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Modulation of ER α transcriptional activity by the orphan nuclear receptor ERR β and evidence for differential effects of long- and short-form splice variants.

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Abstract

Oestrogen receptor related proteins (ERRs) affect target gene expression without binding oestradiol. We investigated the functional activity of two splice variant isoforms of ERR β (ERR β S [short], ERR β L [long]) expressed in human endometrium, where they are

5 coexpressed with the oestrogen receptor alpha (ER α). Over-expression of ERR β L enhanced ER α -dependent ligand-induced activation of an ERE-luciferase reporter construct, altered the induction of c-myc mRNA and increased proliferation of Ishikawa cells whereas ERR β S was found to reduce these endpoints.

10 Fluorescent recovery after photobleaching (FRAP) revealed that intra-nuclear mobility of YFP-ERR β S was more rapid than YFP-ERR β L. Fluorescence resonance energy transfer (FRET) assays revealed a close association between ERR β L and ER α following addition of ligand. We speculate that ERR β L may alter ER α -dependent gene activation by enhancing the recruitment of co-activators. In conclusion, variant isoforms of ERR β have differential effects
15 on ER α -dependent gene expression and this has implications for human endometrial cell function.

1. Introduction

The nuclear receptors (NR) play a key role in the regulation of processes involved in reproduction, development, growth and nutrient uptake [1]. A classical view is that NRs act as transcription factors modulating the expression of target genes defined by NR-selective regulatory elements in their promoters; some are classified as ligand-activated (e.g. steroid receptors), while others for which a natural/endogenous ligand has yet to be identified are known as ‘orphans’ [2]. As many cell types express more than one type of NR an important challenge is elucidating to what extent variations in the intracellular complement of receptors can influence cell or tissue responsiveness to different stimuli.

Oestrogenic ligands bind and activate one of two oestrogen receptors (ER) known as ER α and ER β [3]. They are encoded by separate genes ESR1 (NR3A1) and ESR2 (NR3A2) [4] and arose from a single ancestral receptor by gene duplication [5]. ERs are widely expressed throughout the body and have been implicated in regulation of normal physiological processes including reproduction, cognition and inflammation as well as pathologies including endometriosis and cancer [6]. Members of the NR superfamily share a common arrangement of structural/functional domains including a conserved cysteine-rich region that functions as the DNA-binding domain [7,8]. In addition to the classical ERs three additional oestrogen receptor related genes known as ERR α (*ESRRA/ERR1/NR3B1*), ERR β (*ESRRB/ERR2/NR3B2*) and ERR γ (*ESRRG/ERR3/NR3B3*) have been identified [9,10]. The full-length human *ESRRB* gene contains 12 exons and three ERR β mRNA splice variants have been identified in human tissues; a short form (exons 1-9), a long form (exons 1-12) and a Δ 10 form that results in a shift in the reading frame and a protein with an altered C terminus [11,12]. Genomic sequence alignment suggests that only the short form exists in species other than primates [11].

It has been proposed that ERRs may modulate both cell fate decisions and cell metabolism [13]. For example, studies in mice have demonstrated that ERR α plays a key role in adaptive thermo genesis and mitochondrial biogenesis [14,15] and ERR α null mice have reduced fat mass [16]. ERR γ null mice die during the first week of life due to heart defects [17]. Bookout et al [1] grouped ERR β and ERR γ with steroid hormone receptor (SHR) receptors involved in circadian and basic metabolic functions and reported that in the mouse highest levels of expression of ERR β mRNA could be detected in the eye, kidney and heart with lower concentrations in testis, thyroid and parts of the brain. An essential role for ERR β in placental

development and proliferation of primordial germ cells has been revealed by studies in $ERR\beta$ null mice [18,19]. Recent data has also implicated $ERR\beta$ in regulating genes critical for the formation and function of the inner ear where it is believed to regulate cell fate decisions [20]. Studies in the human have demonstrated immunoexpression of $ERR\beta$ in the cochlea and missense mutations in the $ESRRB$ gene have been detected in affected families diagnosed with nonsyndromic hearing impairment [12]. Studies using cells from the mouse have suggested that $ERR\beta$ may function in conjunction with OCT4 and SOX-2 to promote expression of genes involved in self-renewal and pluripotency [21]. However $ERR\beta$ has not been detected in human ESCs suggesting that fundamental differences may exist between the role played by this gene in functional maturation of embryonic cells [22]. The impact of $ERR\beta$ on cell function in oestrogen-dependent human tissues such as the endometrium remains unexplored.

The classical model for ER-dependent regulation of gene expression involves the direct binding of receptor dimers to palindromic DNA sequences known as oestrogen response elements (EREs) and a large number of genes containing EREs have been identified (reviewed in [23]). Genomic studies using breast cancer cells have discovered that binding of $ER\alpha$ often occurs at enhancer elements and in some cases may depend upon the binding of additional transcription factors such as Sp1 [24]. Once bound to promoter or enhancer elements NR activate transcription by recruitment of additional proteins/protein complexes including members of the general transcription machinery. $ERR\beta$ contains a core DNA binding domain that exhibits high sequence homology to the sequence in the DNA binding domain of $ER\alpha$ and is reported to bind as a monomer at ERE half sites [5'-AGGTCA-3'] in the major groove of DNA and an upstream 5'-TNA-3' site in the minor groove [25]. An interplay between ERs and ERRs in regulation of gene expression has been claimed on the basis of the results of in vitro studies and it has been proposed that ERRs could interfere with ER-dependent gene expression or participate in co-regulation of target genes such as osteopontin and pS2 [26,27].

We have previously demonstrated that $ERR\beta$ is expressed in multiple cell types within the human endometrium and found evidence of expression of both long and short splice variants of $ERR\beta$ [28]. In the present study we used an endometrial epithelial cell line to investigate whether expression of $ERR\beta S$ or $ERR\beta L$ splice variants can alter oestrogen-responsiveness. We have documented differences in the activity of these isoforms and speculate that the

presence of additional sequences at the C-terminus of ERR β L influences intra-nuclear mobility and functional activity of the receptor.

2. Materials and methods

2.1 Constructs

An ERR β cDNA (NM_004452), coding for the long form of ERR β (ERR β L), was purchased from Origene (Rockville, MD, USA). The entire cDNA (ERR β L) was subcloned into the pDC315 plasmid (Microbix, Toronto, ON, Canada) using Bam HI and Eco RI restriction sites. In order to generate the short form of ERR β (ERR β S), a PCR reaction was carried out using primers specific for the sequence encoded by exons 1 to 9. The last amino acid was turned into a valine and a stop codon was introduced, by substituting the ERR β L sequence GCC TGG with the ERR β S-specific sequence GTA TAG. The primers had Bam HI and Eco RI restriction recognition sites incorporated into their 5' ends and the resulting amplicon was cloned into the pDC315 vector as above. In order to generate proteins with fluorescent protein tags ERR β L and ERR β S cDNAs were subcloned between the Eco RI and Bam HI restriction sites in a yellow fluorescent protein vector (pEYFP-C1, Clontech, Mountain View, CA, USA). ER α cDNA was subcloned between the Eco RI and Bam HI sites of a cyan fluorescent protein expression vector (pECFP-C1, Clontech). A short hairpin siRNA sequence specific for ER α was designed using freely available online tools (available at www.ambion.com). Two complementary oligomers (siER α forward 5'-GAT CCC AGA TTG GCC AGT ACC AAT GTT CAA GAG ACA TTG GTA CTG GCC AAT CTT TTT TTG GAA A-3' and siER α reverse 5'-AGC TTT CCA AAA AAA GAT TGG CCA GTA CCA ATG TCT CTT GAA CAT TGG TAC TGG CCA ATC TGG-3') were purchased from MWG Biotech, annealed and cloned into the pSilencer vector (Ambion) between the Bam HI and Hind III sites. Specific knockdown of ER α protein was confirmed by Western blotting using cells co-transfected with 500ng ER α and 1, 5 or 10nM of plasmid expressing an ER α -specific shRNA, or 10nM of a non-specific scrambled shRNA or an shRNA specific for ER β . (Took out mRNA as we don't mention method or results) All constructs were checked by sequencing and purified on caesium chloride gradients using standard methods.

2.2. Cell culture, transfection and adenoviral infection.

ER α -positive endometrium adenocarcinoma Ishikawa cells (ATCC code 99040201; [29]) were grown at 37°C in 5% CO₂, in phenol red DMEM (Gibco), supplemented with 1% non essential amino acids (Sigma), 2mM L-glutamine (Sigma), 10⁵U/L penicillin, 100mg/L

streptomycin (Sigma), 1.25g/L fungizone (Gibco) and 10% fetal bovine serum (Gibco). They were transferred to phenol red free DMEM (Gibco), containing charcoal stripped fetal calf serum for 48 hours prior to use in order to reduce the presence of steroid ligands and growth factors, this media was also used for all subsequent experiments. COS 7 cells, an African
5 green monkey kidney fibroblast line (ATCC code CRL-1651) were cultured using identical conditions.

Luciferase reporter assays were carried out using cells seeded at 2×10^5 per well in 12-well plates on day 1 that were transfected on day 2 with JetPEI (QBiogene, Illkirch, France)
10 following the manufacturer's instructions with a 3xERE-tk-luc reporter plasmid (250ng per well, [30]), a tk-Renilla control plasmid to assess transfection efficiency (100ng per well, Promega, Madison, WI, USA) and pDC315 containing either ERR β L or ERR β S (500ng for the dose response experiments and for the study of ERR β basal effect on ERE, 250-750ng made up to 750ng with empty pDC315). In some experiments the mixture also contained
15 50ng of the shER α construct. After 24h transfected cells were treated with vehicle control (DMSO Sigma, final concentration of 10 ppm), 10^{-11} to 10^{-7} M oestradiol-17 β (E $_2$, Sigma) or the ER α -selective agonist propyl pyrazole triol (PPT, Tocris, Ellisville, MO, USA). After a further 24h the cells were washed in PBS, lysed and assayed for luciferase activity using the dual luciferase reporter system (Promega) following the manufacturer's instructions. A
20 sigmoidal dose-response equation was applied to the data using Prism 4 (GraphPad, SanDiego, CA, USA) and statistical significance was assessed using the 95% confidence interval of the curves generated.

In order to obtain uniform over-expression of ERR β proteins adenoviral vectors containing
25 both the long and short form cDNAs were prepared according to a standard protocol (Microbix). Briefly the cDNA of interest (ERR β S or L) in the pDC315 shuttle vector (see above), was allowed to recombine into the adenoviral genome (pBHGlox Δ E1, 3Cre, Microbix) in Hek293 cells. The resulting viral particles were plaque purified to ensure transgene expression, amplified in Hek293 cells and concentrated using a commercial kit
30 (Vivascience, Hanover, Germany).

For overexpression of cDNAs using adenoviral gene delivery, Ishikawa cells were infected on day 2 with adenovirus at a MOI of 40, after seeding on day 1. Preliminary experiments using a control virus expressing GFP alone indicated that at this MOI ~100% of cells
35 expressed the protein with no evidence of toxicity or cell death. To assess the effect of ERR

overexpression on endogenous gene targets, RNA was harvested 48 hours after infection and transcript levels measured using qRT-PCR.

Transient transfections using identical concentrations of plasmids expressing ERR β S or ERR β L tagged with yellow fluorescent proteins (Figures 5 and 6) resulted in similar intensities of the fluorescence proteins when measured by confocal microscopy (see section 2.5 below), suggesting that the variant isoforms have a similar stability.

2.3 Gene expression analysis

RNA was extracted from cell samples using the RNeasy kit (Qiagen, Crawley, UK). Reverse transcription was carried out with random hexamers and analysed by TaqmanTM qRT-PCR on an ABI 7900 Real Time PCR System. Assay on demandTM primer/probe sets specific for ER α and ER β (Hs00174860_m1 and Hs00230957_m1 respectively) were purchased from Applied Biosystems. Messenger RNA for c-myc was measured using primers designed using the Universal Probe Library (Roche) programme (available at <http://qpcr.probefinder.com/organism.jsp>) and library probe number 75 (c-myc forward: 5' TTTTTCGGGTAGTGGAAAACC 3' and c-myc reverse: 5'TTCCTGTTGGTGAAGCTAACG 3').

All data was normalised by measuring 18S ribosomal RNA (assay 4308329) in the same reactions and transcript abundance was expressed as a ratio in comparison with a standard comparator sample of cDNA made from untreated Ishikawa cells that was run on all plates.

2.4 Cell proliferation assay (MTT)

Ishikawa cells were seeded at a density of 3×10^4 per well in 96 well plates, in charcoal stripped media (DMEM) and allowed to attach and grow for 24 hours. They were infected with adenoviruses (Rad60 [control], ERR β S or ERR β L at a MOI of 40) in serum free media (DMEM) and 24h later medium was replaced with control serum free medium (DMSO alone) or medium containing 10^{-9} M E₂. The impact of E₂ treatment on cell proliferation was assessed after 24 hours using the MTT assay (Roche, Burgess Hill, UK) following the manufacturer's instructions.

2.5 Live cell imaging.

Ishikawa cells (4×10^5) were plated in glass bottom dishes (MatTek, Ashland, MA, USA). Cells were transfected with 2 μ g of plasmid DNA using Jet PEI (as above); plasmids expressed ERR β S or ERR β L tagged at the N-terminus with yellow fluorescent protein (YFP-

ERR β S, YFP-ERR β L). Transfection efficiency was routinely ~ 30%. The intra-nuclear mobility of YFP-ERR β S and YFP-ERR β L was examined using Fluorescence recovery after photobleaching (FRAP) on an LSM510 metaconfocal (Zeiss) with a stage maintained at 37°C. Prior to imaging the media was replaced the PBS with 10mM HEPES buffer (Sigma).

In each cell 2 areas within the nucleus were selected and fluorescence was measured before and after bleaching of one of the 2 areas. Recovery of fluorescence was monitored every 3 seconds for 30 seconds. The fluorescence intensity data was normalised for each cell and used in a non-linear regression model, $Y=Y_{\max} \times (1-e^{-Kx})$ (GraphPad Prism 4), where the regression coefficient r^2 was typically 0.95. The $Y_{\max}$ and half-life of recovery values (0.69/K) were averaged for at least 20 cells per treatment.

In order to determine whether the ERR β variant proteins were capable of forming heterodimers with ER α , fluorescence resonance energy transfer (FRET) assays were conducted using Ishikawa cells that had been transfected with expression plasmids containing YFP-ERR β L or YFP-ERR β S together with a cyan fluorescent protein-tagged ER α (CFP-ER α). The intensity of the CFP signal was monitored following bleaching of the YFP molecules using an Argon2 laser (514 nm). The FRET efficiency (change in CFP signal normalised to the YFP signal) was measured for at least 10 cells per treatment. Cells treated with 10^{-8} M PPT were incubated for 20 minutes before measurements started.

In order to validate the procedure, experiments were carried out with cells transfected with a positive control pCY24 plasmid (a generous gift from Dr Rory Duncan, University of Edinburgh), consisting of the CFP and YFP cDNA sequences cloned in frame with a 72bp (24 amino acid) spacer. Typical FRET efficiency values for this positive control were around 30%. Negative control experiments were carried out with cells co-transfected with the pECFP and pEYFP plasmids, to check no FRET signal was generated when non-interacting fluorophores were present in the same cell.

2.6. Statistical analysis

Data from all experiments were averaged (3 to 5 repeats) where possible. When the results of all experiments were similar but differed greatly in the dynamic range of the response, a representative experiment was shown and the statistics were carried out on the replicates. All statistical analysis was carried out using Prism 4 (GraphPad). Data normality was assessed and where required one- (treatment) or two-way (treatment and time) ANOVA, Kruskal-Wallis or Friedman tests were used, with a level of significance of 5%.

3. Results.

3.1 Impact of *ERRβ* variants on ERE-dependent reporter gene activity

In order to determine what impact *ERRβ*L and *ERRβ*S have on ERE-dependent gene expression *ERα*-positive Ishikawa cells were co-transfected with a luciferase reporter construct under the control of a promoter containing 3 copies of a consensus ERE, together with an expression plasmid containing *ERRβ*S or *ERRβ*L. Overexpression of *ERRβ*S and *ERRβ*L resulted in a modest constitutive expression of luciferase that did not reach statistical significance (Figure 1A). Consistent with expression of *ERα* in this Ishikawa cell line incubation of cells with E2 (Figure 1B) or PPT (Figure 1C) resulted in a dose dependent increase in expression of luciferase to ~15 fold over control (no ligand) in the presence of 10^{-7} M E2 or PPT. Transfection of cells with *ERRβ*S or *ERRβ*L had a differential effect on ligand-induced expression of luciferase. Whereas *ERRβ*S blunted ligand-induced expression of luciferase and caused the plateau (Y_{max}) value to decrease (from 17.4 to 5.5 for E2 and from 16.1 to 7.8 fold for PPT) transfection of cells with *ERRβ*L resulted in an increase in the Y_{max} (to 22 fold for both agonists). The EC_{50} value, a reflection of the receptor-ligand interaction, remained constant between treatments (around 3×10^{-10} M for both ligands). The use of increasing amounts of *ERRβ*L (100, 250 and 500ng) produced a gradual shift upwards of the sigmoid plateau values, suggesting the transcriptional enhancement is *ERRβ*L dependent (Figure 1D). In order to confirm that ligand-dependent gene expression required activation of *ERα* the experiments with *ERRβ*L were repeated in the presence or absence of an *ERα*-specific shRNA (Figure 1E). Partial ablation of the response to PPT, caused by reduced concentrations of *ERα* as a result of the introduction of the specific shRNA resulted in a decrease of the plateau value (from 12.7 to 2.4 fold maximal activation). Overexpression of *ERRβ*L partially rescued this response (from 2.4 to 6.8 fold) (Figure 1F). The validity of using sigmoid curve regression analysis to assess *ERα*-mediated induction was confirmed by demonstrating that the regression coefficient values (r^2) were higher than 0.95.

3.2 Impact of *ERRβ* variants on expression of *c-myc*

In breast cancer cells expression of *c-myc* is rapidly up-regulated in response to oestrogen-treatment. The *c-myc* promoter does not contain an oestrogen-response element [31] and up-regulation by *ERα* appears to involve co-recruitment of Sp1 transcription factors [32]. Over expression of either *ERRβ*S or *ERRβ*L using an adenoviral delivery system did not have a significant impact on the basal expression of *c-myc* mRNA compared with control

(uninfected) cells or those infected with the Rad60 viral control (Figure 2A). However when a time course experiment was conducted in order to explore the impact of ERR β variants on E2-mediated expression of c-myc differences emerged (Figure 2B). In the presence of Rad60 or ERR β S, maximal expression of c-myc mRNA (~4 fold compared with control) was detected at 60 min after the addition of E2. However over-expression of ERR β L increased expression of c-myc induced in response to E2 at 90 min ($p < 0.001$). A similar pattern of response was observed when treatments were carried out using PPT in place of E2, although the data did not reach statistical significance (not shown).

3.3. Impact of ERR β variants on cell proliferation

Oestrogens act as potent mitogens and c-myc plays a key role in the induction of cell cycle progression in response to oestrogen [33] therefore as a complement to the studies presented above the impact of ERR β variants on E2-induced cell proliferation was investigated 24h after addition of ligand (Figure 3). Over expression of ERR β L had a significant ($p < 0.05$) positive impact on cell proliferation in the presence of E2 whereas ERR β S did not.

3.4 Impact of ERR β variants on concentration of ER α and ER β mRNAs

To determine whether overexpression of ERR β was altering oestrogen responsiveness by modulating expression of ER α or ER β total mRNA for each receptor was determined by qRT-PCR using cells infected 48h previously with control adenovirus or that containing ERR β S or ERR β L; no significant differences in total amounts of transcript were detected (Figure 4A, B). Western analysis did not reveal any difference in expression of ER α (not shown). These results are consistent with the lack of effect of ERR β S and ERR β L on the EC₅₀ values obtained in the dose response experiments (see above, Figure 1C).

3.5 FRAP analysis

Intranuclear mobility of ERR β S and ERR β L was compared by performing FRAP analysis using YFP-tagged receptors transfected into COS7 or Ishikawa cells (Figure 5A). A striking difference between the variants was noted when the rate of recovery into the bleached area of the nucleus was compared. Whereas ERR β S was highly mobile the normalised recovery rate of ERR β L was slower in both cell lines studied. Similar results were obtained regardless of whether YFP-ERR β constructs were transfected into ER α negative COS7 (Figure 5B) or ER α positive Ishikawa (Figure 5C) cells. Treatment of Ishikawa cells with the ER α specific PPT (10^{-8} M) had no significant impact on the intra-nuclear mobility of ERR β S or ERR β L (Figure 5C).

3.6 FRET analysis

To investigate whether the impact of ERR β on oestrogen responsiveness of the Ishikawa cells might be due to formation of ERR β /ER α heterodimers FRET analysis was carried out

5 comparing control cells (no ligand) with those incubated in the presence of PPT. In control cells no measurable FRET signal was observed after co-transfection with CFP-ER α and either YFP-ERR β S or YFP-ERR β L. After incubation of cells with PPT, there was a significant increase in FRET signal between ER α and ERR β L (~12%) whereas there was no significant increase in the FRET signal in the presence of ER α and ERR β S (Figure 6). We
10 used PPT for these experiments to ensure that ligand binding was selective for ER α ; a similar but non-significant trend was obtained when E2 was used in the place of PPT (not shown). In line with expectations FRET signals with positive (pCY24) and negative (pECFP and pEYFP) control plasmids were ~30% and 0% respectively (Figure 6)

15 4. Discussion.

The human endometrium is an oestrogen target tissue and during the follicular phase of the cycle rising levels of circulating oestrogens increase the rate of endometrial cell proliferation [34] an observation that can be replicated using explant cultures of postmenopausal
20 endometrium [35]. Studies in rodent models suggest that both proliferation and increased expression of progesterone receptor are mediated by ER α [36]. The current studies were initiated following the observation that mRNAs encoding both ERR β L and S are expressed in human endometrium [28] and by reports in the literature that expression of ERRs can have an impact on ER-dependent gene expression (reviewed in [9]). We used a variety of approaches
25 to determine whether expression of ERR β isoforms in endometrial cells might have an impact on their response to oestradiol and whether ERR β could alter the activity of ER α in these cells.

Several studies have investigated the impact of ERRs on gene transcription [26,37-42]. The
30 majority of these studies have involved transient transfection using a variety of cell lines. For example, Xie et al [37] reported that ERR β (the sequence used corresponded to the ERR β S isoform used in the current study) activated a reporter construct containing a tk-ERE when they were co-transfected into CV-1 cells [10 fold over controls using 2 μ g of plasmid plus 5 μ g reporter]. Activation of the ERE promoter was dependent upon the amount of ERR β
35 transfected into HeLa cells but independent of E2; ERR β was also able to activate a reporter

containing a copy of the palindromic thyroid response element [5'-TCAGGTCATGACCTGA-3'] in a ligand independent manner [37]. In the current study we investigated whether ERR β L and ERR β S could activate expression using a reporter construct containing three copies of the vitellogenin ERE [3xGGTCAcagTGACC] [30] in endometrial Ishikawa cells. We found that over expression of ERR β S and ERR β L (500ng) resulted in a modest yet non-significant induction of the reporter gene. These results contrast with previous work, where constitutive activity was reported [11,27]. The apparent contradictions in the data may reflect differences in the environment of the cells used in the studies. For example, Zhou et al [11] performed their reporter assays in MCF-7 derived ER α negative cells whereas Lu et al [27] used HeLa cells transfected with ERR constructs and a luciferase reporter under the control of the pS2 gene promoter. We speculate that the reduction in the ER α -dependent induction of the ERE-luciferase reporter observed in the presence of ERR β S (Fig. 1B and 1C) may be due to 'squenching' associated with competition for nuclear receptor cofactors [43].

A novel finding of the present study was the differential impact of the two ERR β variant isoforms on ER α -dependent gene activation. Initial observations were based on studies using the 3xERE reporter assay and these were complemented and extended by examining expression of the oestrogen-regulated gene c-myc and the impact on cell proliferation using the MTT assay system. In all cases increased expression of ERR β L in ER α -positive Ishikawa cells augmented the response to E2. We considered a number of different explanations for this observation. Firstly since the Ishikawa cell line we used for these experiments expresses both ER α and ER β (our unpublished results and [29]) we determined whether introduction of ERR β s into the cells might have a direct impact on expression of either receptor subtype but expression remained unchanged. Secondly, we established that the impact of ERR β L on the response to E2 was linked to the presence of ER α in these cells by demonstrating that the augmented response observed in the presence of E2 could be mimicked by the ER α -selective agonist PPT and that this response was reduced when the amount of ER α protein present in the cells was reduced by RNA interference. Notably, in their study Zhou et al [11] used an ER α -negative MCF-7 cell line and in their assays addition of E2 to cells transfected with ERR β constructs had no impact on ERE-dependent reporter gene activation .

Formation of homo- or hetero-dimers is a key feature of NR signalling and recent studies using mouse heart have reported that $ERR\alpha$ and $ERR\gamma$ can bind a common set of promoters and act as non-obligatory heterodimers at these sites [13]. $ER\alpha$ and $ER\beta$ can form heterodimers at ERE sequences [44] and $ER\alpha$ is reported to be unable to bind an ERE as a monomer [45]. Previous studies have reported evidence that a physical association occurs between $ER\alpha$ and $ERR\alpha$, when recombinant proteins were co-incubated *in vitro* [46].

In order to establish whether the impact of $ERR\beta L$ on $ER\alpha$ -dependent gene expression reflected a physical association between the receptors we used two well-established *in vitro* imaging methods, FRAP and FRET [47]. FRAP was used to compare the intra-nuclear mobility of $ERR\beta$ variants in the presence and absence of E2 and PPT in both $ER\alpha$ - positive and $ER\alpha$ -negative cells. In both cell types $ERR\beta S$ and $ERR\beta L$ proteins tagged with YFP were detected within the cell nuclei with no evidence of significant expression in the cytoplasmic compartment for either variant. Following bleaching of a small region of the nucleus recovery of $ERR\beta S$ was more rapid than that of $ERR\beta L$; results were similar in the two cell lines and in the Ishikawa cells there was no evidence that the rate of recovery was changed following addition of E2 or PPT. Parallel studies in our own laboratory using a YFP-tagged $ER\alpha$ construct have demonstrated that the receptor is highly mobile in the absence of ligand but following addition of E2 or PPT the rate of recovery into the bleached area is dramatically reduced (Fitzgerald and Saunders unpublished). These results are in agreement with other studies on the impact of ligand binding on members of the NR family including $ER\alpha$ [48] and androgen receptor [49]. Transient immobilisation within the nucleus of ligand-bound NRs is believed to reflect association of the proteins with the nuclear matrix [49] and studies on activity of ARs have demonstrated that both the ligand binding and DNA binding domains are required for DNA binding (reviewed in [47]).

Mobility of $ERR\beta S$ was not altered by addition of ligands to $ER\alpha$ positive cells and this finding was backed up by FRET analysis showing that there was no increase in FRET efficiency between $ER\alpha$ and $ERR\beta S$ suggesting that the proteins were not closely associated with each other within the nucleus. In contrast, FRET efficiency between $ER\alpha$ and $ERR\beta L$ was increased following addition of E2 or PPT a finding that implies the CYP and YFP protein tags on the proteins were within 10 angstroms of each other. Although this finding might be consistent with formation of a heterodimer between the two proteins alternative explanations need to be considered. For example, is $ERR\beta L$ typically associated with the

chromatin and therefore when ligand-activated ER α becomes more closely associated with the same nuclear sub-compartment does this account for the increase in FRET? Alternatively does the presence of ERR β L augment protein-protein interactions in pre-initiation complex formed by ER α homodimers and receptor co-activators? We speculate that in tissues such as the human endometrium, where ER α , ERR β S and ERR β L are all expressed in multiple cell types [28], the former may act as a repressor of ER α -dependent gene activation, whereas the latter may augment the impact of oestrogens in ER α -positive cells.

Our data suggest that the presence of an F-domain consisting of 67 amino acids at the C-terminus of the ERR β L [11] has a marked impact on the intra-nuclear mobility of the protein and plays a key role in the ERR β -dependent increase in ER α -mediated transcription. Not all members of the nuclear receptor superfamily have an F domain and in those that do its length varies from as little as 11 amino acids in the androgen receptor, to more than 90 amino acids in hepatocyte nuclear factor 4 (HNF4 α , NR2A1). Compared with many papers containing detailed analysis of the ligand-binding domain this region has received relatively little attention. Two of the best-studied F domains are those found in ER α [50] and HNF1 α [51]. The F-domain of ER α contains ~45 amino acids and *in silico* modelling studies suggest that it contains an alpha helix and two extended regions with a similar secondary structure to the F-domain of HNF1 α [52]. Yang et al [53] have reported that removal of 24 amino acids at the C-terminus of the F-domain resulted in a 5 fold increase in dimerisation of E2-bound ER α and increased interactions with SRC-1 in yeast two hybrid assays. In contrast studies using HeLa cells have demonstrated that sequences within this region can have a positive impact on the ability of the receptor to activate ERE-dependent reporter genes [50,54], can promote interactions with co-factors including TAF-1 β [55] and SRC-1 [54] and is essential for interaction with Sp-1 proteins at GC-rich promoters [56]. The large F-domain present on HNF4 α 1 appears to interact directly with the ligand-binding domain acting as a repressor by blocking binding of the transcriptional regulators SRC1a, GRIP1, p300 and CBP. Whereas in the HNF α 2 splice isoform a 10 amino acid insert into the F-domain partially relieves this inhibition [51], highlighting the impact of alternative splicing on receptor activity something also observed in hER β where replacement of 61 amino acids, including the F-domain (~30 amino acids) at the C-terminus of the protein, with an alternative 26 amino acids completely abolishes E2-dependent gene activation [57]. Bioinformatic analysis of the ERR β L F-domain failed to identify any evidence of putative phosphorylation sites (not shown) or secondary structures such as alpha helices using the DAS program

(<http://www.sbc.su.se/~miklos/DAS/>). Therefore additional investigations using methods such as site directed mutagenesis will be necessary if we are to determine which amino-acids within the F domain of ERR β L are critical for ER α -dependent gene activation.

5 The short form of ERR β can repress transcriptional activity of Nrf2 on the antioxidant response element and alter the subcellular distribution of Nrf2 [41]. ERR β and Nrf2 could be co-precipitated consistent with the formation of a protein complex containing both proteins and in the discussion of their paper the authors suggested that ERR β S might have an impact on Nrf2 transcriptional activity by bringing co-repressors into this complex. ERR β L was not
10 investigated in their studies [41]. ERR α , ERR β and ERR γ can also bind to HIF1 α /HIF1 β heterodimers, enhance transcriptional activity of a HIF1-luc reporter gene as well as expression of HIF1 α regulated genes including PGK-1 [42]. The results in these papers are interesting not only because they demonstrate that ERR β may have impacts on gene
15 influence HIF-1 dependent gene expression by boosting the number of co-activators in the transcription complex [42]. A similar mechanism may apply to the enhanced activity of ER α -dependent gene expression we observed in the presence of ERR β L since both ERRs and ER α are reported to bind co-activators including PGC-1 α and SRC-1.

20 In conclusion, our results suggest that expression of ERR β L can augment ER α -dependent gene activation whereas ERR β S cannot. This result is another example of differential regulation of nuclear receptor activity as a result of alternative splicing. The results highlight a critical role for the F domain of ERR β L and we speculate that ERR β L may enhance co-factor recruitment by ligand-activated ER α .

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Figure legends

Figure 1: ERR β L and ERR β S exhibit minimal constitutive activity but have differential effects on ERE-dependent luciferase reporter gene expression following treatment of cells with agonists, in an ER α specific fashion.

Ishikawa cells were transfected with 500ng of plasmids expressing ERR β S or ERR β L (A), together with the ERE-luciferase plasmid. Reporter gene activation (luciferase activity) was measured after 48h. The graph represents the average from 5 experiments. Luciferase activity was not significantly increased: $p=0.23$ by Kruskal-Wallis test. Panels B and C: Ishikawa cells were transfected as above with 500ng of ERR β or the empty pDC vector and treated with increasing doses of E2 (B) or the ER α specific agonist PPT (C). The resulting increase in luciferase activity was converted to fold induction using the basal DMSO luciferase value and a sigmoid regression was applied (see method section). The regression data indicates the 95% confidence of the Ymax (plateau) values do not overlap indicating a statistical difference, with no change in the EC50 values. Each experiment was performed in duplicate, replicated 3 times and gave the same result. Panel D: Ishikawa cells were transfected as above with increasing amounts of ERR β long form (100, 250 or 500ng) or the empty pDC vector. The final plasmid concentration was kept constant to 500ng using pDC vector. The data was analysed as for panels B and C and showed that the enhancement of ERE-luciferase induction was more pronounced with increasing amounts of ERR β L. Panel E: Validation of sh-RNA dependent knockdown of ER α . Image shows Western analysis of ER α protein. Note that transient transfection with an ER α plasmid increased expression of the protein and that this was reduced in concentration dependent manner by co-transfection with a shRNA specific for ER α (shER α) but not by a scrambled non-specific shRNA or a shRNA directed against ER β . Panel F: Ishikawa cells were transfected with 500ng empty pDC vector or ERR β long form, in the presence or absence of 50ng shER α plasmid (see panel E) were treated with various concentrations of PPT for 24 hours. The 95% confidence interval of the Ymax regression values did not overlap, with no change in the EC50 values. This representative experiment was performed 4 times and gave similar results. All data was expressed as mean $\pm$ SEM.

Figure 2: Differential effect of ERR β short and long forms on the induction kinetics of c-myc, in response to treatment of cells with E2.

Ishikawa cells were infected with adenoviral particles at a MOI of 40 and expression of c-myc mRNA was assessed by qRT-PCR; data represents the average of 3 independent experiments (expressed as mean $\pm$ SEM). A) Over expression of Rad60 (control), ERR β S or

ERR β L adenoviral particles had no significant impact on the amount of c-myc mRNA compared with uninfected cells. B) 24h after infection cells were treated with 10⁻⁸M E₂ for 30, 60 and 90 min. At 90 mins the amount of c-myc mRNA was significantly increased in cells infected with ERR β L compared to ERR β S (*, p<0.001 using a 2 way ANOVA).

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Figure 3: Impact of ERR β splice variants on cell proliferation as determined by MTT assay.

Cell proliferation was assessed using the MTT assay 24h after addition of DMSO or 10⁻⁹ M E₂. Over expression of ERR β L significantly increased proliferation compared that recorded in cells overexpressing ERR β S (data expressed as mean $\pm$ SEM; *, p<0.05; Kruskal-Wallis test followed by a Dunn's Multiple Comparison Test).

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Figure 4: Overexpression of ERR β variants does not alter of the amount of ER α or ER β mRNAs

Ishikawa cells were transfected with ERR β L or ERR β S plasmids, total RNA was recovered after 48h and mRNAs determined by qRT-PCR. No significant difference was seen in the expression of ER α (A) and ER β (B) mRNAs compared with controls. The graph represents the average of 3 independent experiments and the data was expressed as mean $\pm$ SEM.

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Figure 5: FRAP analysis revealed a difference in the intra-nuclear mobility of ERR β variants.

Ishikawa or COS7 cells were transfected with YFP-tagged ERR β constructs and the rate of recovery between 3 to 30 seconds after photo-bleaching was determined. A. Fluorescent proteins were detected in the nuclear compartment; ERR β S showed a rapid (<3 sec) recovery after bleaching whereas when using ERR β L the bleached area was still clearly visible 30 seconds after firing of the laser. These observations were similar whether the cells used were: B, COS7 (ER negative, * p<0.05) or C, Ishikawa (ER α positive). In Ishikawa cells the rate of recovery of ERR β L was significantly reduced (p<0.0001, *) compared to ERR β S but the addition of PPT had no impact on the mobility of either splice variant ERR β (hatched bars) and they remained significantly different from each other (**). The data is expressed as mean $\pm$ SEM.

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Figure 6: FRET analysis suggests a ligand-induced physical interaction between ER α and ERR β L.

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Ishikawa cells were co-transfected with CFP-ER α plus YFP-ERR β S (open bars) or YFP-ERR β L (solid bars), FRET analyses were carried out with or without the addition of 10⁻⁸M PPT. A minimum of 20 cells were analysed in each treatment. Positive control cells were transfected with pCY24 whereas negative controls were co-transfected with two different plasmids containing CFP or YFP alone. This experiment was performed 5 times. FRET efficiency was significantly ($p < 0.0001$) increased in cells co-transfected with ER α and ERR β L treated with PPT compared to untreated (*). The results are expressed as mean $\pm$ SEM

Figure 5

A ERR β short

ERR β long

B

C