Scientific report

ESF/ENS@T Exchange visit grant
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Background

Adrenocortical carcinoma (ACC) is a rare tumour with a very poor prognosis and no effective treatment. Currently, in localized ACC only surgery provides a chance for a long-term cure. However, recurrence rates as high as 60-80% after radical resection have been referred (Pommier and Brennan, 1992; Stojadinovic et al., 2002), indicating a need for adjuvant pharmacological therapies. To date, treatment with mitotane remains the common therapy mainly because of its ability to impair adrenal steroidogenesis (Martz and Straw, 1980). The molecular mechanisms underlying ACC development are poorly understood and this has limited the discovery of effective cures. Studies carried out in the past ten years suggest that genetic mutations in the adrenal gland lead to the initiation of a malignant tumour (Soon et al., 2008; Giordano et al., 2009). The most consistent and dominant genetic change in ACC is the perturbation of the insulin-like growth factor II (IGF-II) locus (11p15) that is imprinted (Gicquel et al., 1994). IGF-II is overexpressed in 90% of ACCs together with insulin-like growth factor 1 receptor (IGF1R). The direct involvement of IGF-II/IGF1R system in adrenocortical tumour cell proliferation has also been shown *in vitro* using as a model system the adrenocortical carcinoma cell line H295R (Logiè et al., 1999). Moreover, advanced human ACC show increased levels of IGF1R. This suggests an important role for the IGF system in adrenocortical carcinogenesis (Fottner et al. 2004). An anti-IGF1R monoclonal antibody called figitimumab has been used in phase I clinical trials for the treatment of refractory ACC. However, patients did not display objective responses to the treatment (Haluska et al., 2010), indicating the need for new effective therapies.

The *CYP19* gene encodes for the enzyme aromatase, which is responsible for estrogen synthesis using androgens as substrate (Santen et al., 2009). Although aromatase is not commonly considered as a member of the adrenocortical cytochrome P450 family, it has been shown that this enzyme is overexpressed in human ACC (Barzon et al., 2008). Aromatase has been detected in adrenocortical H295R cells, by mRNA analysis (Staels et al., 1993) as well as enzyme activity (Watanabe and Nakajin, 2004; Montanaro et al., 2005). Estrogens produced by aromatase exert their action by binding to the nuclear receptor family members estrogen receptor (ER)-α and ERβ. Like other members of nuclear receptor family, the ERs are best known as transcription factors, which modulate directly (binding to estrogen-responsive elements) or indirectly (interacting with other transcription factors) the transcriptional activity of target genes in response to the natural hormone 17β-estradiol (E2) or to synthetic non-hormonal agonists and antagonists (Nilsson and Gustafsson, 2010). In recent years, it has emerged that the effects of E2 can have a very rapid onset, within seconds to minutes, and activate cellular responses known as nongenomic steroid signals (Losel et al., 2003). These effects may be mediated by membrane-localized ERα and ERβ respectively (after exposure to ligand stimuli) or by membrane receptors that are genetically and structurally unrelated to the ERs, such as the G-protein coupled receptor 30 (GPR30) (Levin, 2009; Maggiolini and Picard, 2010). Through these signals estrogens activate growth factor signalling. On the other end, growth factors can stimulate ERα activity independently of estrogens. Indeed IGF-I through phosphatidylinositol 3 kinase (PI3K) and extracellular signal-regulated kinase (ERK) pathways increases ERα phosphorylation at serine-167 and 118 (Kato et al., 1995; Lee et al., 1997).

A recent paper published by Sirianni and coworkers established a critical role for ERα in both E2- and IGFII-dependent proliferation of the adrenocortical cancer cell line H295R (Sirianni et al., 2012). The authors provided evidence that E2 and IGF-II induce comparable signalling responses in those cells including activation of IGF1R/AKT signalling and cyclin D1 expression. Moreover,

targeting ERα with a specific small interfering RNA (siRNA) or with the selective estrogen receptor modulator (SERM) hydroxytamoxifen (OHT, the active metabolite of tamoxifen) was effective in controlling E2- and IGF-II- induced H295R proliferation. Finally, H295R xenograft growth was shown to be strongly reduced by tamoxifen. These data provide a rationale for targeting ERα to control the proliferation of ACC. The same group had previously demonstrated that OHT and the ER antagonist ICI 182 780 (ICI) inhibit H295R proliferation by up-regulating ERβ (Montanaro et al., 2005). In particular, OHT induced morphological changes that were characteristic of apoptosis together with a marked expression of the pro-apoptotic factor Fas ligand (FasL) and the cleavage of both caspase-8 and caspase-3. They showed that the apoptotic effects of OHT in H295R cells may be consequent to the enhanced levels of ERβ which stimulate FasL expression interacting with activating protein (AP)-1 sites located within its promoter sequence (Montanaro et al., 2005). This study points towards a role for ERβ as an important mediator of the repressive effects exerted by antiestrogens on H295R cells.

Description of the work carried out during the visit

Based on the observations reported in the last part of the background, the purpose of my visit in the laboratory directed by Dr. Enzo Lalli was to define the ERα and ERβ target genes in H295R cells. To achieve this aim, we proposed to perform chromatin immunoprecipitation experiments coupled to high-throughput sequencing (ChIP-seq). This method was already routinely being used in Dr. Lalli's laboratory to identify chromatin binding sites for different transcription factors.

ChIP-seq is an extremely powerful tool to study how proteins interact with the genome, but there are several features which limit the utility of this technique and make it very challenging. They are:

a) the quality of antibodies that are available for a protein of interest;
b) the number of cells generally needed to obtain a robust result;
c) the high cost of high-throughput sequencing platform;
d) the experimental process long and sometimes challenging to troubleshoot.

Considering this, before starting ChIP-seq experiments I have mainly focused on the validation of the data recently published by Sirianni and coworkers (Sirianni et al., 2012) on the role of estrogens in ACC. In summary, I checked in H295R cells:

a) the mRNA levels of ERα/ERβ by quantitative PCR (qPCR);
b) the protein levels of ERα/ERβ by Western blotting (WB);
c) the intracellular localization of ERα/ERβ by immunofluorescence (IF);
d) the possible effects of ERα/ERβ stimulation or inhibition by selective agonists and antagonists on cell proliferation.

As it will be extensively reported in the section "Description of the main results obtained", the data collected indicate that ERα mRNA and protein levels are extremely low, almost absent, in the cell models used. This implicates that a ChIP-Seq approach to the study of ERα target genes in H295R cells is unfeasible. Modest levels of ERβ mRNA and protein can be detected in this cell line. Nuclear fractionation indicates that ERβ localizes to the nucleus. However, the results of the functional assay aimed at establishing ERβ role in H295R proliferation indicated that ERβ stimulation/inhibition does not affect cell viability. The absence of a functional effect coupled to the modest levels of ERβ in H295R cells (although higher than those of ERα) suggested that also the ChIP-Seq-based study of ERβ target genes cannot be performed in those cells.

Materials and methods

Cell cultures

H295R cells obtained from Dr. W. E. Rainey (Medical College of Georgia, Augusta, GA) (Rainey et al., 1994) were cultured in DMEM/F-12 supplemented with 10% fetal bovine serum (FBS, Gibco, UK), 1% ITS Plus (Becton Dickinson, Franklin Lakes, NJ) and 1% penicillin/streptomycin (P/S). H295R cells obtained from Dr. E. Lalli (IPMC/CNRS, Sophia Antipolis, Valbonne, France) were grown in DMEM/F-12 supplemented with 2% NuSerum (Becton Dickinson, Oxford, UK), 1% ITS Plus (Becton Dickinson, Franklin Lakes, NJ) and 1% P/S. H295R cells from American type Culture Collection (ATCC) Manassas, VA were cultured in DMEM/F-12 supplemented with 10% fetal bovine serum (FBS, Gibco, UK), 1% ITS Plus (Becton Dickinson, Franklin Lakes, NJ) and 1% P/S. MCF7 were grown in DMEM/F-12 supplemented with 5% calf serum (CS, Gibco, UK) and 1% P/S. COS1 cells were cultured in DMEM supplemented with 5% FBS (Gibco, UK), 1% non-essential amino acid (NEAA) and 1% P/S. All cell lines were grown at 37°C in a 5% CO2 atmosphere.

Drug treatment and proliferation assays

To measure proliferation, H295R cells from Dr. Lalli were seeded in triplicate in 96-well plates at the density of 5000 cells/well. The day after they were treated for 5 days in complete growth medium with the indicated concentrations of 17 β-estradiol (E2, dissolved in ethanol) (Sigma, St. Louis, MO), 2,3-bis (4-hydroxyphenyl)-propionitrile (DPN, dissolved in DMSO) (Tocris Bioscience, Bristol, UK), OHT (dissolved in ethanol, Sigma), 4-[2-Phenyl-5,7-*bis*(trifluoromethyl)pyrazolo[1,5-*a*]pyrimidin-3-yl]phenol (PHTPP) (dissoved in DMSO, Tocris Bioscience, Bristol, UK). As controls, cells were treated with medium or vehicles (ethanol for E2- and OHT- treated cells and DMSO for PHTPP- and DPN-treated cells respectively) alone. Cell proliferation was measured using the CellTiter-Glo Luminescent Cell Viability Assay (Promega). To measure proliferation, H295R cells from Dr. Rainey were seeded in triplicate in 96-well plates at the density of 25000 cells/well in DMEM/F-12 w/o phenol red supplemented with 10% FCS, 1% P/S and grown for 2 days. Before experiments, cells were starved overnight (o/n) in DMEM/F-12 w/o phenol red medium containing only 1% P/S and treated with the indicated concentrations of the drugs reported above in the starvation medium for 2 days. Cell proliferation was measured using the CellTiter-Glo Luminescent Cell Viability Assay (Promega).

Immunofluorescence

Cells were fixed (15 min at 22°C) in 4% paraformaldehyde in PBS and permeabilized by 2 treatments with 0,1% Triton X100 PBS (PT) for 10 min each. After blocking (30 min) in 2% BSA in PBS, cells were incubated o/n at 4°C with the following antibodies: 1) mouse monoclonal anti ERα antibody (F-10) (1:50; sc-8002, Santa Cruz Biotechnology); 2) rabbit polyclonal anti ERα antibody (HC-20) (1:50; sc-543, Santa Cruz Biotechnology); 3) mouse monoclonal anti ERβ antibody (6A12) (1:50; GTX70178, GeneTex); 4) mouse monoclonal anti ERβ antibody (14C8) (1:50; GTX70174, GeneTex); 5) mouse monoclonal anti ERβ antibody (7B10.7) (1:50; GTX70182, GeneTex). Cells were washed 3 times with PT and incubated 1 hour at room temperature (rt) with Alexa 594- conjugated goat anti-mouse or anti-rabbit secondary antibodies (1:200; Invitrogen, Cergy-Pontoise, France). Cells were washed 3 times again with PT and mounted in SlowFade Gold antifade reagent with 4′, 6-Diamidino-2-phenylindole (DAPI) (Invitrogen). Images were acquired with a Zeiss Axioplan 2 fluorescence microscope coupled to a digital charge-coupled device camera and processed using ImageJ.

RNA isolation and reverse transcription quantitative PCR (RT-qPCR)

Total cellular RNA was extracted from cells with the RNeasy kit (Qiagen, #74104) according to manufacturer's instructions. Purity and integrity of the RNA was checked spectroscopically and by

gel electrophoresis before carrying out the analytical procedures. 1 µg of the RNA was DNase-treated using the DNA-free™ kit (Ambion, Austin, TX, USA) and reverse transcribed using Superscript III reverse transcriptase (Invitrogen Corp., Carlsbad, CA). RT-qPCR was performed using the SYBR Green I dye assay on a LightCycler 480 (Roche Applied Science, Indianapolis, IN) instrument using TATA-binding protein (TBP) as a reference transcript. Primer sequences used were: TBP, forward 5′-GAACATCATGGATCAGAACAACA-3′ and reverse 5′-ATTGGTGTTCTGAATAGGCTGTG-3′; ERα forward 5′-TTACTGACCAACCTGGCAGA-3′ and reverse 5′-ATCATGGAGGGTCAAATCCA-3′; ERβ forward 5′-ATGATGGGGCTGATGTGG-3′ and reverse 5′-TTCTACGCATTTCCCCTCA-3′. Results were calculated using the ΔΔ threshold cycle method (Livak and Schmittgen, 2001).

Western blot analysis

To obtain total cell protein extracts, cells were lysed in ice-cold RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 2 mM NaF, 2mM EDTA, 0,1% SDS) supplemented with protease inhibitors. Nuclear extracts were prepared as previously described (Andrews ans Faller, 1991). All centrifugations of less than 30 seconds were carried out in a rt centrifuge; between steps, the samples were placed on ice. Adherent cells were scraped into 1.5 ml cold phosphate-buffered saline (PBS); non-adherent cells were pelleted and resuspended in 1.5 ml cold PBS. The cell suspension was then transferred to a microcentrifuge tube. Cells were pelleted for 10 s and resuspended in 400 µl cold buffer A (10 mM Hepes-KOH, pH 7.9 at 4°C, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM PMSF) by flicking the tube. The cells were allowed to swell on ice for 10 min and then vortexed for 10 s. Samples were centrifuged for 10 s and the supernatant fraction (cytosolic fraction) was stored at -20°C. The pellet was resuspended in 50 µl cold buffer C (20 mM Hepes-KOH pH 7.9, 25% glycerol, 1.5 mM MgCl2, 420 mM NaCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM PMSF) and incubated on ice for 20 min for high-salt extraction. Cellular debris was removed by centrifugation for 2 min at 4°C and the supernatant fraction (containing DNA-binding proteins) was stored at -70°C. The yield of both total, cytosolic and nuclear extracts was determined by *DC* protein assay (Bio-Rad) according to manufacturer's instructions. Proteins were separated on 10% SDS/polyacrylamide gel and then electroblotted onto a nitrocellulose membrane.

Immunoblot was performed using a chemiluminescence system for protein detection (ECL Plus; GE Healthcare Bio-Sciences Corp., Piscataway, NJ). Primary antibodies used were: 1) mouse monoclonal anti ERα antibody (F-10) (1:200 dilution, o/n 4°C; sc-8002, Santa Cruz Biotechnology); 2) rabbit polyclonal anti ERα antibody (HC-20) (1:200 dilution, o/n 4°C; sc-543, Santa Cruz Biotechnology); 3) mouse monoclonal anti ERβ antibody (6A12) (1:250 dilution, o/n 4°C; GTX70178, GeneTex); 4) mouse monoclonal anti ERβ antibody (14C8) (1:250 dilution, o/n 4°C; GTX70174, GeneTex); 5) mouse monoclonal anti ERβ antibody (7B10.7) (1:250 dilution, o/n 4°C; GTX70182, GeneTex); 6) mouse monoclonal anti-*β*-tubulin (1:1000 dilution, o/n 4°C; Sigma-Aldrich).

Description of the main results obtained

ERα transcript is poorly expressed in H295R cells

The first step of my investigation was to check the mRNA levels of ERα in three different clones of H295R cells (see Materials and Methods) by qPCR. To this end a couple of primers for ERα was designed by the Universal ProbeLibrary Assay Design Center (Roche, http://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp?id=UP030000) and validated to verify that they met the following critical performance parameters: a) single amplicon specificity and b) consistently high

amplication efficiency. The ERα-positive breast cancer MCF7 cells and the ER-negative transformed African green monkey kidney fibroblast COS1 cells were used as positive and negative controls respectively. The data obtained indicate that ERα is expressed at extremely low levels in the three H295R cell lines tested (Fig. 1 a). As expected, ERα mRNA was detected in MCF7 cells, whereas it was not expressed in COS1 cells (Fig. 1 a). These results are in line with those reported by de Cremoux and coworkers on ERα transcript levels, showing that ERα mRNA is weakly detected in normal human adrenal gland and ACC and significanly lower in ACA (adrenocortical adenoma) (de Cremoux et al., 2008).

ERα protein is undetectable in H295R cells

The mRNA expression results suggested that ERα should be barely expressed or undetectable at the protein level in the same cell lines. To investigate this point, whole cell lysates were prepared from H295R clones. Total proteins were also extracted from MCF7 and COS1 cells. Two different antibodies from Santa Cruz Biotechnology were employed (see Materials and Methods).

A clear band was observed with both antibodies at the predicted molcular weight (about 70 kDa) only in whole cell lysates from MCF7 cells (Fig. 1 b/c). The F-10 anti-ERα antibody also gave a band at a lower molecular weight (between 35 and 55 kDa) in all cell lines tested (Fig. 1 b). A N-terminus–truncated splice isoform of estrogen receptor ERα (46 kDa molecular weight), which triggers membrane-initiated signals, has been described for ERα (Haynes et al., 2003; Li et al., 2003). However, the presence of this band also in the negative controls (COS1) suggested that it was a non-specific signal instead of the truncated ERα isoform. The pattern shown by the clone HC-20 anti-ERα antibody was very similar to the one reported in the datasheet provided by the company. A non-specific band of about 100 kDa was present in all extracts tested (Fig. 1 c). Furthemore, other non-specific bands between 35 and 55 kDa were seen. They were present with various intensities in all whole lysates (Fig. 1 c).

Collectively these data indicate that ERα, clearly expressed in MCF7 cells as expected, is undetectable at the protein level in H295R cells. They also show that the two antibodies tested give different non-specific signals, suggesting that those tools are not entirely reliable. These data correlate with the qPCR analysis described in the previous section and agree with data obtained by de Cremoux and collegues (de Cremoux et al., 2008) and Montanaro and coworkers (Montanaro et al., 2005).

To definitely confirm these results, I monitored the ERα intracellular localization by IF using the same antibodies employed for WB. A clear nuclear signal was present in MCF7 cells, as expected (using both the antibodies) (Fig. 2 and 3). This signal was specific, as it was not present in COS1 cells and it was clearly distinguishable from that observed in MCF7 cells stained with the secondary antibody alone (Fig. 2 and 3). A diffuse, undefined, non-specific staining was detectable in all three H295R clones using both antibodies (Fig. 2 and 3). It was comparable to that observed in COS1 cells or in H295R cells incubated only with the secondary antibody (Fig. 2 and 3). These data further confirm that ERα protein is undetectable in H295R cells.

Conclusions I

I have shown by three different approaches (qPCR, WB and IF) that ERα protein is undetectable in three different clones of H295R cells, similarly to a cell line which is a known negative control for ERα expression (COS1). This clearly implicates that a ChIP-Seq approach to study ERα target genes in H295R cells is not feasible.

ERβ transcript is expressed in H295R cells

Besides the identification of ERα target genes, we proposed to define by ChIP-seq analysis also ERβ target genes in H295R cells. It has been previously shown by Montanaro and coworkers that in those cells the protein levels of ERβ were significantly higher than those of ERα (Montanaro et al., 2005). Furthermore, de Cremoux and collegues showed by qPCR that ERβ transcript levels were very high in normal adrenal tissues, ACA and ACC (de Cremoux et al., 2008).
Thus, as a first investigation step I checked ERβ mRNA levels in the different H295R clones by qPCR. To this end, a couple of primers for ERβ was designed by the Universal ProbeLibrary Assay Design Center (Roche) and validated for their specificity and efficiency (see above). COS1 cells were used as negative control. The results indicated that the ERβ transcript is expressed in the H295R clones tested (Fig. 4 a). As expected, it was not expressed in COS1 cells (Fig. 4 a). These data indicate that ERβ is expressed in H295R cells at least at the mRNA level, differently from ERα and according to previous reports (de Cremoux et al., 2008; Montanaro et al., 2005).

ERβ protein is faintly expressed in H295R cells at the nuclear level
ERβ protein levels were then investigated. Whole cell lysates prepared from the three H295R clones and COS1 cells were subjected to WB analysis. Three different antibodies from GeneTex were used (see Materials and Methods). Among them only one (the clone 7B10.7) gave a band at an unexpected molecular weight (between 70 and 100 kDa) in all H295R lines tested (Fig. 4 b). The predicted molecular weight of ERβ is 55 kDa, thus we hypothesised that it was a protein non-specifically recognized by the antibody. Conversely, the other two antibodies gave no signals.
Considering the data obtained from qPCR analysis and the literature evidence on the expression of ERβ protein in H295R cells (Montanaro et al., 2005), I then investigated ERβ expression in nuclear/cytosolic extracts from the three H295R clones and COS1 cells. I detected a faint band at the expected molecular weight in the H295R nuclear fraction by two of the three antibodies used (clones 7B10.7 and 14C8) (Fig. 4 c/d). This band was absent in the cytosolic extracts prepared from the same cells (Fig. 4 c/d) and in both nuclear and cytosolic extracts obtained from COS1 cells (Fig. 4 c/d). I wanted to confirm the nuclear localization of ERβ in H295R cells by IF analysis. The anti-ERβ antibodies used for WB were not reported to work in IF analysis. I thus tested them for this application and I showed that only the clone 14C8 gave a nuclear staining in the H295R tested (Fig. 5). The signal was distinguishable from that displaied by cells stained only with the secondary antibody (fig. 5), however it was present also in COS1 cells, that literature evidence, my qPCR and WB results showed to not express the protein (fig. 5). This indicates that this antibody likely recognizes a nuclear protein in IF different from ERβ that is expressed also in COS1 cells.
Collectively the above data indicated that ERβ protein is expressed in the nuclear fraction of H295R cells, however its levels appear to be too low to perform a ChIP-seq for the identification of potential target genes.

ERβ stimulation/inhibition does not affect H295R proliferation
Despite the fact that levels of ERβ in H295R cells were likely not high enough to carry on the ChIP-seq technique, we decided to investigate whether ERβ plays a functional role in H295R cells. Most *in vitro* studies that have analysed the biological effects of ERβ expression in cancer cells showed that ERβ has antiproliferative effects, althought the mechanisms that govern the cancer biological responses of ERβ have not been fully elucidated. ERβ expression was found to inhibit cell growth and to induce G1 cell cycle arrest in various types of cancer cells by regulating the expression of cyclin D1, CDC25A, MYC, FOXO1, p53 and p14, which act on the same cell cycle checkpoint (Hartman et al., 2009; Lin et al., 2007; Nakajima et al., 2011; Ström et al., 2004). Conversely, a few *in vitro* studies have proposed a proliferative and anti-apoptotic role of ERβ in cancer cells (Hershberger et al., 2009; Hou et al., 2004). Montanaro and collegues showed that ERβ is a mediator of the repressive effects of antiestrogens on H295R proliferation (Montanaro et al., 2005).

I thus studied the effects of various doses of the ERβ-specific agonist DPN or the selective ERβ antagonist PHTPP on cell viability through the CellTiter-Glo Luminescent Cell Viability Assay (Promega). This is a homogeneous method to determine the number of viable cells in culture based on ATP quantitation, which signals the presence of metabolically active cells. In addition to DPN and PHTTP treatment, cells were also subjected to treatment with different doses of the non-selective ERs agonist E2 or the SERM OHT. This last compound has been shown by Montanaro and coworkers to up-regulate ERβ expression and dose-dependently inhibit basal and E2-induced H295R cell proliferation (Montanaro et al., 2005).
H295R from Dr. Lalli were treated for 5 days with various doses of the reported drugs without any starvation and in complete culture medium (see Material and Methods) according to the protocol set up in Dr. Lalli's laboratory to measure H295R cell proliferation (Doghman et al., 2012). The long time of treatment is explained by the low proliferation rate characterizing these cells. None of the drugs tested had significant effects on cell proliferation (Fig. 6). The highest doses used (100 μM) was extremely toxic for cells treated with PHTPP, E2 and OHT, but not DPN (Fig. 6). Sirianni and coworkers showed that the ERβ selective agonist DPN (1 μM) had no effect on cell proliferation (measured by 3-[4,5-Dimethylthiaoyl]-2,5-diphenyltetrazolium bromide, MTT assay) (Sirianni et al., 2012). Thus, our results confirm these data, however they disagree with the published data on the effects of E2 (100 nM) and OHT (10 μM), which indicate that E2 promotes H295R proliferation through ERα, whereas OHT has the opposite effect due to ERα inhibition (Sirianni et al., 2012). Our results are consistent with the evidence that ERα is undetectable in H295R cells, as shown by multiple approaches in this report.
Similar experiments were also performed on H295R cells from Dr. Rainey (the same clone used by Sirianni et al. for their study) following the same protocol set up by Sirianni and collegues (detailed in Material and Methods) apart from the proliferation detection method, which was based on the CellTiter-Glo Luminescent Cell Viability Assay. Our results indicate that also under these alternative experimental settings the drugs tested did not significantly affect cell viability (fig. 7), confirming previous results.
Collectively these data suggest that ERβ has not a role in H295R proliferation.

Conclusions II

I have shown the ERβ transcript is expressed in H295R cells. A little amount of protein has been detected in the H295R nuclear fraction. Both stimulation and inhibition of ERβ did not alter H295R cells proliferation. Considering the expression levels of ERβ (although higher than those of ERα) and the absence of a role in H295R proliferation we decided to not proceed with ChIP-Seq analysis.

Our collaboration with Dr. Lalli is still continuing thanks to a new ESF/ENS@T exchange visit grant, which I have obtained to carry on a new project aimed at defining the role of VAV2, a novel target of transcription factor SF-1, in the pathogenesis of adrenocortical tumors.

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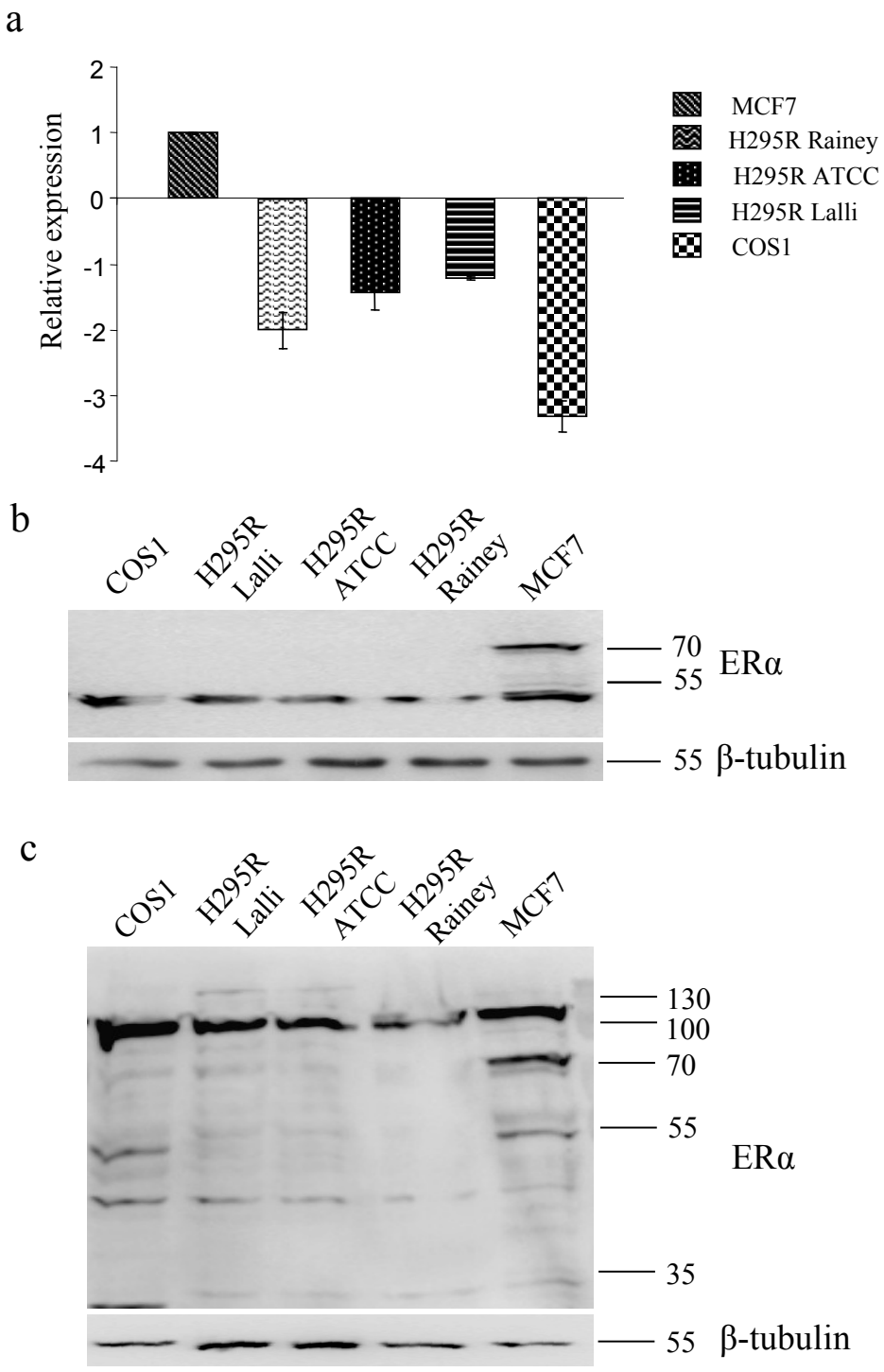

Figure 1. ERα transcript and protein are barely detectable in H295R cells. **a.** ERα levels were measured in MCF7 cells, H295R clones (from Rainey, Lalli and ATCC) and COS1 cells by quantitative RT-PCR. Results were calculated using the ΔΔ threshold cycle method and represented in Log 10 scale. MCF7 sample was used as calibrator. **b-c.** Immunoblot analysis was performed on 35 µg of total protein extracted from MCF7, H295R clones and COS1 cells. Blots were incubated with an anti-ERα antibody, clone F-10 (b) or an anti-ERα antibody, clone HC-20 (c) from Santa Cruz Biotechnology. β-tubulin was used as a loading control.

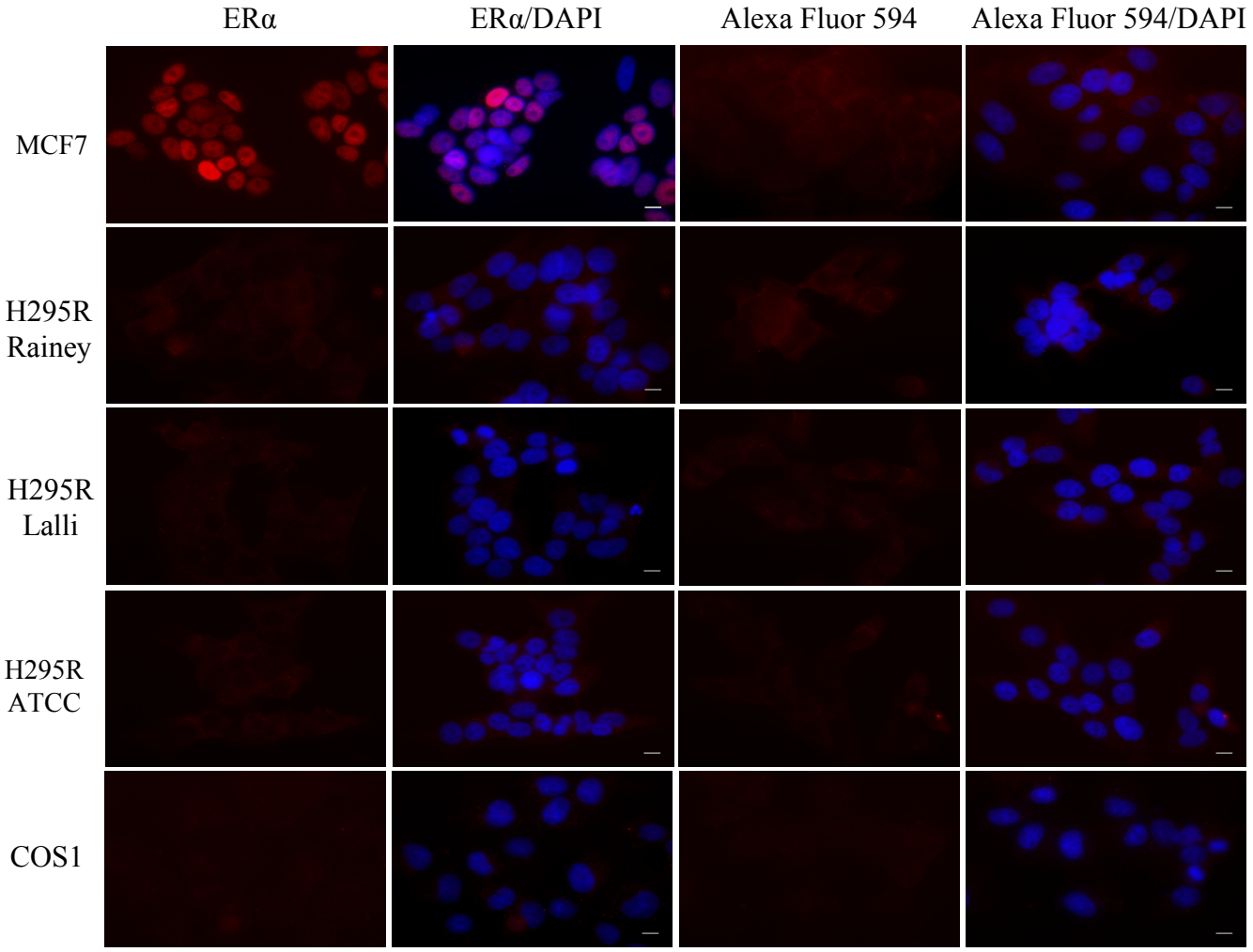

Figure 2. ERα intracellular localization. MCF7, H295R (from Rainey, Lalli and ATCC) and COS1 cells were double-stained for ERα (F-10 clone, red) and DAPI (blue). The same cell lines were also co-stained with the Alexa 594-conjugated goat anti-mouse secondary antibody alone (Alexa Fluor 594) and DAPI (blue). Images of red and blue signals are shown (ERα/DAPI; Alexa Fluor 594/DAPI). Scale bars, 5 μm.

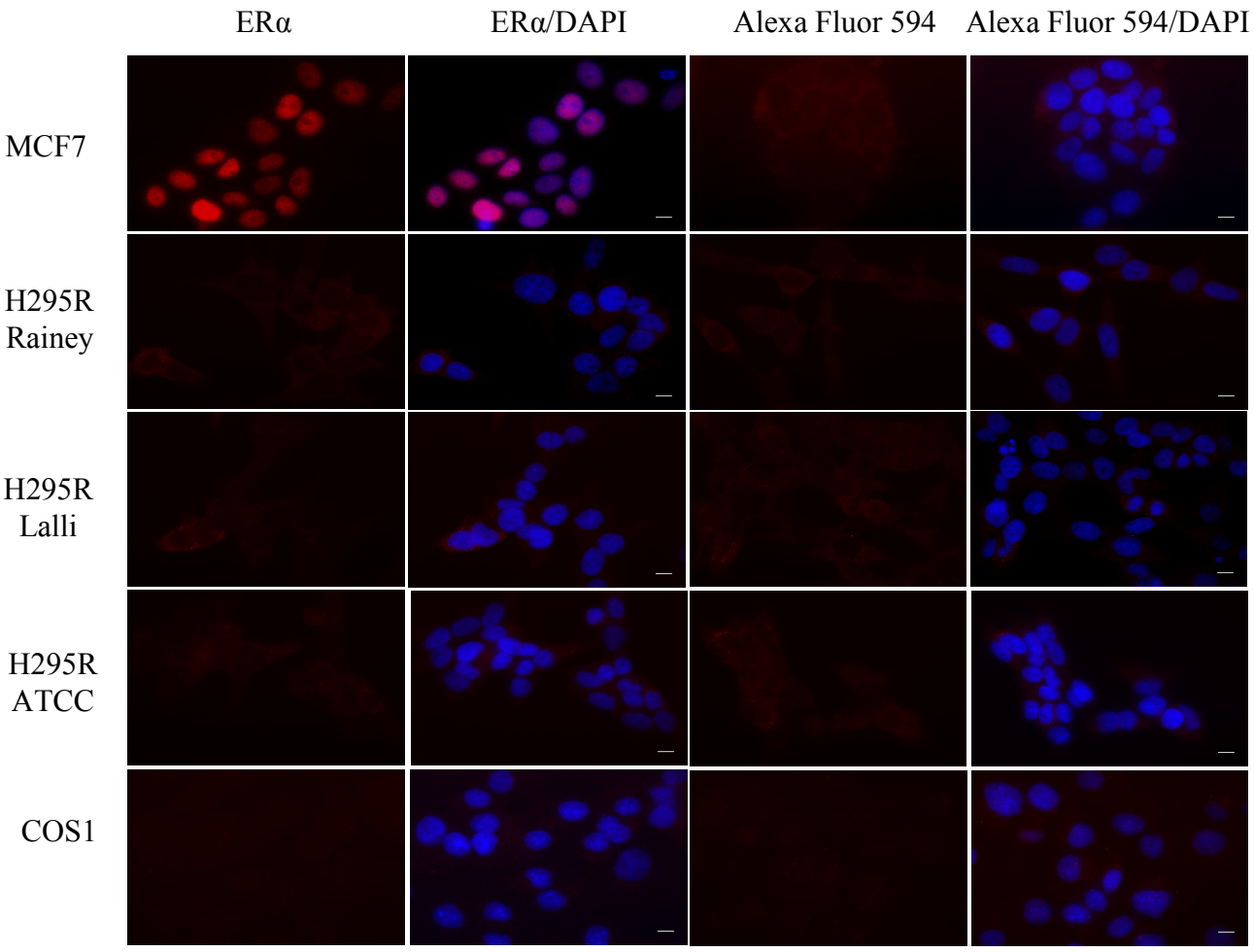

Figure 3. ERα intracellular localization. MCF7, H295R (from Rainey, Lalli and ATCC) and COS1 cells were double-stained for ERα (HC-20 clone, red) and DAPI (blue). The same cell lines were also co-stained with the Alexa 594-conjugated goat anti-rabbit secondary antibody alone (Alexa Fluor 594) and DAPI (blue). Images of red and blue signals are shown (ERα/DAPI; Alexa Fluor 594/DAPI). Scale bars, 5 μm.

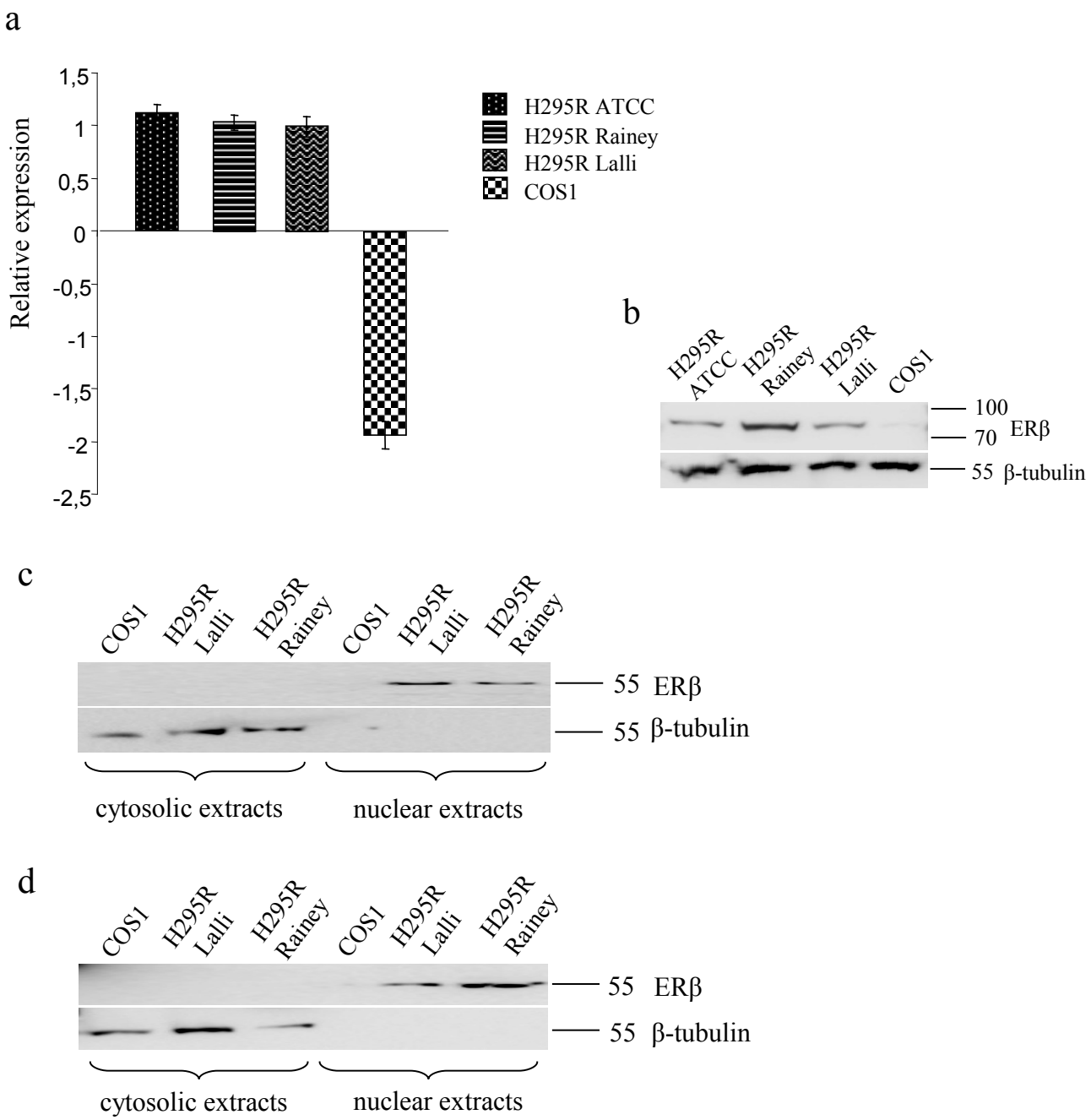

Figure 4. ERβ transcript and protein are expressed in H295R cells. **a.** ERβ levels were measured in H295R (from ATCC, Rainey and Lalli) and COS1 cells by quantitative RT-PCR. Results were calculated using the ΔΔ threshold cycle method and represented in Log 10 scale. H295R ATCC sample was used as calibrator. **b.** Immunoblot analysis was performed on 35 µg of total protein extracted from H295R (from ATCC, Rainey and Lalli) and COS1 cells. Blots were incubated with an anti-ERβ antibody, clone 7B10.7 from GeneTex. β-tubulin was used as a loading control. **c-d.** Immunoblot analysis was performed on 35 µg of cytosolic and nuclear extracts from H295R (from Lalli and Rainey) and COS1 cells. Blots were incubated with an anti-ERβ antibody, clone 7B10.7 (c) or an anti-ERβ antibody, clone 14C8 (d) from GeneTex. β-tubulin was used as a loading control for cytosolic extracts.

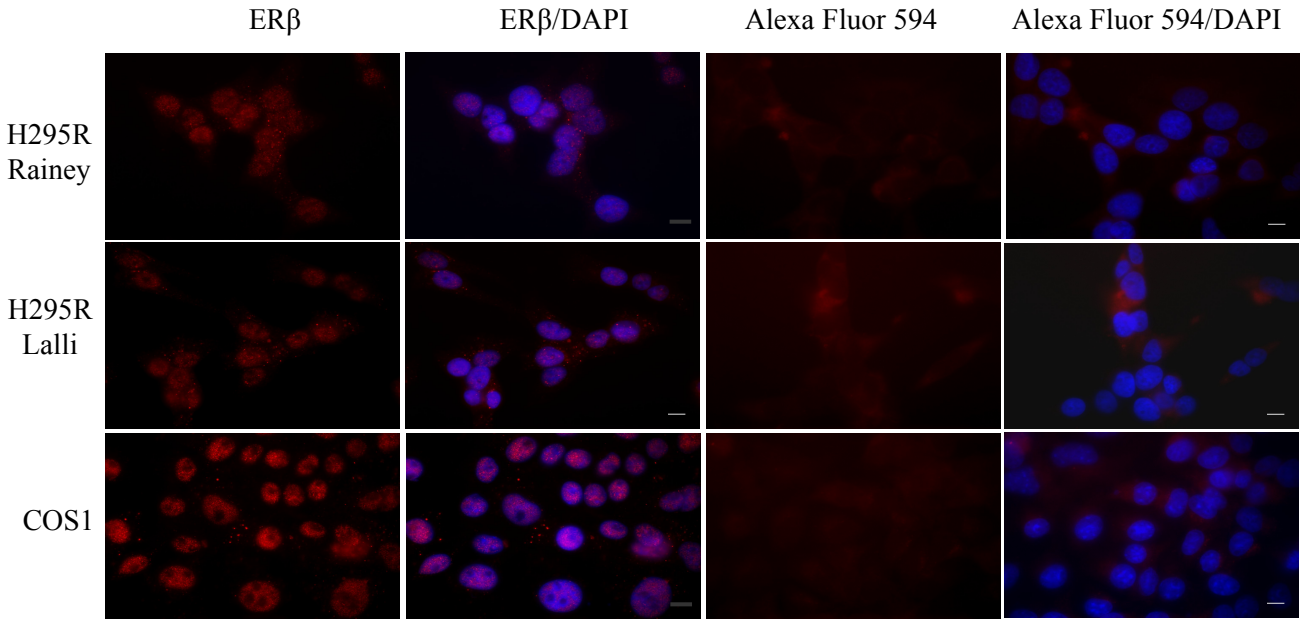

Figure 5. ERβ intracellular localization. H295R (from Rainey and Lalli) and COS1 cells were double-stained for ERβ (14C8 clone, red) and DAPI (blue). The same cell lines were also co-stained with the Alexa-594 conjugated goat anti-mouse secondary antibody alone (Alexa Fluor 594) and DAPI (blue). Images of red and blue signals are shown (ERβ/DAPI; Alexa Fluor 594/DAPI). Scale bars, 5 μm.

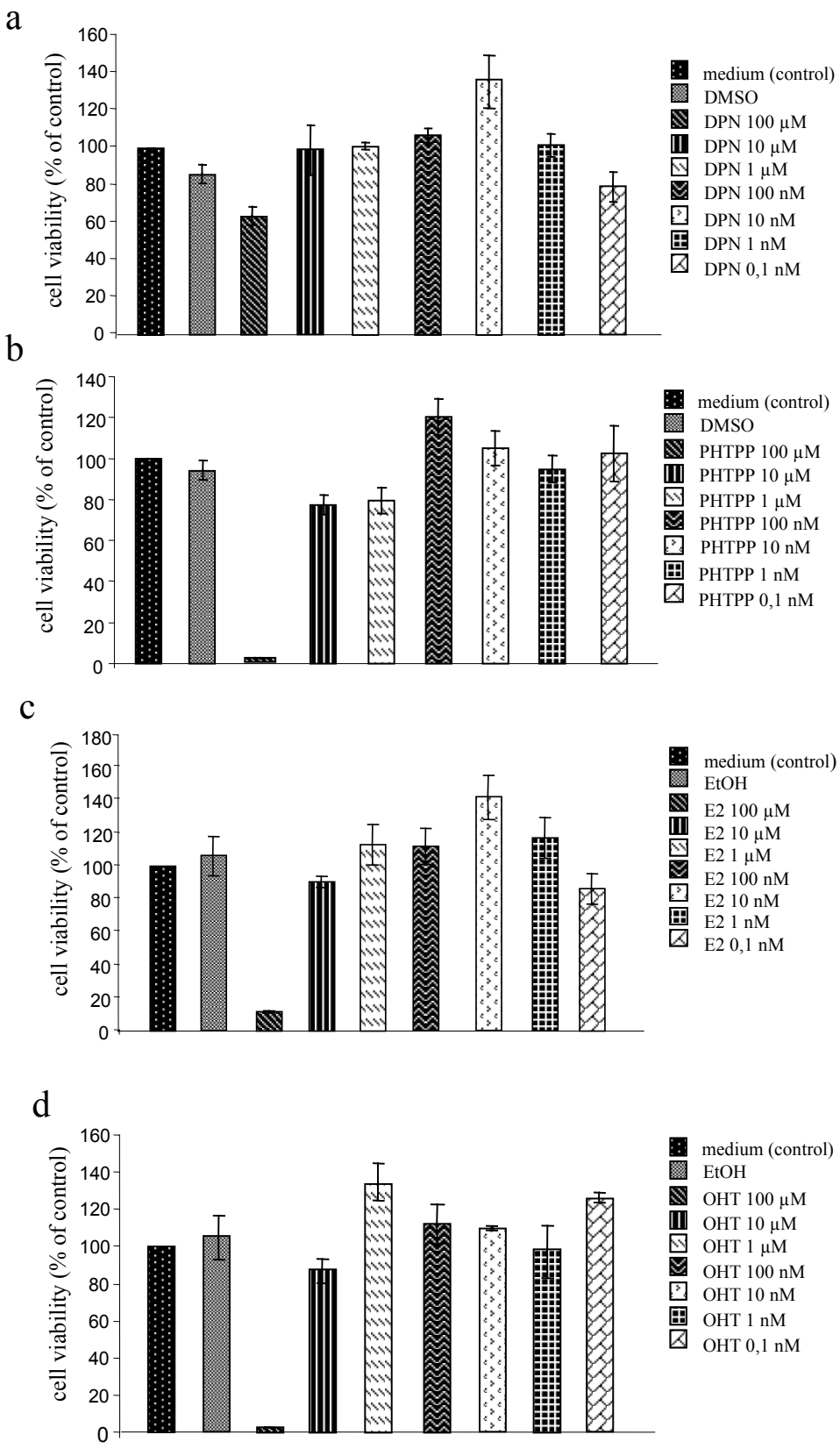

Figure 6. Effects of various concentrations of ER agonist/antagonists on H295R cell proliferation. H295R cells (Lalli's clone) were untreated (medium or vehicle alone) or treated with different concentrations of DPN (a), PHTPP (b), E2 (c) or OHT (d). Proliferation was measured 5 days after the beginning of treatments by CellTiter-Glo Luminescent Cell Viability Assay. Results are expressed as a percentage of control (medium alone).

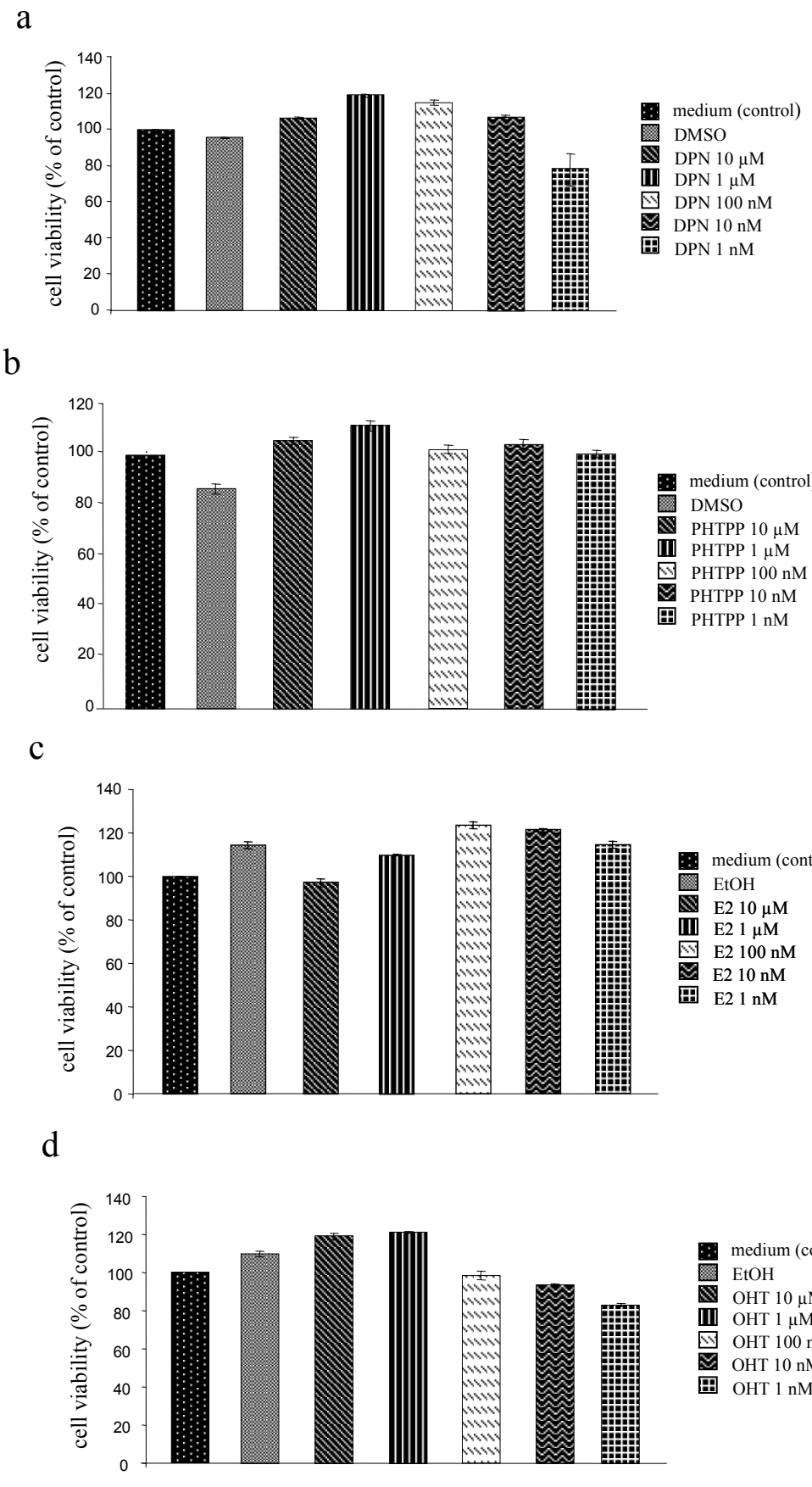

Figure 7. Effects of various concentrations of ER agonist/antagonists on H295R cell proliferation. H295R cells (Rainey's clone) were untreated (medium or vehicle alone) or treated with different concentrations of DPN (a), PHTPP (b), E2 (c) or OHT (d). Proliferation was measured 48 h after the beginning of treatments by CellTiter-Glo Luminescent Cell Viability Assay. Results are expressed as a percentage of control (medium alone).