Differential regulation of oestrogen receptor β isoforms by 5′ untranslated regions in cancer

Laura Smith [a], **Rebecca A. Brannan** [b], **Andrew M. Hanby** [b], **Abeer M. Shaaban** [b], **Eldo T. Verghese** [b], **Mark B. Peter** [a], **Steven Pollock** [a], **Sampoorna Satheesha** [a], **Marcin Szynkiewicz** [a], **Valerie Speirs** [a], **Thomas A. Hughes** [a], *

[a] *Leeds Institute of Molecular Medicine, Leeds University, Leeds, UK*
[b] *Department of Histopathology, St James's University Hospital, Leeds, UK*

Abstract

Oestrogen receptors (ERs) are critical regulators of the behaviour of many cancers. Despite this, the roles and regulation of one of the two known ERs – ERβ – are poorly understood. This is partly because analyses have been confused by discrepancies between ERβ expression at mRNA and proteins levels, and because ERβ is expressed as several functionally distinct isoforms. We investigated human ERβ 5′ untranslated regions (UTRs) and their influences on ERβ expression and function. We demonstrate that two alternative ERβ 5′UTRs have potent and differential influences on expression acting at the level of translation. We show that their influences are modulated by cellular context and in carcinogenesis, and demonstrate the contributions of both upstream open reading frames and RNA secondary structure. These regulatory mechanisms offer explanations for the non-concordance of ERβ mRNA and protein. Importantly, we also demonstrate that 5′UTRs allow the first reported mechanisms for differential regulation of the expression of the ERβ isoforms 1, 2 and 5, and thereby have critical influences on ERβ function.

Keywords: breast cancer ● ERβ ● 5′UTRs ● translation ● uORFs ● RNA structure ● alternative splicing

Introduction

Oestrogen receptors (ERs) are key mediators of oestrogen function and play roles in many pathological processes, including carcinogenesis [1]. Although the roles of ERα in carcinogenesis are relatively well understood, the roles of ERβ remain uncertain. This is partly because analyses have been confused by consistent discrepancies between ERβ expression at mRNA and protein levels in breast tissue [2–3], leading to difficulties in interpreting mRNA expression data. In addition, ERβ is expressed as five functionally distinct isoforms [4], yet their roles have frequently been studied in combination at the level of total ERβ. ERβ appears to be anti-proliferative and anti-apoptotic, and has been discussed as a tumour suppressor [5–9]. However, the specific effects of each isoform and whether these result from independent actions as transcription factors, or *via* hetero-dimerization with other receptors remain undetermined [10–11]. An understanding of how ERβ mRNA and protein levels relate, and how expression of each individual isoform is determined would provide useful background from which to assess the importance of each isoform more effectively.

In recent years, evidence has accumulated that deregulation of gene-specific translation plays a role in oncogenic transformation and tumour progression [12]. Translation occurs mainly by a cap-dependent mechanism with most regulation during initiation [13]. Initiation involves translational machinery binding to the mRNA cap and scanning along the 5′ untranslated region (UTR) to the reading frame, where an initiation codon is recognized and protein synthesis starts [14]. 5′UTRs are thus placed in unique positions to regulate the efficiencies of recruitment and scanning of the translational machinery [15]. In particular, translation can be inhibited by the presence within 5′UTRs of upstream open reading frames (uORFs) or regions that form stable secondary structures [16–17]. Many mRNAs have short 5′UTRs (composed of <50 nucleotides) that lack these motifs and consequently these 5′UTRs are thought to have little regulatory effect on translation [18–19]. A substantial minority of mRNAs have long 5′UTRs

*Correspondence to: Thomas A. HUGHES,
Leeds Institute of Molecular Medicine,
St. James's University Hospital,
Leeds, LS9 7TF, UK.
Tel.: 44 113 3438422
Fax: 44 113 3438431
E-mail: t.hughes@leeds.ac.uk

(>200 nucleotides) and/or contain these regulatory sequences [20]. Interestingly, many oncogenes and tumour suppressor genes express these atypically long or complex 5′UTRs, highlighting the importance of 5′UTRs in carcinogenesis [19]. Various cellular activities impact on the degrees of translational inhibition specified by these 5′UTRs allowing them to act as sites of regulation; for example changes in activities of translation factors eIF2α and eIF4E modify the influences of uORFs and secondary structures, respectively [21–22]. It is also clear that further layers of regulation involving 5′UTRs exist, since many genes allow expression of alternative 5′UTRs resulting from use of multiple transcriptional start sites or from differential splicing [23]. Alternative 5′UTRs allow tissue- and/or stimuli-specific gene regulation by control of relative proportions of 5′UTRs with different translational properties in the total pool of mRNAs from that gene [24]. Given the wide range of cancer-related genes that allow expression of potentially regulatory 5′UTRs and the frequency of expression of multiple 5′UTRs, deregulation of translation *via* inappropriate expression of UTR-regulatory factors and/or changes in relative proportions of alternative 5′UTRs is thought to play a key role in carcinogenesis [12].

Our hypothesis was that regulation of ERβ translation is responsible for the non-concordance of ERβ mRNA and protein levels, and provides an important level of regulation of total ERβ expression and expression of specific ERβ isoforms. Accordingly, we have investigated the influences of ERβ 5′UTRs. We show that ERβ 5′UTRs have potent differential influences on expression of ERβ isoforms, and that these are modulated by cellular context and in carcinogenesis.

Materials and methods

Cell culture, transfection and flow-cytometry

Cell lines were obtained from Dr. Valerie Speirs (University of Leeds; BT-20, HB2, MCF7, MDA-MB-231 and MDA-MB-453), Prof Pamela Rabbitts (Univesity of Leeds; A549) or ATCC (H1299). Culture and transfection conditions are shown in Table S1. For flow-cytometry, cells were removed from wells with trypsin and resuspended in fresh medium containing 1% serum. GFP expression was quantified (mean fluorescent intensity of 10^4 events after exclusion of debris/dead cells on the basis of forward activated light scatter *versus* side scatter) at 525 nm (LSRII, BD Biosciences, Oxford, UK). Gates were set so that <1% of untransfected cells were defined as expressing GFP.

Plasmids

pTH-GFPa and pcDNA3HA-eIF4E have been described previously [25–26]. UTR reporters were created by cloning UTRs upstream of the GFP ORF in pTH-GFPa using a cloning strategy described previously [27]. Importantly, this strategy results in the removal of the 5′ end of the multiple cloning site, therefore inserted 5′UTRs are immediately adjacent to the transcriptional start site with no restriction sites intervening. QuikChange Lightning

(Stratagene, La Jolla, CA, USA) was used for mutagenesis. All primers are listed in Table S2.

cDNA synthesis and PCR

All primers are listed in Table S2. RNA was purified from cells with RNeasy kits (Qiagen, Crawley, UK) or tissues with Trizol (Sigma, Poole, UK); contaminating DNA was removed with Turbo DNase I (Applied Biosystems, Warrington, UK). First strand cDNA was synthesized using SuperScript II (according to the manufacturer's protocol) and oligo(dT) or random hexamers, or SuperScript III and ERβ isoform specific primers. The Superscript III protocol was modified to enhance primer specificity. RNA and primers (0.4 pmol) were heated (80°C, 2 min.) and cooled to 55°C (0.03°C/s). A master mix (RNase OUT [Invitrogen, Paisley, UK], dNTPs, DTT, buffer, Superscript) was incubated at 55°C (5 min.) and then mixed with RNAs. Reactions were incubated (55°C 50 min.; 70°C 10 min.). cDNAs panels for normal human tissues and matched normal and tumour lung tissues were purchased (Clontech, Mountain View, CA, USA; 637260, 631765). Thorough semi-quantitative PCR analyses were performed at least twice for each cDNA sample exactly as described previously [27]; in particular, PCR reactions were analysed after at least three different numbers of cycles in order to establish that the products shown were taken from reactions within the linear range of amplification; representative data are shown. Note that products in lanes marked with asterisks were undetectable after 55 cycles of amplification. In some lanes, products were only detectable after this level of amplification – these products are not shown, in order to preserve the linear PCR dynamics of the other reactions, but are not marked with asterisks to indicate that they can be detected. Triplicate real-time PCR analysis was performed (Applied Biosystems SYBR® Green PCR Master Mix and 7900HT machine). Dissociation curves and serial cDNA dilutions were performed to ensure primer specificities and equal amplification efficiencies. Reactions were also performed with template lacking reverse transcriptase (RT): products were either undetectable or greatly reduced (>30,000 fold less products than the equivalent RT^+) hence genomic DNA contamination was not considered to interfere with our data. 5′RACE reactions were performed with 5′RACE System2 (Invitrogen). Products were analysed on 2.5% agarose (0.5 μg/ml ethidium bromide, 1×TBE) and visualized on an UV trans-illuminator. Products were excised from gels and cloned into pGEM-Teasy (Promega, Madison, WI, USA); up to five clones for each were sequenced.

Western blotting

This was performed as previously [27] using SDS 4–15% polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA, USA) and Hybond-ECL membrane (Amersham Biosciences, Buckinghamshire, UK). Membranes were incubated (4°C overnight) with rat anti-HA (Roche, Basel, Switzerland, 3F10; 1:1500), anti eIF4E (Santa Cruz, USA, sc9976, 1:500) or β-actin (Sigma AC-15; 1:10,000) and HRP-conjugated secondaries (Santa Cruz Biotech, Santa Cruz, CA, USA; 1:1000; 1 hr), before being visualized with Supersignal pico (Pierce, Northumberland, UK).

Patient samples and immunohistochemistry (IHC)

Ethical approval was obtained (Leeds East 05/Q1206/136, 00/102 and 02/029). For initial semi-quantitative PCR analyses, breast tumour

samples were collected prospectively, matched normal and tumour cells were dissected on the basis of histology under guidance of breast pathologists (AMH, AMS) and RNA was extracted immediately. For IHC, archival breast cancer specimens were obtained for 424 patients who were treated at the LTH NHS Trust from 1983 to 2006. Tissue microarrays (TMAs) were constructed from these cases using 0.6 mm cores selected from the most representative tumour area (determined by haematoxylin and eosin staining). IHC analyses for ERβ isoforms were performed exactly as we have previously validated and described [28]. For eIF4E, antigens were retrieved by 2 min. pressure-cooking (boiling and maximum pressure) in antigen un-masking solution (Vector, Burlingame, CA, USA) and sections were stained with anti-eIF4E (sc-9976, Santa Cruz Biotech; 1:100 TBS; 4°C; overnight). This antibody has previously been validated for IHC [29]. Envision detection was used (DAKO, Glostrup, Denmark). Negative controls (primary antibodies omitted) were included in each IHC run; adjacent normal epithelium, lymphocytes and blood vessel endothelium served as internal controls. Slides were scanned into Spectrum Web Viewer (Aperio Technologies, Vista, CA, USA). Cores were scored for immunoreactivity by two independent individuals taking into account both average staining intensity and percentage positively stained tumour cells (ERβ isoforms [30], eIF4E [31]), under supervision of consultant breast pathologists (AMH, AMS). Where independent scores differed consensus scores were determined. Some data were not available due to TMA core loss during processing (a well-recognized problem associated with TMA IHC), meaning that a total cohort of 408 with follow up data was available.

RNA structure and statistical analyses

Modelling was performed with mfold v3.1 as previously [27]. This algorithm predicts potential secondary structures for RNA molecules by finding base-pairing solutions that are sterically possible and release the greatest amount of free energy (G). ΔG represents the free energy released during structural folding; more stable structures release more energy as they form and therefore have greater ΔG values [32]. The Student's t-test or Spearman correlation was used for statistical analyses using Microsoft Excel or SPSS (SPSS, Inc., IL, USA). Correlation coefficients were interpreted as follows: <0.2 little (if any) correlation, 0.2–0.5 weak correlation, 0.5–0.8 moderate correlation. All P-values were two-sided; $P < 0.05$ was considered significant.

Results

Alternative ERβ 5′UTRs are differentially expressed in human cells

We identified four alternative 5′UTRs for ERβ from the literature [33] and from Genbank, which we have termed UTRa, UTRa-long, UTRb and UTRc. Figure 1(A) shows an alignment of mRNAs containing these 5′UTRs with the 5′ end of the human ERβ gene on 14q23. The gene includes two promoters (black arrows) that allow expression of different untranslated first exons (E0K and E0N), while a further section of untranslated sequence and the translational start site are within exon 1 (E1). Alternative splicing and transcriptional start sites allow expression of UTRa (*e.g.* accession BC024181), UTRa-long (*e.g.* NM_001040276), UTRb (*e.g.* AB006589) and UTRc (*e.g.* NM_001437). These 5′UTRs contain motifs that provide potential mechanisms for regulation of ERβ translation, including uORFs and regions capable of forming substantial secondary structures. UTRa, a-long, b and c have 6, 9, 33 and 2 uORFs, respectively. We used computer modelling with the mfold algorithm to determine potential UTR secondary structures [32]. The degree and stability of structures was quantified using theoretical change in free energies (ΔG); more stable structures release more energy as they form and have greater ΔG values. UTRa, a-long, b and c have predicted secondary structures with ΔGs of –84, –305, –400 and –166 kcal/mole, respectively. For comparison, the ΔG of the non-regulatory β-actin 5′UTR is only –24 kcal/mole. *In vitro* studies have revealed that structures with ΔGs greater than –30 kcal/mole can be sufficient to inhibit translation [34]. Our hypothesis was that these alternative 5′UTRs play a role in regulating ERβ translation. As a first step in testing this hypothesis, we have investigated the cell types in which these 5′UTRs are expressed.

We performed 5′RACE reactions to determine which 5′UTRs were expressed in primary breast tumour cells and in breast cell lines. We identified 5′UTR sequences representing UTRa and UTRc, but not UTRa-long or UTRb. In subsequent analyses we were unable to detect expression of UTRa-long or UTRb in any sample tested (human tissues and a panel of human cell lines; data not shown); therefore we do not believe these UTRs have a role in most human cells and they were not investigated further. Representative RACE reactions using RNA prepared from two independent breast tumours are shown in Fig. 1(B). Variable 5′ ends were identified for UTRa and UTRc; these may represent 5′ mRNA truncations but are more likely to represent variation in transcriptional start site as is typical from promoters lacking TATA boxes. We designed primers for analysis of UTRa and UTRc expression based on sequences common to all the sequenced UTRa and UTRc RACE products (forward primers positioned in the shaded regions in Fig. 1B; filled arrows in Fig. 1A). Semi-quantitative PCR analyses of expression of UTRa and UTRc were performed on cDNA from human tissues (Fig. 1C). Reactions were also performed with primers specific for a sequence encoded by exon 1 that is within the ERβ reading frame as a measurement of total ERβ expression, and for glyceraldehyde-3-phosphate dehydrogenase to allow assessment of relative amounts of overall cDNA template. ERβ mRNA was present in all tissues tested and was expressed most strongly in brain. Clear differential expression of UTRa and UTRc was observed between tissues; UTRa was expressed in pancreas and brain but was undetectable in other tissues (*asterisks;* Fig. 1C), while UTRc showed a wide distribution, although it was expressed at very low levels in heart (only detectable following many amplification cycles; not visible in Fig. 1C as lesser amplification was used to preserve the linearity of the other reactions). We also examined whether 5′UTR expression is altered during carcinogenesis by comparing expression in
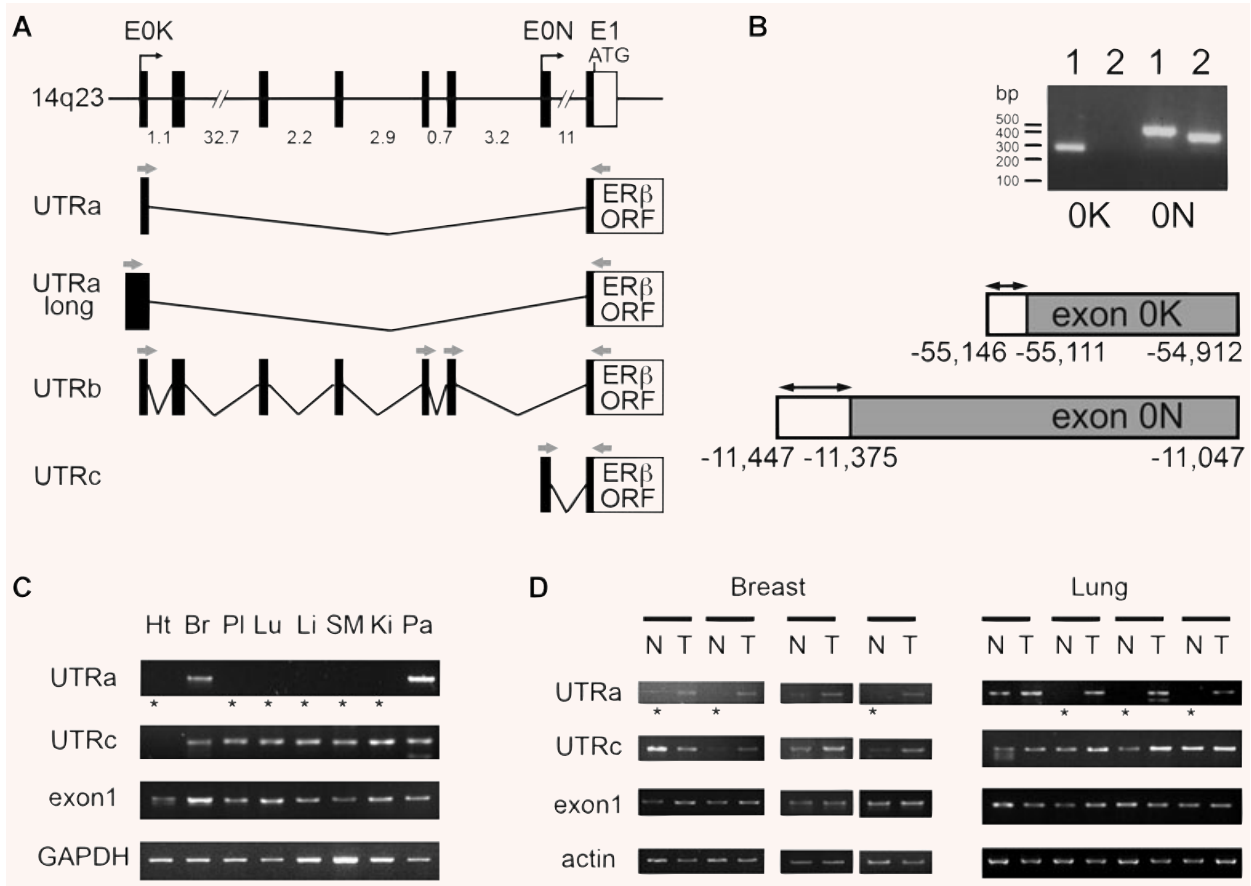
Fig. 1 Alternative ERβ 5′UTRs are differentially expressed in human tissues and in cancer. (**A**) Alignment of the 5′ end of the human ERβ gene with mRNAs containing different 5′UTRs. UTR exons (filled boxes), coding regions (open boxes), transcriptional (black arrows) and translational (ATG) start sites, intron sizes (kbp), and primers used for PCR analysis (grey arrows) are shown. (**B**) 5′ ends of ERβ mRNAs correspond to variable positions in exon (E) 0K and E0N. 5′RACE reactions were performed on two breast tumour samples (1 and 2). Products corresponding to the 5′ end of E0K (UTRa) or 0N (UTRc) are shown; UTRa was only detected in tumour 1. These (and other) products were sequenced; the diagram shows sequence consistently included in transcripts (grey boxes) and sequence containing variable start sites (open boxes and double-headed arrows; variation for UTRa and UTRc spans 35 or 72 bases), with genomic locations with respect to the E1 ATG codon. (**C**) and (**D**) PCR analyses showed that UTRa and UTRc have tissue specific distributions in normal cells and are differentially expressed between breast and lung matched normal and tumour tissues. Heart, brain, placenta, lung, liver, smooth muscle, kidney, pancreas; patients 1–4 (**D**), normal, tumour) *species not detected. Note: species not visible in (**C**) and (**D**) but lacking * were detected after 55 amplification cycles but this is not shown in order to preserve the linear PCR dynamics of the other reactions.

matched normal and tumour samples from patients with either breast or lung cancers. PCR analyses were performed on cDNA prepared from these tissues as before (using β-actin to determine relative amounts of total cDNA) (Fig. 1D). As before, ERβ mRNA was present in all tissues tested. Interestingly, UTRa was consistently up-regulated in breast and lung tumours relative to matched normal tissues. In contrast, changes in UTRc expression were less consistent. We concluded that UTRa and UTRc have tissue specific distributions in normal cells and are differentially expressed between normal and tumour tissues of breast and lung.

5′UTRs define ERβ translational efficiency

Next, we investigated whether ERβ 5′UTRs directly influence translational efficiencies of downstream ORFs using an established reporter assay [27]. UTRa and UTRc were cloned upstream of the GFP reading frame in expression vectors. We cloned sequences that were common to all identified UTRa and UTRc RACE products (shaded boxes, Fig. 1B). Following on from our analysis of breast and lung tumours (Fig. 1D), breast and lung cell lines were used for our analyses. Cells were transiently transfected

with equal numbers of copies of either empty expression vector (negative control; data not shown) or vector to allow expression of GFP mRNAs with non-regulatory 5′UTRs (positive control), or with reporters for UTRa or UTRc (Fig. 2A). GFP protein expression was measured by flow-cytometry and GFP mRNA expression was measured by real-time PCR. Protein and mRNA levels were used to calculate translational efficiencies for each GFP message: these are amounts of GFP protein produced per unit mRNA and were utilized to account for differences in GFP transcription from vectors (Fig. S1). A minimum of two independent experiments were performed and within each experiment three technical replicates were included. Representative translational efficiencies are shown relative to positive controls in each cell type (Fig. 2B). Each ERβ 5′UTR inhibited GFP translation; in particular, UTRa was strikingly inhibitory in nature. For example, in HB2 cells, UTRa allowed a translational efficiency of only 6% of the positive control. A consistent pattern of relative influences for each UTR was seen across the cell line panel, with UTRa being more, and UTRc less inhibitory. However, substantial differences were also observed in absolute degrees of inhibition between cell lines. For example, UTRa showed a 2.6-fold difference in translational efficiency between HB2 and MDA-MB-231 cells. We concluded that ERβ 5′UTRs specify the efficiencies with which downstream ORFs are translated, and also that these efficiencies are further modulated by the cellular context.

Upstream ORFs reduce ERβ translational efficiency

Next, we were interested in which regulatory motifs in the UTRs were responsible for specifying translational inhibition. First, we examined uORFs, which can reduce translational efficiency by stopping some scanning ribosomes from reaching the true mRNA initiation codon [35]. UTRa contains six, and UTRc two uORFs. One uORF is encoded within exon 1 (E1), therefore is common to UTRa and UTRc, while the others are unique to each UTR. Start codons of uORFs are required for their inhibition of expression of downstream reading frames. We mutated the start codon of each uORF within our GFP reporters to test whether particular uORFs were responsible for translational inhibition (Fig. 3A). Cells were transfected with equal copy numbers of the vectors and translational efficiency was analysed as before (Fig. 3B). Mutation of the start codons of the first, second, third/fourth and fifth uORFs significantly increased translation from UTRa by 2.0-fold ($P = 2.7 \times 10^{-5}$), 4.4-fold ($P = 2.7 \times 10^{-6}$), 1.3-fold ($P = 0.02$) and 1.1-fold ($P = 0.03$). Mutation of the sixth uORF had no significant effect. For UTRc, mutation of the start codons of the first and second uORFs also significantly increased translation by 1.1-fold ($P = 0.01$) and 1.3-fold ($P = 5.8 \times 10^{-5}$). The Kozak context was generally weak for all uORFs. The first uORF in UTRa showed the strongest consensus and was shown to have an influence on translation. More surprisingly, the second uORF in UTRa had a weaker consensus yet demonstrated a stronger translational effect. In order to be confident that these changes related mainly
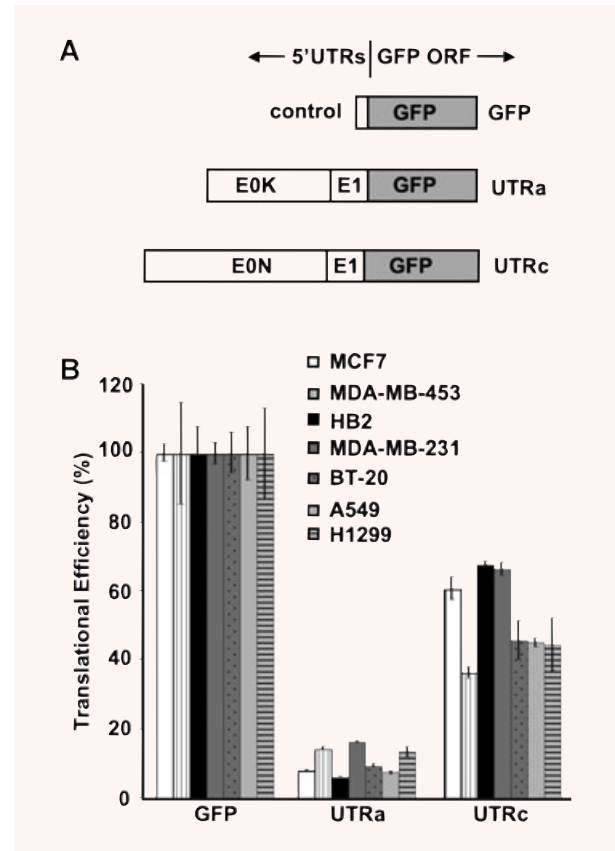

Fig. 2 5′UTRs regulate ERβ translational efficiency. (**A**) Reporters were constructed to express mRNAs containing the GFP reading frame (grey box) preceded by different 5′UTRs (white boxes). Sequences encoded by E0K, 0N and 1 are shown. (**B**) The translational efficiency specified by each UTR varies in different cell lines. Cell lines were transiently transfected with equal copy numbers of either control or experimental constructs. GFP protein and mRNA were quantified by flow-cytometry and real-time PCR, respectively. Translational efficiency (protein synthesized per unit mRNA) is presented relative to the GFP control that lacks a specialized 5′UTR. Error bars show the standard deviation of technical triplicates within a representative experiment.

to loss of uORFs, we also examined how these mutations influenced the potential secondary structure formed by the UTRs (using mfold; see above). Each mutation within UTRa caused small increases in the extent of structure as assessed by ΔG, while mutations within UTRc caused either a small decrease (m1) or a small increase (m2) in the extent of structure (Table S3). These analyses underline the role of the uORFs since the striking increases in translational efficiency seen with UTRa m1 and m2, and with UTRc m2 each occurred despite the presence of small increases in potentially inhibitory secondary structure. We have not examined the effect of combined mutations on uORF function
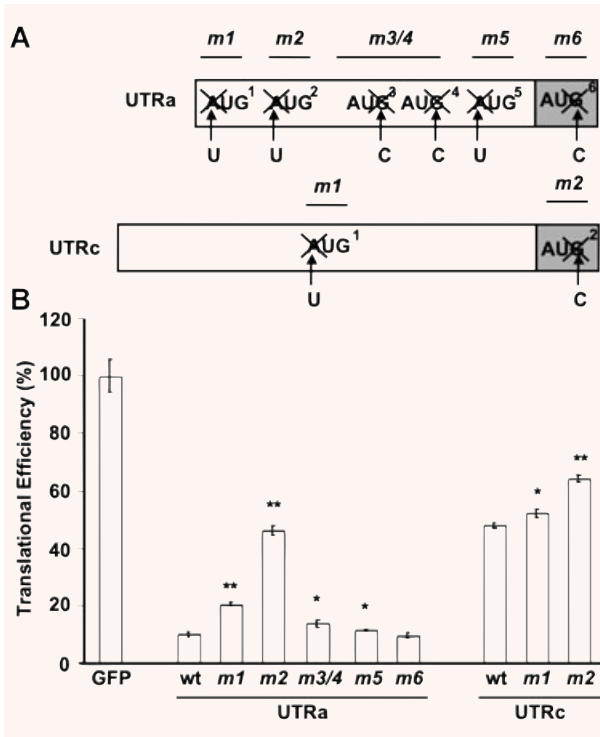
Fig. 3 uORFs within UTRa and UTRc are responsible for inhibition of translation. (**A**) The start codons within the uORFs of UTRa or UTRc were mutated to UUG or AUC as shown in the context of UTR-GFP reporter constructs. UTRa and UTRc are shown with sequences encoded by E0K or 0N as open boxes (with unique uORFs) and by E1 as shaded boxes (containing uORF common to UTRs). (**B**) MCF7 cells were transfected with reporter constructs and translational efficiencies determined relative to GFP control. Error bars show the standard deviation of technical triplicates within a representative experiment. The statistical significance of the difference between the wild-type UTRs and each mutant is shown: *$P < 0.05$, **$P < 0.001$.

since structural predictions suggest that these mutations may cause more substantial changes in overall secondary structure. We concluded that that uORFs, particularly the first and second in UTRa, and the second in UTRc, play a role in the translational inhibition specified by these UTRs.

5′UTR secondary structure reduces ERβ translational efficiency

Secondly, we were interested in whether RNA secondary structure within these UTRs was responsible for translational inhibition. We have already shown *in silico* that these 5′UTRs may form potentially inhibitory secondary structures (see above). The inhibitory influence of 5′UTR structures is especially important in carcino-

genesis since translation of these mRNAs can be specifically de-repressed by the action of the oncogenic translational factor eIF4E [36]. eIF4E is frequently over-expressed in cancers, including those of breast [37] and lung [38], leading to enhanced translation of a wide range of cancer-related transcripts. We examined whether ERβ 5′UTRs specify responsiveness to eIF4E by co-transfecting our 5′UTR GFP reporters with an expression vector for eIF4E, and examining translational efficiency as before. For these analyses we have used two cell lines, HB2 and MCF7, since these have different constitutive eIF4E activities (HB2s express low eIF4E levels compared to MCF7s; Fig. S2) and therefore are likely to respond differently to over-expressed eIF4E.

Cell lines were co-transfected with GFP reporters (Fig. 2A) and either empty expression plasmids or plasmids allowing eIF4E over-expression. Western blot analysis was used to confirm expression of exogenous eIF4E in the appropriate co-transfected cells (Fig. 4A). Translational efficiencies of reporters were determined as before (Fig. 4B). As expected, the translational efficiency of the control reporter (GFP), which expresses a non-specialized 5′UTR, was not significantly altered in either cell line by eIF4E over-expression. This demonstrated that over-expression of eIF4E did not result in non-specific changes in translation. In contrast, translational efficiencies specified by each ERβ 5′UTRs were increased. In HB2 cells, exogenous eIF4E enhanced translation from UTRa by 1.2-fold ($P = 0.004$) and from UTRc by 1.8-fold ($P = 0.0008$) (Fig. 4B). Increases were also seen in MCF7 cells (UTRa, 1.2-fold [$P = 0.047$], UTRc 1.3-fold [$P = 0.004$]) (Fig. 4B). It is worth emphasizing that the inhibitory effect of UTRc was relieved by exogenous eIF4E so effectively in HB2 cells that transcripts were translated as efficiently as uninhibited controls (Fig. 4B). We therefore concluded that secondary structure within these 5′UTRs was responsible for inhibition of translation, and that this inhibition, especially that specified by UTRc, can be overcome by highly active eIF4E – delineating a potential pathway for translational de-regulation of ERβ in cancer.

Different 3′ spliced isoforms are associated with particular 5′UTRs

Having defined influences of ERβ 5′UTRs and mechanisms involved using reporter assays, we were interested to examine whether 5′UTRs impacted on endogenous ERβ protein. However, this question is complex since the ERβ gene gives rise to multiple different ERβ mRNA isoforms that code for different proteins (ERβ1–5). ERβ1, 2 and 5 are particularly relevant in breast cell lines and cancers, since ERβ3 is thought to be testis-specific [4] and ERβ4 is not expressed in the breast [39], and we focused on these. The protein isoforms have identical N-termini but different C-termini due to differential 3′ splicing of their transcripts (Fig. 5A). Importantly, the proteins are functionally distinct being associated with differential responses to anti-oestrogens and patient survival [28, 40–42]. We have investigated whether alternative ERβ 5′UTRs are differentially associated with mRNAs for each ERβ

isoform, and therefore would have *separate* influences on expression of each isoform.

To perform these analyses we synthesized cDNA using primers specific for ERβ1, 2 or 5 and a protocol to enhance primer specificity, thereby creating cDNA pools greatly enriched for each specific message of interest (Fig. 5A). ERβ isoform specific cDNA pools were prepared from MCF7, HB2 and MDA-MB-231 cells. First, we determined whether enrichment for each isoform was successful using real-time PCR analysis of levels of ERβ1, 2 and 5 cDNA (Fig. 5B). In each case, cDNA pools were enriched for target isoforms, therefore in subsequent analyses we were able to examine relative expressions of 5′UTRs on transcripts for each isoform in isolation. Relative expression levels of 5′UTRs within these enriched cDNAs were then determined using real-time PCR for UTRa and UTRc (Fig. 5C–E). Clear differences in associations of 5′UTRs with mRNAs for specific isoforms were seen. For example, in MCF7 cells (Fig. 5C), ERβ2 mRNAs mainly contain UTRc (compare lanes 3 and 4) while ERβ1 and ERβ5 mRNAs mainly contain UTRa (lanes 1 and 2, or 5 and 6). Furthermore, these associations varied in different cell lines; for example, in contrast to MCF7 cells, ERβ1 messages mainly contained UTRc in both HB2 (Fig. 5D) and MDA-MB-231 cells (Fig. 5E), and UTRa containing ERβ5 mRNAs could not be detected in MDA-MB-231 cells. We concluded that mRNAs for each isoform have different and cell-type specific proportions of the two 5′UTRs. Since we have also shown that ERβ 5′UTRs determine greatly differing translational efficiencies (Fig. 2) we concluded that the proportions of the UTRs may define the overall translational efficiencies for each isoform, and thereby contribute to defining each expression level.

5′UTRs define how eIF4E impacts on expression of ERβ isoforms in tumours

Since ERβ 5′UTRs specified different degrees of response to eIF4E (Fig. 4), we expected that the relative proportions of these UTRs expressed on transcripts for each isoform would define different extents of translational enhancement given by eIF4E overexpression in cancer. We examined this complex regulation in tumour tissues by determining whether expression of ERβ1, 2 or 5 correlated with eIF4E expression. We predicted that correlations would differ depending on cancer-cell specific factors. For example, in tumour cells with similar regulation acting as is the case in HB2 cells, high eIF4E expression would stimulate translation of ERβ, effecting UTRc containing transcripts most strongly (Fig. 4), and since these were over-represented in mRNAs for ERβ1 (Fig. 5), we expected a stronger positive correlation between eIF4E expression and ERβ1 than with other isoforms. In contrast, in MCF7 or MDA-MB-231 cells UTRc was associated with either ERβ2 (MCF7) or with all three isoforms (MDA-MB-231), therefore we predicted that tumour cells with similar behaviour to these cell lines, would show more varied correlations, with the strongest positive correlation with ERβ2.
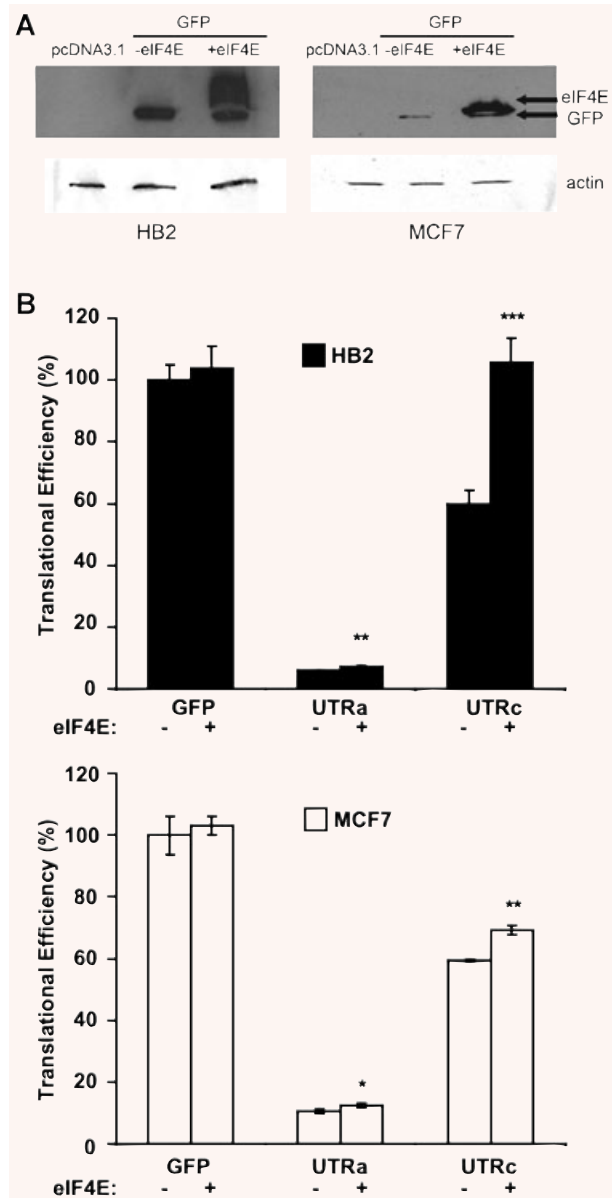

Fig. 4 Inhibition of translation by UTRa and UTRc is mediated by regions of stable secondary structure, and can be relieved by eIF4E. HB2 and MCF7 cells were transiently transfected with equal copy numbers of control or experimental GFP reporter constructs, without (using empty vector as a control plasmid) or with co-transfection to allow eIF4E overexpression. (**A**) Expression of exogenous proteins was examined within transfected HB2 and MCF7 cells, as shown, by Western blot analysis. Exogenous GFP and eIF4E both include the HA-epitope. (**B**) Translational efficiencies of reporters were determined relative to GFP control. Error bars show the standard deviation of technical triplicates within a representative experiment. The statistical significance of the difference between the UTRs with and without exogenous eIF4E is shown: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.
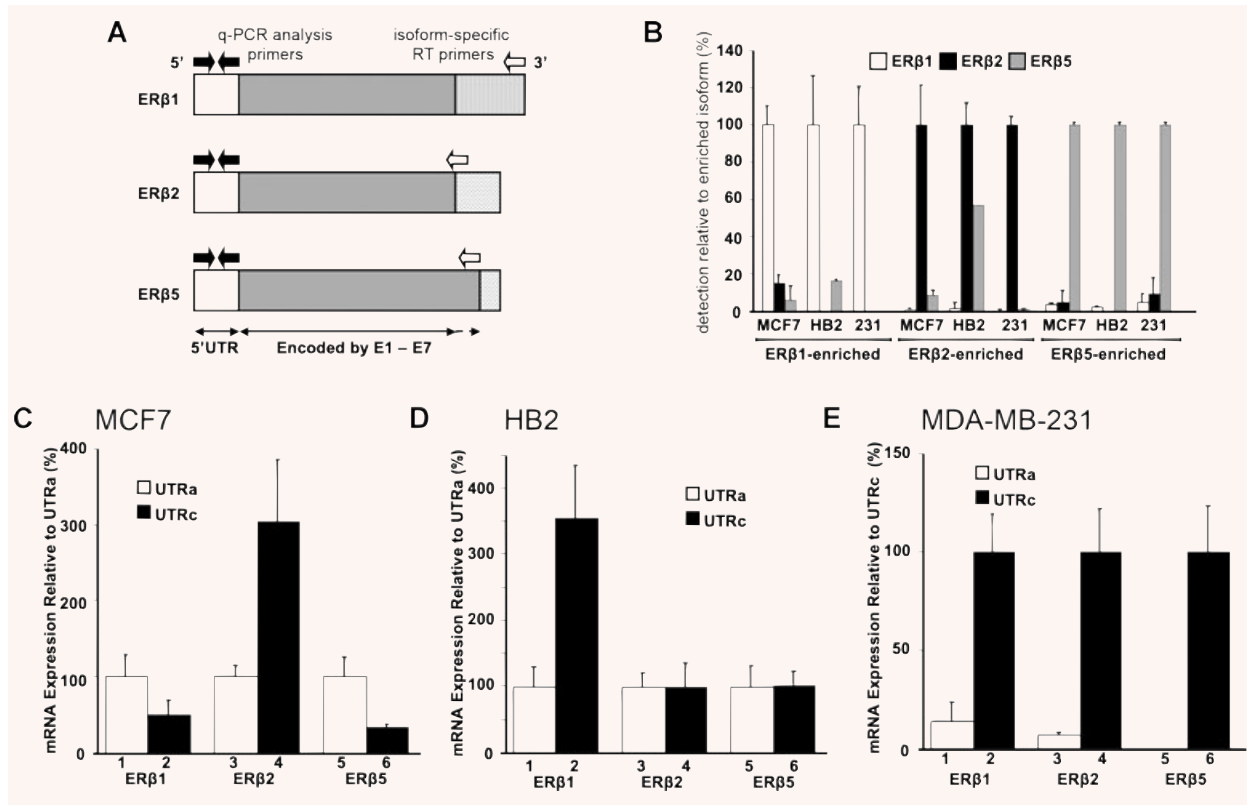
Fig. 5 Transcripts for ERβ1, 2 and 5 have cell-type specific associations with 5'UTRs. (**A**) Strategy for analysis of 5'UTRs of transcripts for ERβ1, 2 and 5. Isoform specific primers (open arrows) were designed to prime reverse transcriptase reactions specifically from only transcripts of ERβ isoforms of interest. Expression of UTRa and UTRc was examined within these isoform-enriched cDNA pools (black arrows). (**B**) cDNA synthesized using isoform specific primers was greatly enriched for the ERβ isoform of interest. ERβ isoform enriched cDNA pools were synthesized from RNA of cells as shown and real-time PCR was used to determine apparent expressions of ERβ1, 2 and 5. (**C–E**) Real-time PCR was used to determine expression of UTRa and UTRc within each highly-enriched cDNA pool. UTR expression is expressed relative to each other as indicated. Error bars indicate the standard deviation of technical triplicates within a representative experiment.

TMAs containing samples from 424 breast tumours were established with mean patient follow up, in terms of disease-free survival, of 91.9 months. We performed IHC for eIF4E, ERβ1, 2 and 5 on sections of these TMAs and assessed immunoreactivity within the tumour cells in each core, taking into account both proportions of tumour cells staining positively and average intensity. We, and others, have previously extensively validated the specificity of the antibodies we have used for IHC applications [28–29, 31]. Due to core loss during processing; a well recognized problem associated with TMAs, scores were available for 408 cases. Representative staining patterns are shown in Fig. 6. For eIF4E, tumour stroma and normal tissue were negative while positive staining within tumour cells was generally cytoplasmic, although nuclear staining was noted in some cases (data not shown [29]). All ERβ isoforms investigated were expressed in epithelial and some stromal cell nuclei. In some cases, additional cytoplasmic

ERβ1 expression was seen and cytoplasmic ERβ2 was occasionally detected in the absence of nuclear immunoreactivity (data not shown, but shown and discussed in our previous work [28]). The full range of scores was observed for each antigen, with scores of 5, 6, 8, and 8 occurring most frequently for eIF4E, ERβ1, ERβ2 and ERβ5, respectively. Associations between markers were examined using Spearman's correlation tests (Table 1). Over the whole cohort ($n = 408$), eIF4E expression showed a positive association with ERβ1 expression (correlation co-efficient $r = 0.28$; $P < 0.0001$), although no association with ERβ2 or ERβ5. We next examined associations between these markers in two patient groups, those who experienced recurrences ($n = 74$) and those that did not ($n = 334$), since we expected correlations to differ with cell-specific factors (Fig. 5), and since both eIF4E [36] and ERβ [28] influence the likelihood of recurrences. In the non-recurrence group ($n = 334$), eIF4E expression showed a positive

Fig. 6 Representative breast tumour TMA cores showing immunoreactivity for eIF4E (**A–B**), ERβ1 (**C–D**), ERβ2 (**E–F**) and ERβ5 (**G–H**). These cores were scored: A 3, B 7, C 3, D 7, E 3, F 7, G 3, H 7. Examples of areas of stromal cells or epithelial cancer cells are labelled 'S' or 'Ep', respectively.
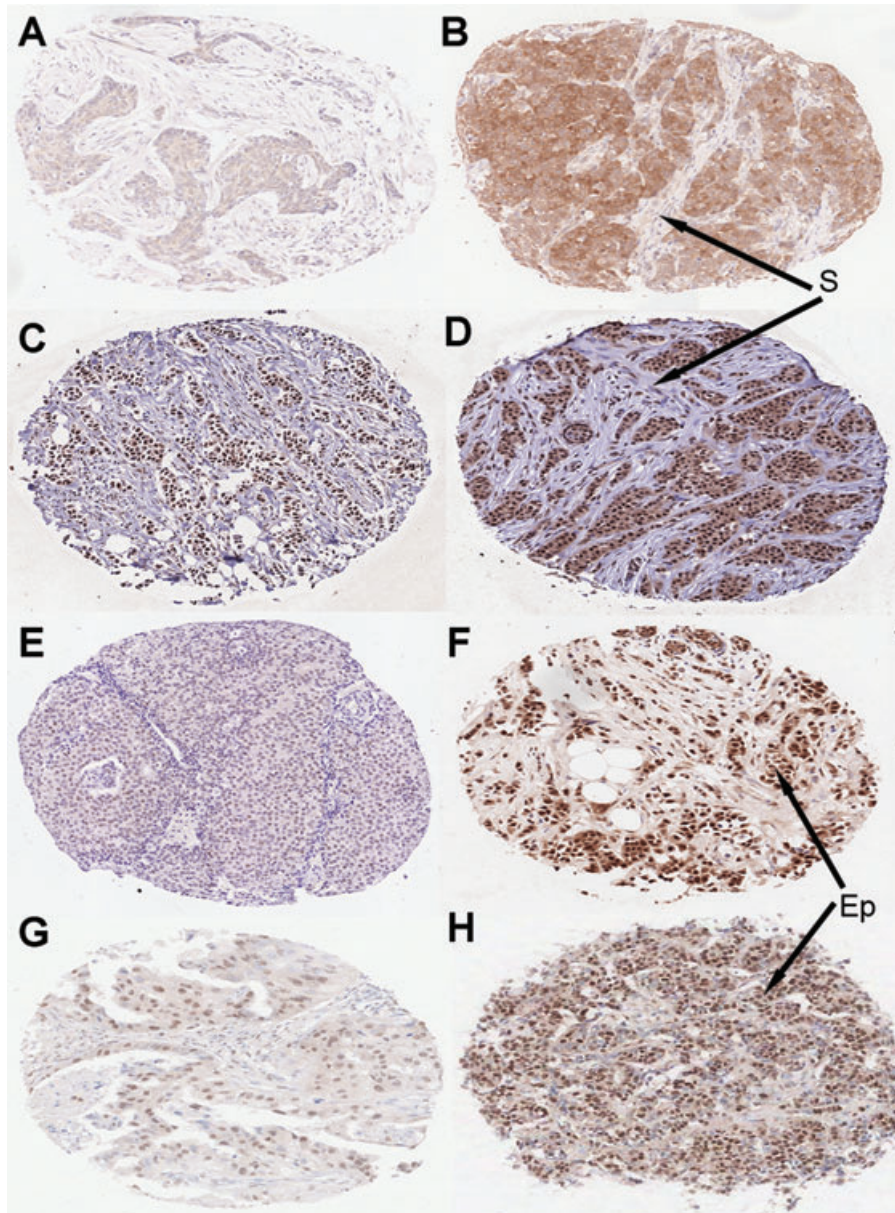
association with expression of only ERβ1 ($r = 0.28$; $P < 0.01$). In contrast, in the recurrence group ($n = 74$), eIF4E expression showed a positive association with expression of ERβ2 ($r = 0.27$; $P < 0.05$), and weaker non-significant associations with ERβ1 and ERβ5. We concluded that correlations seen in the patient groups mirrored the differences we expected based on analysis of cell lines; with regulation occurring within tumours that did not recur resembling that in the transformed, but non-malignant HB2 cells, while tumours that did recur resembled the weakly (MCF7) and strongly (MDA-MB-231) invasive cancer cell lines of metastatic origin.

Discussion

We have investigated the abilities of ERβ 5′UTRs to allow post-transcriptional regulation of ERβ expression, and – *via* differential effects on ERβ isoforms – ERβ function. Initially, we studied four alternative 5′UTRs (Fig. 1A), although we were only able to show expression of two and we believe these, UTRa and UTRc, to be the majority species (Fig. 1B–D). Unlike most vertebrate 5′UTRs, these are relatively long (>200 nucleotides) and contain sequences with potential to confer translational regulation upon

Table 1 eIF4E expression is differentially associated with ERβ isoforms in breast tumours that did or did not recur. Spearman's rho analyses were performed to examine correlations between expression of eIF4E and ERβ1, 2 and 5 in a cohort of breast tumours. Correlation coefficients are shown for the whole cohort (*n* = 408), and for the cohort split into patients who did (*n* = 74), or did not suffer recurrences (*n* = 334). The three strongest and most significant associations are highlighted in bold. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

eIF4E	ERβ1	ERβ2	ERβ5
Whole cohort: (*n* = 408)	**0.28***	0.15*	0.16*
Non-recurrences: (*n* = 334)	**0.28**	0.12	0.15*
Recurrences: (*n* = 74)	0.24	**0.27***	0.21

their mRNAs. We have demonstrated that these 5′UTRs directly inhibit translation of downstream reading frames (Fig. 2), and we have determined that uORFs (Fig. 3) and stable secondary structures (Fig. 4) are responsible for these effects. Importantly, we have found that these 5′UTRs act as sites of regulation of expression, rather than of invariant repression, by at least two separate mechanisms. First, we found that the degree of translational inhibition specified by each 5′UTR depended on the cellular context; UTRa was generally strongly inhibitory, but the degree of translation varied in different cell lines by up to 2.6-fold when comparing the least inhibited to the most. UTRc was generally much less inhibitory, although similar variation was seen (up to 1.9-fold) between cell lines. It is clear that changes in expression or activity of cellular factors (including, for example, eIF4E; Fig. 4) can alter the degree of translation from ERβ transcripts. Secondly, the relative proportions of each UTR within either the total pool of ERβ transcripts (Fig. 1C and D) or within transcripts for a specific ERβ isoform (Fig. 5C–E) varied substantially, therefore altering the net translational efficiency for that isoform. ERβ would be more highly expressed in cells that express a greater proportion of UTRc (for example, ERβ2 transcripts in MCF7 cells; Fig. 5C), since these transcripts would be translated relatively efficiently (Fig. 2), as compared to lower expression from transcripts containing a majority of UTRa (for example, ERβ5 transcripts in MCF7 cells; Fig. 5C). Thus, ERβ expression is defined not only by the total amount of mRNA, but also by the proportions of the different 5′UTRs within these messages, and by the cell-type specific translational efficiency specified by each UTR. ERβ joins a growing list of potential oncogenes and tumour suppressor genes for which this has been reported [27, 43–44]. The fact that proportions of ERβ 5′UTRs differ for transcripts coding for each ERβ isoform *separately* (Fig. 5) demonstrates the potential complexity of these regulatory mechanisms.

It is important to note that differing proportions of these 5′UTRs are associated with mRNAs with different 3′ splice patterns and this has fundamental implications for transcription and mRNA processing dynamics. It appears that in MCF7 cells, if transcription initiates at E0K the mRNAs contain UTRa and are likely to be spliced at their 3′ ends into transcripts for ERβ1 or ERβ5, and are relatively unlikely to become ERβ2 transcripts (Fig. 5C). In contrast, if transcription initiates at E0N the mRNAs contain UTRc and are more likely to be spliced at their 3′ ends to form ERβ2. An

interesting question is how could either the transcriptional start site and/or the presence of a particular 5′UTR influence 3′ splicing taking place mega-bases of DNA away? It is also evident that this influence is itself regulated by cellular context as a different relationship between the transcriptional start site/5′UTR and 3′ splicing was observed in HB2 cells (Fig. 5D). Cross-talk between mRNA 5′ ends and 3′ splicing has been reported only rarely and various models have been proposed [45–46]. These include recruitment at specific promoters and maintenance within the extending polymerase complex of transcription factors with functions in both transcription and splicing [47–48]. Alternatively, 5′ mRNA structure may influence alternative splicing by controlling polymerase processivity [46, 49–50] or by binding directly to splicing factors. It has been estimated that at least 10–18% of genes have multiple promoters [24, 51] allowing expression of alternative 5′UTRs and that the majority of genes show differential splicing within their coding regions [48], therefore this regulatory cross talk may be extremely influential.

ERβ deregulation in cancer

The role and expression of ERβ in carcinogenesis is poorly understood. ERβ appears to be anti-proliferative and pro-apoptotic, although details differ as to whether effects of each isoform result from their independent actions as transcription factors, or *via* hetero-dimerization with other receptors [10–11]. Expression of ERβ1 is down-regulated during carcinogenesis in breast and colon, as one might expect for an anti-proliferative molecule, and consequently ERβ has been discussed as a tumour suppressor [52–54], although this may be misleading given the different functions of the other isoforms. ERβ2 appears to be up-regulated during carcinogenesis [55–56], while little is known about these patterns of expression of ERβ5. In terms of prognostic value, conflicting reports abound, with expression of ERβ1 but not ERβ2 [42], or ERβ2 and 5 but not ERβ1 [28, 40–41] being associated with good prognosis, or with ERβ2 associated with poor prognosis [57]. This suggests that these isoforms should be considered as functionally distinct receptors. Even less is known about the regulatory mechanisms responsible for the expression changes in cancer, although hypermethylation of the ERβ promoter has been suggested as a mechanism for loss of ERβ1 expression [5, 54,

58]. It is well established that changes in translational efficiency of key oncogenes and tumour suppressor genes are associated with and contribute to carcinogenesis [12]. Here, we provide evidence that translational regulation allows deregulation of ERβ expression in cancer by three related mechanisms.

First, we show that UTRa, which specifies very inefficient translation (Fig. 2B), is up-regulated in breast and lung tumours relative to matched normal tissues in the absence of up-regulation of total message (Fig. 1D). The result is a higher proportion of translationally repressed transcripts within the total ERβ mRNA pool, and therefore protein expression would be down-regulated within tumours, as has been reported [53–54, 56]. A change in ratio of differentially acting 5′UTRs has also been implicated in the deregulation of other tumour suppressors and oncogenes, including BRCA1 [44] and Mdm2 [43]. Secondly, the translational efficiencies of ERβ mRNAs were increased by eIF4E (Fig. 4). This translation factor is overexpressed in a wide range of cancers [37–38, 59–60], and contributes to carcinogenesis by derepressing translation of cancer-related transcripts that are otherwise inefficiently translated on account of 5′UTR secondary structures [21]. This mechanism allows up-regulation of ERβ expression during carcinogenesis and is dependent on the degree of eIF4E activity. Since ERβ1 has been discussed as a tumour suppressor [52–54], it may seem surprising to uncover a mechanism for its up-regulation in cancer. However, this up-regulation could act as a driving force for expression loss by other mechanisms during the molecular evolution of tumours. In addition, and supported by our work, eIF4E is likely to act differentially on the different ERβ isoforms – some of which have no reported tumour suppressor function. With this in mind, we finally examined how these mechanisms combine to allow changes in expression of specific ERβ isoforms. We showed that 5′UTRs were differentially associated with transcripts for ERβ1, 2 and 5 (Fig. 5) and demonstrated a differential response of the UTRs to eIF4E (Fig. 4), allowing us to infer that overexpression of eIF4E in cancer would cause different degrees of induction of each isoform. Therefore, we examined associations between expressions of eIF4E and ERβ1, 2 and 5 in a large cohort of invasive breast cancers (Fig. 6, Table 1). The associations between ERβ isoforms and eIF4E varied between patients that had a recurrence and those that did not, suggesting that eIF4E not only had a differential influence on translation of each isoform, but that this influence was dependent on further tumoral factors. In particular, eIF4E apparently stimulated the expression of ERβ1 in tumours that did not recur, and the expression of ERβ2 in tumours that did recur. These observations mirrored our observations in cell lines where eIF4E would preferentially stimulate expression of ERβ1 in HB2 cells, an immortalized breast cell line without malignant or metastatic properties, since the 5′UTR associated with ERβ1 transcripts responds most strongly to eIF4E (Figs 4 and 5D). Similarly, the 5′UTR expression patterns for each ERβ isoform in MCF7 cells, a more aggressive breast cell line of malignant and metastatic origin, would mean that eIF4E would preferentially stimulate ERβ2 expression in these cells (Figs 4 and 5C). Breast cancer cell lines are likely to reflect features of cancer cells *in vivo* [61], therefore this correlation also validated our use of these *in vitro* models within the study. Unfortunately, reliable quantitative detection of either the protein isoforms in cell lines, or the mRNA species in archival tumour tissues is not possible, hence we are unable to confirm that the regulation occurring within HB2 and MCF7 cells is truly representative of these tumours. It is worth noting that eIF4E expression did not significantly associate with expression of all three isoforms simultaneously, as would be predicted if the tumours' 5′UTR expression patterns were as seen in MDA-MB-231 cells (Fig. 5E). A probable explanation is that MDA-MB-231 cells have a basal phenotype and these tumours were rare within our cohort.

Conclusion

We have defined complex regulatory mechanisms for ERβ that have a role in deregulation of ERβ expression in cancer. Importantly, these are the first reported insights into separate regulation of expression of the functionally distinct ERβ isoforms, and are therefore likely to be critical in defining ERβ function. Interestingly, our data also provide novel evidence of a role for promoter or 5′UTR elements in the regulation of alternative splicing downstream.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Translational efficiencies (**C**) from GFP mRNA expression levels, as determined by real-time PCR analyses and normalized to control (**A**) and GFP protein expression levels, as determined from the mean fluorescence intensity of cell populations and normalized to control (**B**). Representative data from transfection of MCF7 cells with either the control GFP reporter, or the reporters for UTRa and UTRc are shown.

Fig. S2 HB2 cells express lower levels of endogeneous eIF4E protein than MCF7 cells. Expression of eIF4E, or beta-actin, was examined within protein lysates of HB2 and MCF7 cells by Western blotting.

Table S1 Culture and transfection conditions

Table S2 Primer details

Table S3 Mutations of each uORF within UTRa caused small increases in the extent of structure relative to the wild-type sequence as assessed by ΔG, whereas mutations within UTRc

caused either a small decrease or small increase in the extent of structure relative to the wild-type.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

References

1. **Sommer S, Fuqua S.** Estrogen receptor and breast cancer. *Semin Cancer Biol.* 2001; 11: 339–52.
2. **O'Neill P, Davies M, Shaaban A, et al.** Wild-type ERβ1 mRNA and protein expression in Tamoxifen-treated post-menopausal breast cancers. *Br J Cancer.* 2004; 91: 1694–702.
3. **Speirs V.** ERβ in breast cancer: good, bad, or still too early to tell? *J Pathol.* 2002; 197: 143–7.
4. **Moore J, McKee D, Slentz-Kesler K, et al.** Cloning and characterization of human ERβ isoforms. *Biochem Biophys Res Commun.* 1998; 247: 75–8.
5. **Bardin A, Boulle N, Lazennec G, et al.** Loss of ERβ expression as a common step in estrogen-dependent tumor progression. *Endocr Relat Cancer.* 2004; 11: 537–51.
6. **Galluzzo P, Caiazza F, Moreno S, et al.** Role of ERbeta palmitoylation in the inhibition of human colon cancer cell proliferation. *Endocr Relat Cancer.* 2007; 14: 153–67.
7. **Lazennec G.** Estrogen receptor beta, a possible tumor suppressor involved in ovarian carcinogenesis. *Cancer Lett.* 2006; 231: 151–7.
8. **Stettner M, Kaulfuss S, Burfeind P, et al.** The relevance of estrogen receptor-beta expression to the antiproliferative effects observed with histone deacetylase inhibitors and phytoestrogens in prostate cancer treatment. *Mol Cancer Ther.* 2007; 6: 2626–33.
9. **Treeck O, Lattrich C, Springwald A, et al.** Estrogen receptor beta exerts growth-inhibitory effects on human mammary epithelial cells. *Breast Cancer Res Treat.* 2009; DOI 10.1007/s10549–009-0413–2.
10. **Leung Y, Mak P, Hassan S, et al.** ERβ isoforms: a key to understanding ERβ signalling. *PNAS.* 2006; 103: 13162–7.
11. **Zhao C, Matthews J, Tujague M, et al.** ERβ2 negatively regulates the transactiva-

tion of ERα in human breast cancer cells. *Cancer Res.* 2007; 67: 3955–62.
12. **Audic Y, Hartley R.** Post-transcriptional regulation in cancer. *Biol Cell.* 2004; 97: 479–98.
13. **Gray N, Wickens M.** Control of translation initiation in animals. *Annu Rev Cell Dev Biol.* 1998; 14: 399–458.
14. **Pain V.** Initiation of protein synthesis in eukaryotic cells. *Eur J Biochem.* 1996; 236: 747–71.
15. **Gebauer F, Hentze M.** Molecular mechanisms of translational control. *Nat Rev Mol Cell Biol.* 2004; 5: 827–35.
16. **Kozak, M.** Regulation of translation *via* mRNA structure in prokaryotes and eukaryotes. *Gene.* 2005; 361: 13–37.
17. **Morris D, Geballe A.** Upstream ORFs as regulators of mRNA translation. *Mol Cell Biol.* 2000; 20: 8635–42.
18. **Kozak, M.** An analysis of 5′-noncoding sequences from 699 vertebrate messenger RNAs. *Nucleic Acids Res.* 1987; 15: 8125–48.
19. **Kozak, M.** An analysis of vertebrate mRNA sequences: intimations of translational control. *J Cell Biol.* 1991; 115: 887–903.
20. **Pesole G, Mignone F, Gissi C, et al.** Structural and functional features of eukaryotic mRNA UTRs. *Gene.* 2001; 276: 73–81.
21. **Koromilas A, Lazaris-Karatzas A, Sonenberg N.** mRNAs containing extensive secondary structure in their 5′ non-coding region translate efficiently in cells overexpressing initiation factor eIF4E. *EMBO J.* 1992; 11: 4153–58.
22. **Wek R, Jiang H, Anthony T.** Coping with stress: eIF2 kinases and translational control. *Biochem Soc Trans.* 2006; 34: 7–11.
23. **Hughes, T.** Regulation of gene expression by alternative UTRs. *Trends Genet.* 2006; 22: 119–22.
24. **Zhang T, Haws P, Wu Q.** Multiple variable first exons: a mechanism for cell- and tissue-specific gene regulation. *Genome Res.* 2004; 14: 79–89.

25. **Hughes T, Brady H.** Cross-talk between pRb/E2F and Wnt/β-catenin pathways: E2F1 induces axin2 leading to repression of Wnt signalling and to increased cell death. *Exp Cell Res.* 2005; 303: 32–46.
26. **Pyronnet S, Imataka H, Gingras A, et al.** Human eIF4G recruits mnk1 to phosphory-late eIF4E. *EMBO J.* 1999; 18: 270–9.
27. **Hughes T, Brady H.** Expression of axin2 is regulated by the alternative 5′UTRs of its mRNA. *J Biol Chem.* 2005; 280: 8581–8.
28. **Shaaban A, Green A, Karthik S, et al.** Nuclear and cytoplasmic expression of ERβ1, 2 and 5 identifies distinct prognostic outcome for breast cancer patients. *Clin Cancer Res.* 2008; 14: 5228–35.
29. **Coleman LJ, Peter MB, Teall TJ, et al.** Combined analysis of eIF4E and 4E-binding protein expression predicts breast cancer survival and estimates eIF4E activity. *Br J Cancer.* 2009; 100: 1393–9.
30. **Allred D, Harvey J, Berado M, et al.** Prognostic and predictive factors in breast cancer by immunohistochemical analysis. *Mod Pathol.* 1998; 11: 155–68.
31. **Zhou S, Wang G, Liu C, et al.** eIF4E and angiogenesis: prognostic markers for breast cancer. *BMC Cancer.* 2006; 6: 231–43.
32. **Zuker M.** Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res.* 2003; 31: 3406–15.
33. **Hirata S, Shoda T, Kato J, et al.** The multiple untranslated first exons system of the human ERβ gene. *J Steroid Biochem Mol Biol.* 2001; 78: 33–40.
34. **Gray N, Hentze M.** Regulation of protein synthesis by mRNA structure. *Mol Biol Rep.* 1994; 19: 195–200.
35. **Meijer H, Thomas A.** Control of eukaryotic protein synthesis by upstream ORFs in the 5′UTR of an mRNA. *Biochem J.* 2002; 367: 1–11.
36. **Clemens M.** Targets and mechanisms for the regulation of translation in malignant transformation. *Oncogene.* 2004; 23: 3180–8.

37. **Kerekatte V, Smiley K, Hu B, et al.** The protooncogene/translation factor eIF4E: a survey of expression in breast carcinomas. *Int J Cancer*. 1995; 64: 27–31.

38. **Rosenwald I, Hutzler M, Wang S, et al.** Expression of eIF4E and eIF2alpha is increased frequently in bronchioloalveolar but not in squamous cell carcinomas of the lung. *Cancer*. 2001; 92: 2164–71.

39. **Poola I, Abraham J, Baldwin K, et al.** ERβ4 and β5 are full length functionally distinct ERβ isoforms: cloning from human ovary and functional characterization. *Endocrine*. 2005; 27: 227–38.

40. **Sugiura H, Toyama T, Hara Y, et al.** Expression of ERβ wild-type and its variant ERβ2 is correlated with better prognosis in breast cancer. *Jpn J Clin Oncol*. 2007; 37: 820–8.

41. **Vinayagam R, Sibson D, Holcombe C, et al.** Association of ERβ2/ERβcx with outcome of adjuvant endocrine treatment for primary breast cancer-a retrospective study. *BMC Cancer*. 2007; 7: 131–40.

42. **Honma N, Horii R, Iwase T, et al.** Clinical importance of ERβ evaluation in breast cancer patients treated with adjuvant tamoxifen therapy. *J Clin Oncol*. 2008; 26: 3727–34.

43. **Okumura N, Saji S, Eguchi H, et al.** Distinct promoter usage of mdm2 gene in human breast cancer. *Oncol Rep*. 2002; 9: 557–63.

44. **Sobczak K, Krzyzosiak W.** Structural determinants of BRCA1 translation regulation. *J Biol Chem*. 2002; 277: 17349–58.

45. **Gendra E, Colgan D, Meany B, et al.** A sequence motif in the SV40 early core promoter affects alternative splicing of transcribed mRNA. *J Biol Chem*. 2007; 282: 11648–57.

46. **Russcher H, Dalm V, de Jong F, et al.** Associations between promoter usage and alternative splicing of the GR gene. *J Mol Encrinol*. 2007; 38: 91–8.

47. **Auboeuf D, Dowhan D, Kang Y, et al.** Differential recruitment of nuclear receptor coactivators may determine alternative RNA splice site choice in target genes. *PNAS*. 2004; 101: 2270–4.

48. **Auboeuf D, Honig A, Berget S, et al.** Coordinate regulation of transcription and splicing by steroid receptor coregulators. *Science*. 2002; 298: 416–9.

49. **Nogues G, Kadener S, Cramer P, et al.** Transcriptional activators differ in their abilities to control alternative splicing. *J Biol Chem*. 2002; 277: 43110–4.

50. **Rosonina E, Bakowski M, McCracken S, et al.** Transcriptional activators control splicing and 3′-end cleavage levels. *J Biol Chem*. 2003; 278: 43034–40.

51. **Trinklein N, Aldred S, Saldanha A, et al.** Identification and functional analysis of human transcriptional promoters. *Genome Res*. 2003; 13: 308–12.

52. **Foley E, Jazaeri A, Shupnik M, et al.** Selective loss of ERβ in malignant human colon. *Cancer Res*. 2000; 60: 245–8.

53. **Roger P, Sahla M, Mäkelä S, et al.** Decreased expression of ERβ protein in proliferative preinvasive mammary tumors. *Cancer Res*. 2001; 61: 2537–41.

54. **Skliris G, Munot K, Bell S, et al.** Reduced expression of ERβ in invasive breast cancer and its re-expression using DNA methyl transferase inhibitors in a cell line model. *J Pathol*. 2003; 201: 213–20.

55. **Esslimani-Sahla M, Kramar A, Simony-Lafontaine J, et al.** Increased ER beta2 expression during mammary carcinogenesis. *Clin Cancer Res*. 2005; 11: 3170–4.

56. **Shaaban A, O'Neill P, Davies M, et al.** Declining ERβ expression defines malignant progression of human breast neoplasia. *Am J Surg Pathol*. 2003; 27: 1502–12.

57. **Saji S, Omoto Y, Shimizu C, et al.** Expression of ER betacx protein in ERalpha-positive breast cancer: specific correlation with PgR. *Cancer Res*. 2002; 62: 4849–53.

58. **Rody A, Holtrich U, Solbach C, et al.** Methylation of ERβ promoter correlates with loss of ERβ expression in mammary carcinoma and is an early indication marker in premalignant lesions. *Endocr Relat Cancer*. 2005; 12: 903–16.

59. **Nathan C, Franklin S, Abreo F, et al.** Expression of eIF4E during head and neck tumorigenesis: possible role in angiogenesis. *Laryngoscope*. 1999; 109: 1253–8.

60. **Rosenwald I, Chen J, Wang S, et al.** Upregulation of protein synthesis initiation factor eIF4E is an early event during colon carcinogenesis. *Oncogene*. 1999; 18: 2507–17.

61. **Lacroix M, Leclercq G.** Relevance of breast cancer cell lines as models for breast tumours: an update. *Breast Cancer Res Treat*. 2004; 83: 249–89.

62. **Giard DJ, Aaronson SA, Todaro GJ, et al.** In vitro cultivation of human tumors: establishment of cell lines derived from a series of solid tumors. *J Natl Cancer Inst*. 1973; 51: 1417–23.

63. **Nelson-Rees WA, Flandermeyer RR, Hawthorne PK.** Distinctive banded marker chromosomes of human tumor cell lines. *Int J Cancer*. 1975; 16: 145–50.

64. **Marot D, Opolon P, Brailly-Tabard S, et al.** The tumor suppressor activity induced by adenovirus-mediated BRCA1 overexpression is not restricted to breast cancers. *Gene Ther*. 2006; 13: 235–44.

65. **Berdichevsky F, Alford D, D'Souza B, et al.** Branching morphogenesis of human mammary epithelial cells in collagen gels. *J Cell Sci*. 1994; 107: 3557–68.

66. **Soule HD, Vazquez J, Long A, et al.** A human cell line from a pleural effusion derived from a breast carcinoma. *J Natl Cancer Inst*. 1973; 51: 1409–16.

67. **Cailleau R, Olivé M, Cruciger QV.** Long-term human breast carcinoma cell lines of metastatic origin: preliminary characterization. *In Vitro*. 1978; 14: 911–5.