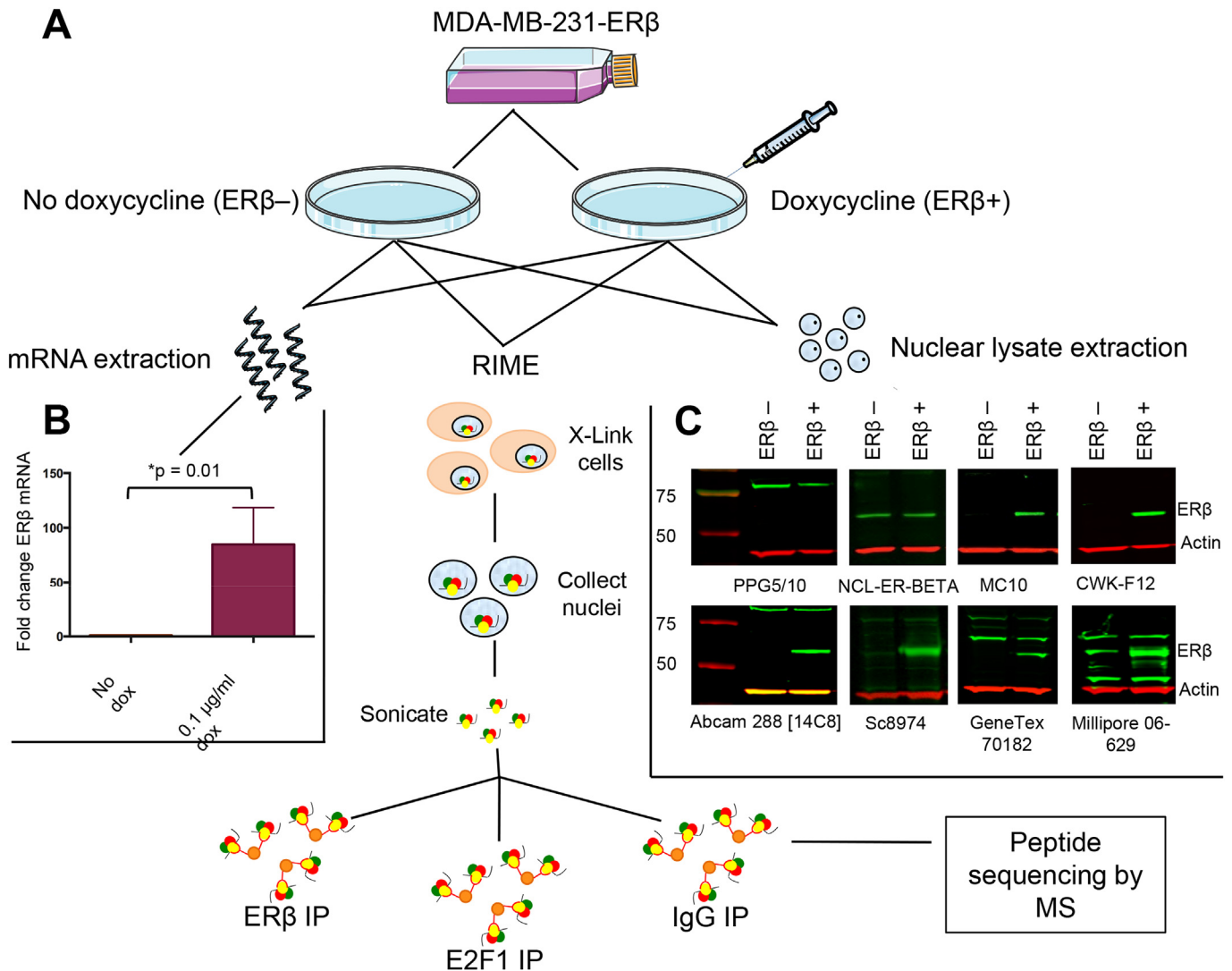


Fig. 1. Validation of ER β antibodies using doxycycline-inducible MDA-MB-231-ER β cells. (A) MDA-MB-231-ER β cells were treated with doxycycline to induce ER β expression. Untreated cells provided an ER β -negative control. Messenger RNA was extracted for qRT-PCR and protein for Western blotting. MDA-MB-231-ER β + and MDA-MB-231-ER β - cells were crosslinked and immunoprecipitated with antibody for RIME. (B) qRT-PCR confirmed 100-fold induction of ER β mRNA in MDA-MB-231-ER β + cells versus untreated MDA-MB-231-ER β - cells. Data are mean $\pm$ S.D. of technical triplicate experiments. (C) Western blots of MDA-MB-231-ER β + and MDA-MB-231-ER β - cells with the 8 antibodies undergoing assessment. The MC10, CWK-F12, Abcam 288 [14C8] and sc8974 antibodies detected bands of 59 kDa, with differential signal in the ER β + versus ER β - conditions, indicating specificity to ER β . GeneTex 70182 detected ER β , although there was non-specific signal at 65 kDa. Millipore 06-629 appears to detect ER β , although there is also a 59 kDa band in the ER β - condition. Review of the RIME data suggests this may be cross-reactivity with LACTB. NCL-ER-BETA, the most commonly used ER β antibody, gives bands of the correct size for ER β , but there is no difference between ER β - and ER β + conditions, confirming that this antibody is not specific to ER β .

decrease in expression associated with increasing cancer grade (Guo et al., 2014; Omoto et al., 2002). Conversely, a non-statistically significant trend towards higher ER β expression in Grade 3 tumors has also been reported (Myers et al., 2004). In our breast TMA (Fig. 5F–I), we observed greatest expression of ER β in benign epithelium, with a trend towards decreasing ER β expression associated with increasing cancer grade.

One potential explanation for the inconsistencies in ER β tissue expression is the presence of ER β splice-variant isoforms, which are fully conserved in exons 1–6, but have differing C-terminal domains (Leung et al., 2006). Different antibodies that bind either to the conserved regions or only to the C-terminal domain of the full-length ER β protein may therefore give differing results (Supplementary Fig. 1). This may particularly be the case in prostate cancer, where it has been reported that ER β isoform expression increases with the development of CRPC (Dey et al., 2012; Leung et al., 2010). Whilst this is likely to have an impact, our data

suggest that some of the differing conclusions around ER β expression in primary tissues are a direct result of certain investigations utilizing non-specific reagents that lack specificity for ER β .

4. Discussion

Despite a large body of published literature, the role of ER β in cancers of the prostate and breast is not clear. Contradictions between IHC findings and antibody-dependent molecular biology methods have contributed to this uncertainty, particularly the lack of clear consensus regarding correlation between tissue expression of ER β and clinico-pathological parameters (Asgari and Morakabati, 2011; Attia and Ederveen, 2012; Bouchal et al., 2011; Dey et al., 2014; Esslimani-Sahla et al., 2004; Guo et al., 2014; Hieken et al., 2015; Horvath et al., 2001; Leav et al., 2001; Leygue and Murphy, 2013; Myers et al., 2004; Omoto et al., 2002; Risbridger et al., 2007;

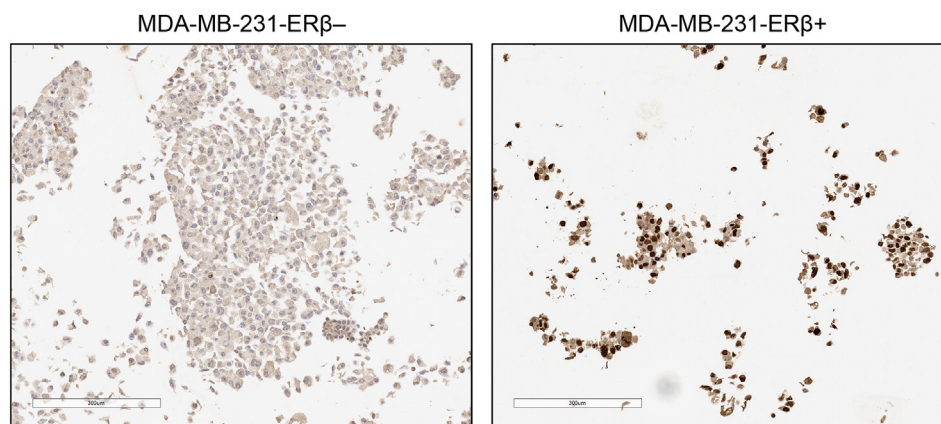


Fig. 2. IHC validation of CWK-F12 ER β antibody in MDA-MB-231-ER β cell pellets. Nuclear staining is evident in MDA-MB-231-ER β + cells and absent from the MDA-MB-231-ER β - control, confirming the specificity of CWK-F12 to ER β .

Roger et al., 2001; Umekita et al., 2006; Zellweger et al., 2013; Zhu et al., 2004).

Our results have demonstrated marked variation in the ability of commonly used commercially available ER β antibodies to accurately detect ER β by Western blotting and protein purification-MS based methods. Most notably, NCL-ER-BETA, a commonly used antibody (Ellem et al., 2014; Hussain et al., 2012; McPherson et al., 2007; 2010; Morais-Santos et al., 2015; Oliveira et al., 2007; Umekita et al., 2006; Yang et al., 2015; Zellweger et al., 2013) did not detect ER β by any methodological approach. This antibody consistently yields bands on Western blots of the appropriate size for ER β (59 kDa) in all tested conditions (Figs. 1C and 3B), but we have confirmed that this protein band is not ER β through the use of the MDA-MB-231-ER β inducible cell line system and the RIME technique. As such, this non-specific ~59 kDa band is likely to be the source of much of the controversy and confusion surrounding the study and characterization of ER β . The PPG5/10 antibody targets the C-terminus of wt ER β , and as such may be useful for distinguishing wt ER β from expression of ER β isoforms. PPG5/10 identified ER β in the MDA-MB-231-ER β cell line by RIME, and has previously been shown to be ER β -specific by IHC in both an inducible cell line model (Wu et al., 2012) and in breast tissue (Carder et al., 2005). However, in our study this antibody did not show specificity by Western blot analysis (Fig. 1C). In their antibody validation study, Carder et al. also assessed the Abcam 288[14C8] antibody and found it to be ER β -specific for IHC in tissue (Carder et al., 2005). Whilst our Western blotting data support this assertion (Fig. 1C), our RIME data suggest that this antibody also purifies additional, non-specific peptides, and as such should be used with caution for IP-based methods (Fig. 3B). Taken together, these findings reassert the importance of validating antibodies for individual experimental approaches, rather than assuming general applicability across methodological platforms (Baker, 2015; Bordeaux et al., 2010).

RIME was initially developed as a discovery tool to study the interacting proteomes of transcription factors in an unbiased manner (Mohammed et al., 2013). The advantage of using RIME in antibody validation arises from being able to identify specific, named peptides purified by an antibody, rather than relying on the presence of a protein band of approximate size on a Western blot. This is typified by the NCL-ER-BETA antibody, which gave bands on Western blot in both ER β - and ER β + conditions and no ER β peptides identified by RIME. Taken together, these data confirm that this antibody is not specific to ER β . The non-commercially available ER β antibodies tested (MC10 and CWK-F12) have been previously

validated by other approaches (Choi et al., 2001; Wu et al., 2012) and our results add further confidence in their specificity using multiple independent assays. By comparing the peptide coverage of each antibody along with the ER β ranking (as a surrogate of specificity) RIME facilitated an informed decision-making process in selecting which antibody to carry forward to the cell-line characterization. Our multi-modal approach to cell-line characterization using both antibody-dependent (Western blotting and RIME) and antibody-independent (qRT-PCR and PRM) approaches has shown that low-passage, genotyped LNCaP and MCF-7 cell lines do not express detectable ER β , despite numerous publications making conclusions about ER β biology using these cell line models (Abd Elmageed et al., 2013; Al-Bader et al., 2011; Bouchal et al., 2011; Chen et al., 2009; Dey et al., 2014; Ellem et al., 2014; Fuqua et al., 1999; Hinsche et al., 2015; Kim et al., 2002; Lau et al., 2000; Mak et al., 2013; Weng et al., 2013; Yang et al., 2012, 2015). Whilst we acknowledge that immortalized cell lines may have variable expression of certain factors across passage numbers and laboratories (Masters, 2000), our data suggest the need for caution in making this assumption with respect to ER β . Reassuringly, we have confirmed expression of ER β in prostate and breast tissue using the validated CWK-F12 antibody. Our IHC study is not intended to be an exhaustive analysis of ER β expression in prostate and breast tissue, and we acknowledge the limitations presented by our small sample size and lack of statistical correlation with clinico-pathological parameters. We have however, demonstrated that the CWK-F12 ER β antibody is validated for IHC and in principle can be used for larger scale assessment of ER β expression in tissue.

Epidemiological evidence suggests that estrogen and its receptors have important roles in the development and progression of prostate cancer. Japanese men are known to have a very low incidence of prostate cancer (Ross et al., 1992), and it has been proposed that their traditional diet, which is high in ER β selective phytoestrogens may exert a protective role (Andres et al., 2011; Attia and Ederveen, 2012; Hori et al., 2011; Messina, 2010; Reiter et al., 2011; Shen et al., 2000; Stettner et al., 2007; Thelen et al., 2007, Thelen et al., 2005; Wuttke et al., 2002). Further evidence from studies of ER β knockout mice (β ERKO) shows a clear phenotype and tumor-suppressive effect of ER β (Ricke et al., 2008). However, clinical trials of agents seemingly effective *in vitro* have demonstrated no clinical benefit of estrogen-selective agents in prostate cancer (Bergan et al., 1999; Kim et al., 2002). There are numerous explanations as to why this might be, for example, expression of ER β in non-epithelial cell types (Gargett et al., 2002; Pierdominici et al., 2010) modulating the tissue response to these

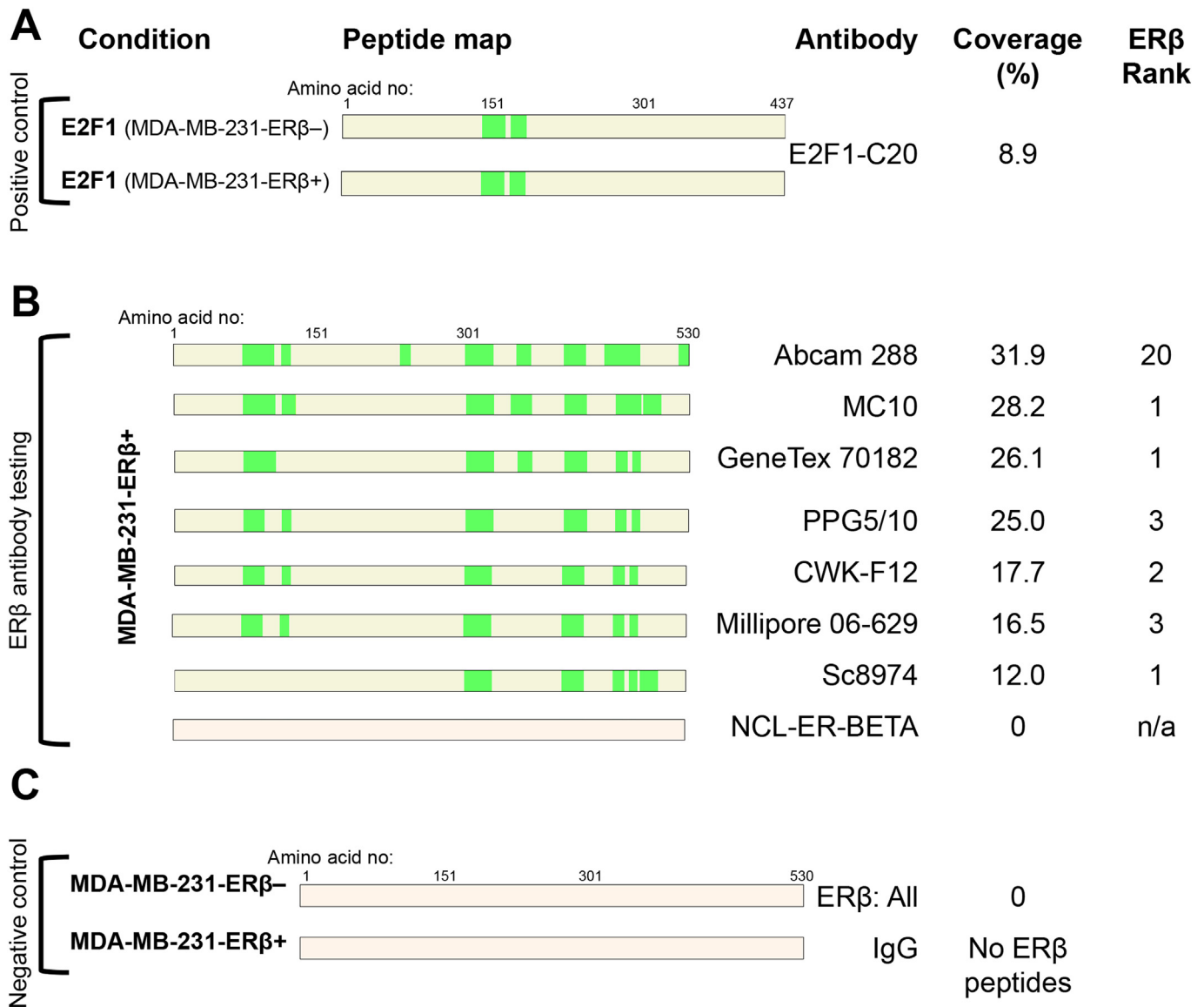


Fig. 3. RIME demonstrates specificity and peptide coverage of ER β antibodies. Eight ER β antibodies were assessed by RIME in MDA-MB-231-ER β +/- cells. Coverage of the protein relates to green areas on the peptide maps, indicating peptides identified by MS with false discovery rate of $\leq 1\%$ (mean of 2 biological replicates). (A) E2F1 antibody was applied to MDA-MB-231-ER β - and MDA-MB-231-ER β + conditions as a positive control, as E2F1 is a ubiquitously expressed protein. (B) ER β antibody tests: 'ER β ranking' indicates where ER β features in a list of proteins purified by the antibody, ranked by number of unique peptides identified in MS, giving an indication of antibody specificity. NCL-ER-BETA failed to identify ER β . (C) Negative controls: All of the ER β antibodies were tested in MDA-MB-231-ER β - cells, to confirm absence of ER β expression in the non-induced condition. Mouse IgG antibodies were used to identify non-specific peptides pulled down by the IP. None of the IgG antibodies purified ER β .

agents, but in light of our findings we would suggest that use of poorly validated reagents and inadequately characterized cancer cell line models is an important contributing factor.

In the presented study, detailed validation of commonly used ER β antibodies has demonstrated that some of these reagents either detect ER β in specific experimental conditions only or lack any specificity for ER β across multiple assays. ER β has been investigated in numerous cancers including prostate, breast, kidney (Yu et al., 2013), colon (Dey et al., 2013), endometrium (Han et al., 2015), ovary (Ciucci et al., 2014; Suzuki et al., 2008) bladder (Hsu et al., 2013) and non-small cell lung cancer (He et al., 2015; Luo et al., 2015) but in many cases, the findings are predicated on non-specific reagents. As such, a re-evaluation of ER β expression and biology is needed using reliable, specific reagents. Our determination of ER β antibody specificity will contribute towards clarifying existing, conflicting data on the role of ER β in these diverse cancers

and provide the necessary, validated tools with which to move forward our understanding of ER β biology.

Ethics

Prostate tissue included in the tissue microarray was obtained under approval granted for the ProMPT study (MREC/01/4/061). Breast tissue collection and assessment was approved by the University of Adelaide Human Research Ethics Committee (#s H-2005-065).

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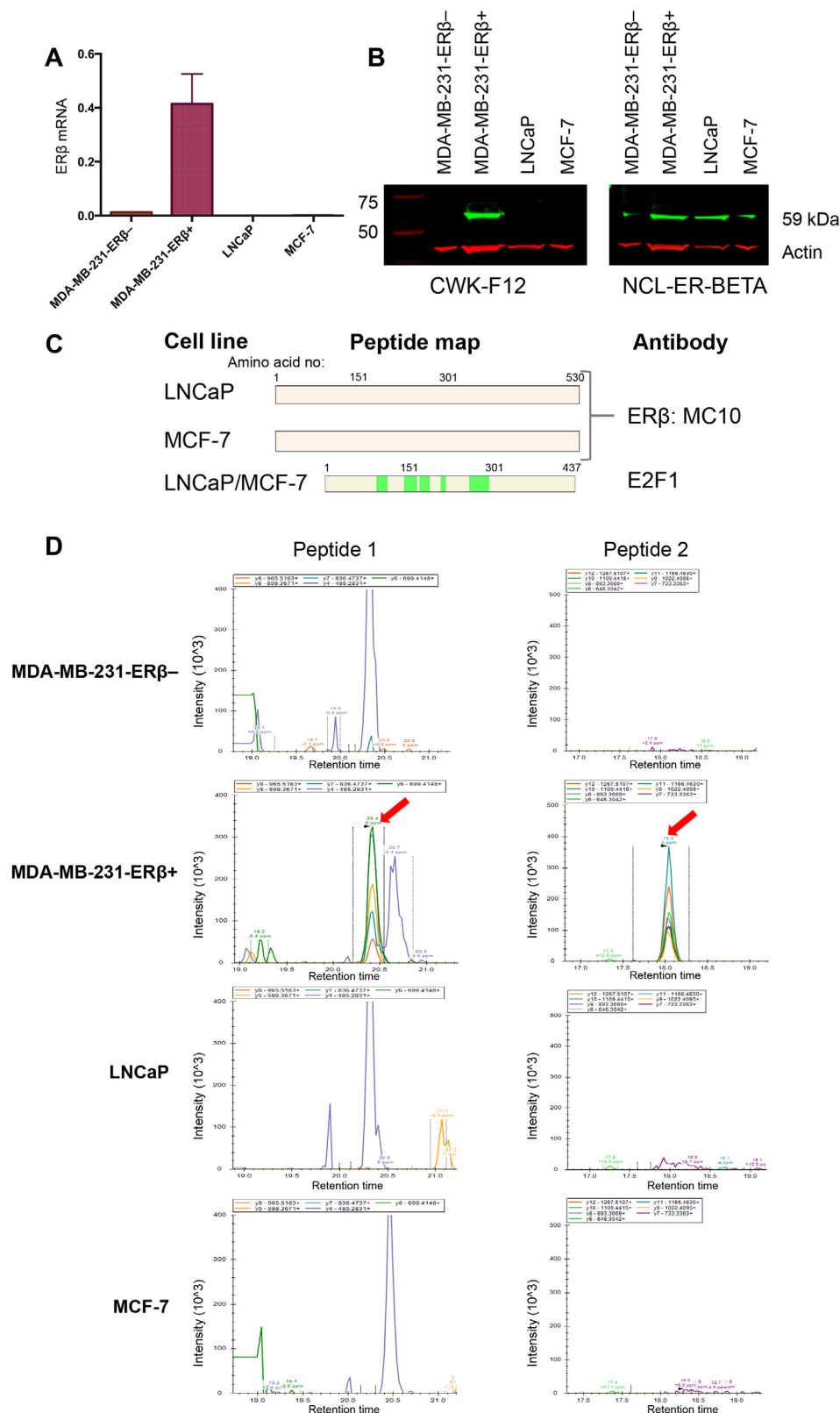


Fig. 4. Multimodal characterization of LNCaP and MCF-7 cell lines confirms absence of ERβ expression. LNCaP and MCF-7 are cell lines commonly used to study ERβ. We detected no ERβ expression in either cell line at mRNA level by qRT-PCR (A) or at protein level by western blot (B) or RIME (C) using validated CWK-F12 and MC10 antibodies respectively. Western blot of the same cell lysates using the NCL-ER-BETA antibody clearly shows how some of the conflicting data in the literature has arisen, as this antibody shows bands of the correct size for ERβ in all conditions including MDA-MB-231-ERβ⁻ negative control. (D) PRM confirms, using an antibody-independent technique, the absence of ERβ protein expression in LNCaP and MCF-7 cells. The ERβ peptides (peptide 1 is SLEHTLPVNR and peptide 2 is SSITGSECSPAEDSK) identified in the MDA-MB-231-ERβ⁺ positive control (red arrows) are absent in the other cell lines. Data shown are representative of 2 independent biological replicates.

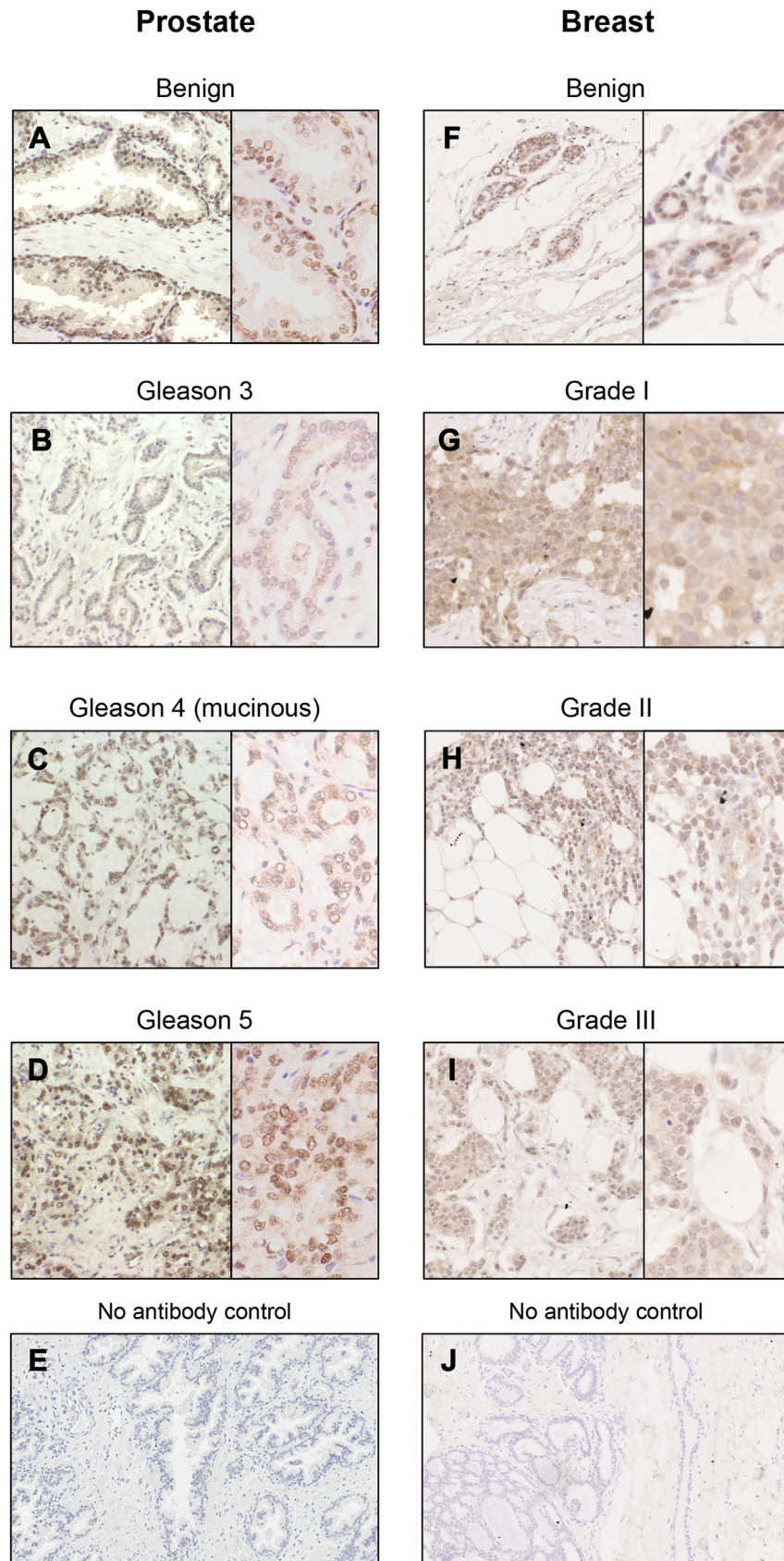


Fig. 5. IHC of prostate and breast tissue with validated CWK-F12 ER β antibody. Demonstration of variable ER β expression in differing grades of prostate (A–D) and breast (F–I) cancer. In prostate, ER β was highly expressed in basal and luminal epithelial cells of benign glands (A), whereas there was no nuclear staining in Gleason grade 3 cancer (B). In Grade 4 mucinous tumor (C) and high grade tumor (D) nuclei showed weak to moderate expression of ER β . In breast, ER β expression was greatest in nuclei of benign epithelial cells (F), which was observed to decrease with increasing grade of cancer (G, H and I – Grade 1, 2 and 3 respectively). The greatest difference in expression was observed between benign (F) and Grade 3 cancer (I). E and I – no primary antibody negative controls.

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Conflicts of interest

The authors confirm there are no conflicts of interest to disclose.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.mce.2016.11.016>.

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