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Oestrogen receptor (ERβ) regulates osteogenic differentiation of human dental pulp cells.

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Abstract

Human dental pulp cells (hDPCs) express oestrogen receptor (ER) isoforms ERα, ERβ1 and ERβ2, as well as a 7-transmembrane G protein-coupled receptor, GPR30, that mediates rapid oestrogen signalling. Following osteogenic differentiation of these cells ERβ1 and ERβ2 were up regulated approximately 50-fold while ERα and GPR30 were down regulated, but to a much lesser degree (approximately 2-fold). ERβ was characterised as a 59 kDa protein following SDS-PAGE. ERβ was detected in both nuclear and cytoplasmic cell compartments following immunofluorescence of cultured cells. Furthermore isoform specific antibodies detected both ERβ1 and ERβ2 in DPC cultures and *in situ* analysis of ERβ expression in decalcified tooth/pulp sections identified the odontoblast layer of pulp cells juxtaposed to the tooth enamel as strongly reactive for both ERβ isoforms. Finally the use of isoform specific agonists identified ERβ as the main receptor responsible for the pro-osteogenic effect of oestrogenic hormones in this tissue.

Introduction

Tooth loss (edentulism) is associated with reduction of alveolar bone mass in post-menopausal women [1-3] and can be counteracted by oestrogen replacement therapy [4-7]. Similarly alterations in local and systemic oestrogen concentrations as seen during use of chemical contraception or in patients undergoing adjuvant therapeutic strategies in hormone dependant breast cancer is associated with challenges to periodontal health [8-10]. Despite these important epidemiological findings very little is known about the mechanism of action of oestradiol (E_2) within the cells and tissues of the oral cavity. In most cells E_2 binds to cytosolic oestrogen receptors (ERs) which translocate to the nucleus and regulate gene expression in association with other trans and cis acting factors. In addition a cell membrane associated 7TM G protein linked ER (GPR30) is associated with the acute E_2 action in many cells.[11, 12] Several tissues within the oral cavity contain populations of mesenchymal stem cells (MSCs) [13-16] which are able to differentiate into a matrix mineralising phenotype and this can be stimulated by the action of E_2 [17, 18]. Accordingly, ER mRNA and protein is expressed in niche MSC populations in the oral cavity including those from periodontal ligament- PDLCs- [19, 20] and dental pulp- DPCs [21, 22] although the proteins remain rather poorly characterised in these tissues. Two classical ER isoforms have been described- ERα and ERβ- which are structurally and functionally related but which represent two different gene products [23, 24]. Although PDLCs and DPCs are reported to express both ER isoforms, the literature is conflicted with regard to which ER isoform(s) are involved in the differentiation of cells to a matrix mineralising phenotype [25-28] and there is little information on the contribution of ER isoforms to the differentiation of stem cell populations from other tissue niches within the oral cavity. For these reasons we have characterised ER isoform expression and function in DPCs isolated from healthy third molars at the cellular and whole tissue level using qRT-PCR, Western blotting, IHC and IF. In addition we have used isoform specific agonists to determine the activity of ERα and ERβ during differentiation of these cells to a matrix mineralisation phenotype.

Materials

Phenol red free α-MEM, dextran charcoal stripped serum (DCS) and phosphate buffered saline (PBS) was from Lonza (Slough, UK); penicillin/streptomycin (PS), L-glutamine, L-ascorbic acid and dexamethasone, were from Sigma (Dorset, UK). Collagenase (Type I), was from Life Technologies (UK). Tissue culture plastic was from Corning (Amsterdam, Netherlands). 4,4',4''-(4-Propyl-[1*H*]-pyrazole-1,3,5-triyl) *tris*phenol (PPT) and 2,3-*bis*(4-Hydroxyphenyl)-propionitrile (DPN) were from Tocris Bioscience (Abingdon,UK). Details of anti-ERβ antibodies are provided in Supplementary Table 1. HRP conjugated secondary antibodies anti-mouse (ab97046), anti-rabbit (ab6721) and anti-βactin (ab8227) were from Abcam .For IF, Texas Red – goat anti-mouse IgG (T6390), Alexa Fluor 594 goat anti-rabbit IgG (A11037) and Alexa Fluor 488 donkey anti-mouse IgG(A21202) were from Life Technologies, UK. NP40 cell lysis buffer (FNN0021) was from Invitrogen UK. BCA protein assay reagent, WB stripping buffer (46430) and TaqMan primers and probes for qRT-PCR were from ThermoScientific, UK. Reagents for WB including 4-15% gradient gels, PVDF membranes and molecular weight markers were from BioRad, UK. Tissue culture treated glass slides for IF were from Falcon, UK. All other reagents were of analytical grade or better.

Methods

Tissue culture

Isolation, growth and differentiation of DPCs were essentially as described previously [29] with the exception that cells were maintained and passaged in Phenol Red free αMEM containing, 10% dextran charcoal-stripped serum (PR-DCS). Similarly all experiments (qRT-PCR, WB, IF, IHC and *in vitro* bioassay) were done in cells grown in this medium. Unless otherwise stated experiments were performed on DPCs derived from healthy pulps from three separate donors and triplicate technical replicates were performed in each instance.

qRT-PCR

Details of mRNA purification, cDNA synthesis, and qRT-PCR and data analysis have also been described previously [29, 30]. Routinely osteogenic markers ALP, OCN, Runx2 and oestrogen receptor isoforms ERα, ERβ1, ERβ2 and GPR30 were assayed using TaqMan probes and primers. GAPDH or HGPRT were used as house- keeping genes. Assay identifiers for TaqMan qRT-PCR reactions are presented in Supplementary Table 2.

Western blot

DPCs were cultured for 7, 14 or 21 day under basal or osteogenic conditions and cell lysates were prepared using NP40 cell lysis buffer. Protein was quantified using Pierce™ BCA protein assay and lysates were stored at -20 ºC prior to analysis. Lysates containing equivalent amounts of protein (typically 30-50 ug) were diluted in x4 Laemmli sample buffer containing β-mercaptoethanol (5% final concentration) and electrophoresed through 4-15% gradient SDS-PAGE gels for 1h at 120 V. Proteins were transferred to PVDF semi dry membranes using the Trans-Blot Turbo (BioRad) for 10 minutes. Membranes were blocked with TBS containing 5% BSA and 0.1% Tween20 (blocking buffer) for at least 1hour on a shaker at room temperature. Subsequently, membranes were incubated overnight room temperature (RT) with anti-ER-β antibodies Ab 288 and MC10 (1:200; a kind gift from Dr John Hawse [31]) in TBS containing 0.1% Tween20 (TBS-T). These antibodies have been extensively validated for specificity in WB applications [31, 32] Membranes were washed x5 with TBS-T and incubated with anti-mouse horseradish peroxidase (HRP) conjugated secondary antibody (1:100000) for 2 hours at room temperature in TBS-T. Membranes were washed x4 with TBST and x1 with TBS, and developed with ECL substrate. Images were processed and stored on the Gel-Doc system (Bio-Rad). For quantitation and densitometry blots were stripped with WB stripping buffer and re-probed with anti-βactin.

Immunofluorescence (IF)

DPCs at passage 4 were seeded in PR-DCS into a 4- chamber polystyrene vessel containing tissue culture treated glass slides (Falcon). At 70 % confluence cells were washed once with PBS and fixed using ice cold methanol for 10 min. Methanol was aspirated and slides left to air dry at RT for 15 min then stored at 4C. Prior to IF slides were rehydrated in PBS for 5 min. Each chamber was blocked using 4 drops of 0.5% skimmed milk in PBS (blocking buffer - BB) for 1 hour and 100µl of diluted primary antibody (ab 78946; 1:50 in BB) was applied for 1 hour followed by 3 x 5 min washes in PBS. A control in which primary antibody was omitted was included. Secondary antibody (Alexa Fluor or Texas-Red conjugated) diluted 1:300 in BB was applied for 30 min. After 3x15 min washes with PBS slides were mounted using Prolong gold mountant plus DAPI. Slides were allowed to cure overnight at RT and covered with tin foil prior to imaging with a Zeiss microscope.

Immunohistochemistry (IHC)

Dental pulp tissues were obtained from freshly extracted human third molars as described previously [29]. Pulp tissue was gently separated by sterilized tweezers and the tissue then preserved in 4% formaldehyde for paraffin embedding and sectioning. Tissues were

processed in a VIP Tissue processor (Sakura) through 70 % EtOH, 90 % EtOH, 4 changes of 100 % EtOH, 3 changes of 100 % xylene and 3 changes of wax, prior to paraffin embedding. 5 µm sections were cut using a Leitz rotary microtome and mounted on glass slides using a 40 ℃ water bath and slides incubated at 37 ℃ overnight prior to staining. Slides were de-waxed at 70 ℃ on hot plates for 30 minutes prior to antigen retrieval. This was performed in order to enhance the detection of antigen using Mena Path Revelation buffer solution (cat no MP-607-X500) in the Mena Path pressure cooker containing 500 ml distilled water for 40 minutes. Slides were then immersed in PBST (1x PBS containing 0.2% Tween 20) buffer and all remaining steps performed at room temperature. Slides were washed in PBST and blocked with 100 µl Novacastra peroxidase (cat no RE7101) for 15 minutes followed by PBST wash. 100 µl of protein blocking solution (Novacastra - cat no RE7102) was added for 15 minutes. Primary anti-ERβ antibody (PPG5/10 or 57/3 both Serotec) was diluted in Zymed antibody diluent and applied for 1 hour and followed by 3x5 min washes in PBST. Both antibodies have been extensively validated for use in IHC [31, 33, 34]. Post primary antibody block (Novolink polymer cat no RE7112) was applied for 30 min followed by 3x5 min PBS washes. The slides then were incubated in diluted DAB chromogen (1:20 v/v) (cat no RE7105) for 5 min. The slides were counterstained in Mayer's Haematoxylin for 1 min and washed under running tap water, then in Scott's tap water substitute for 2 minutes followed by a further wash in running tap water. The slides were dehydrated in a series of graded ethanols (25% for 15 seconds, 50% for 2 minutes, 70% for 5 minutes, and 100% for 5 minutes) and were immersed in xylene 3 times for 3 minutes. The slides were mounted in DPX and left overnight to set prior to scanning using the ScanScope™ system at x20 magnification, and then visualised using ImageScope™ software. For staining of serial sections from demineralised tooth freshly extracted human third molars were washed with $dH_2O$and any attached tissue was removed. Teeth were placed in 10% EDTA solution (300 mg of NaOH was added to the solution to enhance the dissolving process). The solution was changed every 2-3 days. The decalcification process was monitored weekly by x-ray until teeth were completely translucent. Decalcified teeth were paraffin embedded and sectioned as described for pulp tissue.

In vitro bioassay

ALP activity was assayed as described previously [35] with some modifications. Briefly DPCs were grown to confluence in PF-DCS. ER agonist (DPN or PPT) was added at the indicated concentrations in osteogenic differentiation medium. Medium (inclusive of ER agonists) was changed at days 4, 7 and 10 and cultures were terminated at day 14. Cells were lysed by the addition of 200ul 0.1% Triton X-100 followed by three cycles of freeze-thawing. Lysates were centrifuged (5 min; 10000g) and 20ul was assayed for ALP activity.

Data are presented as nmol p-nitrophenol (pNP) formed per ug DNA and represent mean ± SD (n=3).

Statistics

Data were analysed by Student's unpaired t-test (densitometry) or by two way Anova followed by Bonferroni's multiple comparisons test (bioassay) (GraphPad Prism v 7.0) In both instance differences were considered significant at $p < 0.05$.

Results

Treatment of DPCs with dexamethasone (Dx) and ascorbic acid (AA) caused osteogenic differentiation of DPCs as evidenced by up regulation of early (ALP) and late (OCN) markers of osteogenesis. The transcription factor RunX2 important for the process of osteogenic differentiation was also up regulated at both early (1wk) and late (3wk) time points (Fig 1). In addition differentiated cell cultures stained positively for both ALP and Alizarin Red an indicator of matrix mineralisation (data not shown). Therefore under our culture conditions differentiation of DPC s was achieved and confirmed recent findings from our laboratories [29]. We next investigated the expression of ER isoforms during differentiation of DPCs. As well as ERα and ERβ we also investigated the expression of the more recently described membrane associated G protein linked ER – GPR30. We found that there was a consistent up regulation of both isoforms of ERβ which we examined (-β1 and -β2) at each time point. Therefore ERβ1 was up regulated 27±7, 57±21 and 63±23 fold at days 7, 14 and 21 respectively in differentiating DPCs (mean ± SEM; n=3). For ERβ2 corresponding values were 32±9, 53±15 and 53±8 fold at days 7, 14 and 21 (mean ± SEM; n=3). – Fig2. For the other two isoforms of ER examined there was a tendency for down regulation especially at the later time points. Therefore at 21 days in culture ERα and GPR30 were down regulated to 62±15 and 64±0.5% respectively of the levels seen in undifferentiated DPCs.

Because ERβ protein has been largely uncharacterised in DPCs we used 2 extensively validated Mabs to detect ERβ in lysates prepared from undifferentiated (B) and differentiated (O) cell cultures - see Methods section. WBs indicated a protein of Mr 59kDa which is close to the theoretical Mr for ERβ1 (59.2 kDa) - Fig3 .A non-specific cross reacting species was also apparent at Mr ~ 65 kDa. This has previously been described in the ERβ inducible cell line used to validate Ab288 [32]. Of note however densitometric analysis (Fig 3b) indicated that ERβ levels in lysates from osteogenically differentiated cells did not differ from lysates prepared from undifferentiated cells ($p=0.46$). As such this data is not consistent with qRT-PCR data reported in Fig2 indicating increased ERβ1 and β2 mRNA in differentiated cells (see Discussion section). The presence of ERβ protein was confirmed by IF using clearly showing the presence of the protein in the cytosol and nucleus of DPCs (Fig4). In addition the presence of both β1 and β2 isoforms was confirmed by IHC using validated and non-cross reactive ERβ isoform specific antibodies (Fig 5) and both ERβ isoforms were detected in whole pulps obtained after sectioning decalcified third molars (Fig 6). Interestingly in these whole tooth sections staining for ERβ2 was particularly evident in the peripheral pulp (sub-enamel) layer of tissue which contains mature and precursor odontoblast cells.

Finally we investigated a possible functional role for ER during differentiation of DPCs using alkaline phosphatase (ALP) activity as a marker of osteogenic differentiation as described previously in our laboratories [29]. PPT is a selective agonist of ERα (410-fold v β) whereas DPN is an ERβ selective agonist (70-fold v α) [36, 37]. Fig 7 shows that DPN stimulates ALP activity in a dose dependent fashion over the concentration range 10^{-8} – 10^{-5}M whilst PPT had little effect on ALP activity. These data suggest that the osteogenic promoting activity of E_2 in DPCs is mediated by the ERβ isoform.

Discussion

Our observation of ER expression in DPCs confirms previous reports of expression in these cells [21, 22]. Although we report for the first time up regulation of both ERβ1 and ERβ2 in DPCs during osteogenic differentiation, increased expression of ERβ during osteogenic differentiation of closely related periodontal ligament stem cells (PDLCs) has been described previously [38]. We characterised ERβ protein in DPC lysates by WB and in cells and whole pulp sections by IF and IHC with a panel of validated antibodies directed towards different epitopes of ERβ (Supplementary Table 1). In WB we found a main reactive band with a Mr for the ERβ species in DPC of 59 kDa (Figure 3). In addition a higher Mr species was identified in WB studies (approx.66kDa). Higher Mr non-specific reactivity with both Ab288 and MC10 has been described although specific reactivity was confirmed at 59kDa using doxycycline inducible ERβ in the MDA-MB-231 breast cancer cell line [32] . The epitopes recognised by each of the antibodies used in WB lie within the N-terminal portion of ERβ protein and as such would be expected to recognise both ERβ1 and ERβ2 isoforms. However we found no evidence of ERβ2 species (Mr 55.5 kDa). Despite the fact that many studies have examined ERβ isoforms expression by WB in different tissues [38-42] in most instances these antibodies have not been validated for specific isoform detection using this technique and this area requires further investigation. Although previous studies have assumed that current commercially available pan-ERβ antibodies detect mainly the ERβ1 isoform in WB of cell lysates we note that in our experiments β1 and β2 specific antisera were unreactive with DPC lysate despite previous reports of specific reactivity of these reagents in human Leydig cells [43], colorectal carcinoma cells [44] and breast tumour tissue [45]. Although there may several reasons for this (sensitivity, specificity, and epitope availability) we note that the lack of reactivity of the ERβ1 specific antibody on WB confirms the findings of an extensive validation study reported recently by Nelson et al [32]. In contrast both ERβ1 and ERβ2 species were detected by IHC (Fig5), a methodology for which the antibodies used have been extensively validated (see above) and this confirms our qRT-PCR data.

There are some functional studies describing E_2 action in cells and tissues within the oral cavity [25, 26, 46-48]. PDLCs isolated from ovariectomized (OVX) rats showed decreased matrix mineralisation compared to cells derived from sham operated controls and E_2 treatment of OVX derived cell cultures enhanced the osteogenic differentiation of these cultures [28]. Subsequent siRNA transfection of PDLCs suggested a role for both ERα and ERβ isoforms in E_2 mediated action [27, 28].However, in direct contrast two studies have used siRNA transfection to show that ERβ is the main ER isoform involved in E_2 stimulated osteogenic differentiation of PDLCs [25, 26]. Although these latter studies are consistent with our findings of ERβ activity in DPCs using isoform specific agonists (Fig 7) this area requires further investigation. There is also conflicting data concerning the signalling pathways used by E_2 during stimulation of osteogenic differentiation of DPCs with reports that activation [18] or inhibition [17] of NF-κB is required for differentiation of DPCs to a matrix mineralising phenotype. In stem cells from apical papillae (SCAP) E_2 is reported to stimulate osteogenesis via activation of the MAPK pathway [49] although whether this pathway is activated in DPCs is currently unknown. Although there are no other reports on GPR30 expression in dental tissues recent studies using membrane impermeable E2 conjugates and phytoestrogens suggest that this cell membrane ER can be activated in DPCs and PDLCs [46, 50] and this is an area worthy of further study.

We report dissociation between ERβ mRNA and protein expression. ERβ1 and β2 mRNA is up regulated several fold during osteogenic differentiation of DPCs but such changes are not translated into differences in protein levels (Figs 2 and 3). A disconnection between ERβ mRNA and protein levels has been reported by other workers [51, 52], with work on breast cancer suggesting this may be as a result of translational control [53, 54].This is also an area worthy of further investigation. Finally differentiation of DPCs in our laboratories is achieved with dexamethasone and ascorbic acid and it is important to confirm that changes in ERβ expression are due to differentiation per se and not to the independent action of either agent. Although a previous report suggested that dexamethasone had no effect on ERβ mRNA expression in discrete rat CNS nuclei [51] it will be important to establish that ERβ is up regulated under dexamethasone free differentiating conditions. The osteogenic differentiation of stem cells derived from subcutaneous adipose tissue (ASCs) and bone marrow (BM-MSCs) in the absence of dexamethasone has been described previously [55] and such experiments on DPCs are currently underway in our laboratories.

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Figure Legends

Fig 1 qRT-PCR analysis of osteogenic marker expression in differentiating DPCs**.** *ALP, OCN* and *Runx2* expression was determined at 7 and 21 day time points and show up regulation of early (ALP), late (OCN) and time independent (RunX2) osteogenic markers.

.Fig 2 qRT-PCR analysis of ER isoform expression in differentiating DPCs. *ERα, ERβ1*, *ERβ2* and *GPR30* expression was determined at 7, 14 and 21 day time points in basal and differentiated cultures of DPCs. ERβ1 and β2 isoforms were up regulated following differentiation and ERα and GPR30 were down regulated to a lesser degree.

Fig 3 (a) WB of ERβ in lysates of basal (B) and osteogenically differentiated (O) DPCs at 7 and 14 days. Blots were probed with ab 288. Mr for main reactive species is indicated. Similar results were obtained with Mab MC10. This experiment was repeated 3 times for each antibody and representative data is shown. (b) Densitometric analysis of ERβ protein in basal (b) and osteogenically differentiated (o) DPC lysates. WBs were stripped and re-probed with βactin as loading control. Triplicate tracks from each of 4 separate blots were analysed and data are represented as mean ± SD n=10 basal; n=11 osteogenic AU = arbitrary units. P=0.46.

Fig 4 IF detection of ERβ expression in DPCs. Cells were grown in tissue culture treated glass slides and processed for IF detection of ERβ. DAPI (top), ERβ (middle) and merged (bottom) images are shown

Fig 5 IHC detection of ERβ expression in dental pulp tissue. Pulp tissue was isolated from healthy third molars and processed for IHC. Pan-ERβ, ERβ1 and ERβ2 isoform detection is shown (arrowed) in relevant figure panels. In negative controls primary antibody was omitted.

Fig 6 Demineralised tooth sections were stained with H&E, pan ERβ, ERβ1 and ERβ2. For H&E panel the location of residual enamel matrix (O) along with dental pulp (P) is indicated. Strong staining of ERβ2 in the odontoblast layer is indicated by arrows.

Fig 7 *In vitro* bioassay of selective ER agonists. AP activity was used as a marker of osteogenic differentiation in DPCs. ERα selective (PPT) and ERβ selective (DPN) activity was determined over the concentration range 0-10 uM. Data represent triplicate technical replicates and this experiment was repeated 3 times. Data are presented as mean ± SD;

n=3. In some instances symbol size is larger than SD. Analysis was by two-way Anova followed by Bonferroni's multiple comparisons test * $p < 0.05$;** $p < 0.0001$.

Supplementary Tables

Table 1S Details for ERβ antibodies used in the current study.

Table 2S TaqMan assay identifiers for qRT-PCR Further details are available at http://www.appliedbiosystems.com

Figure 1

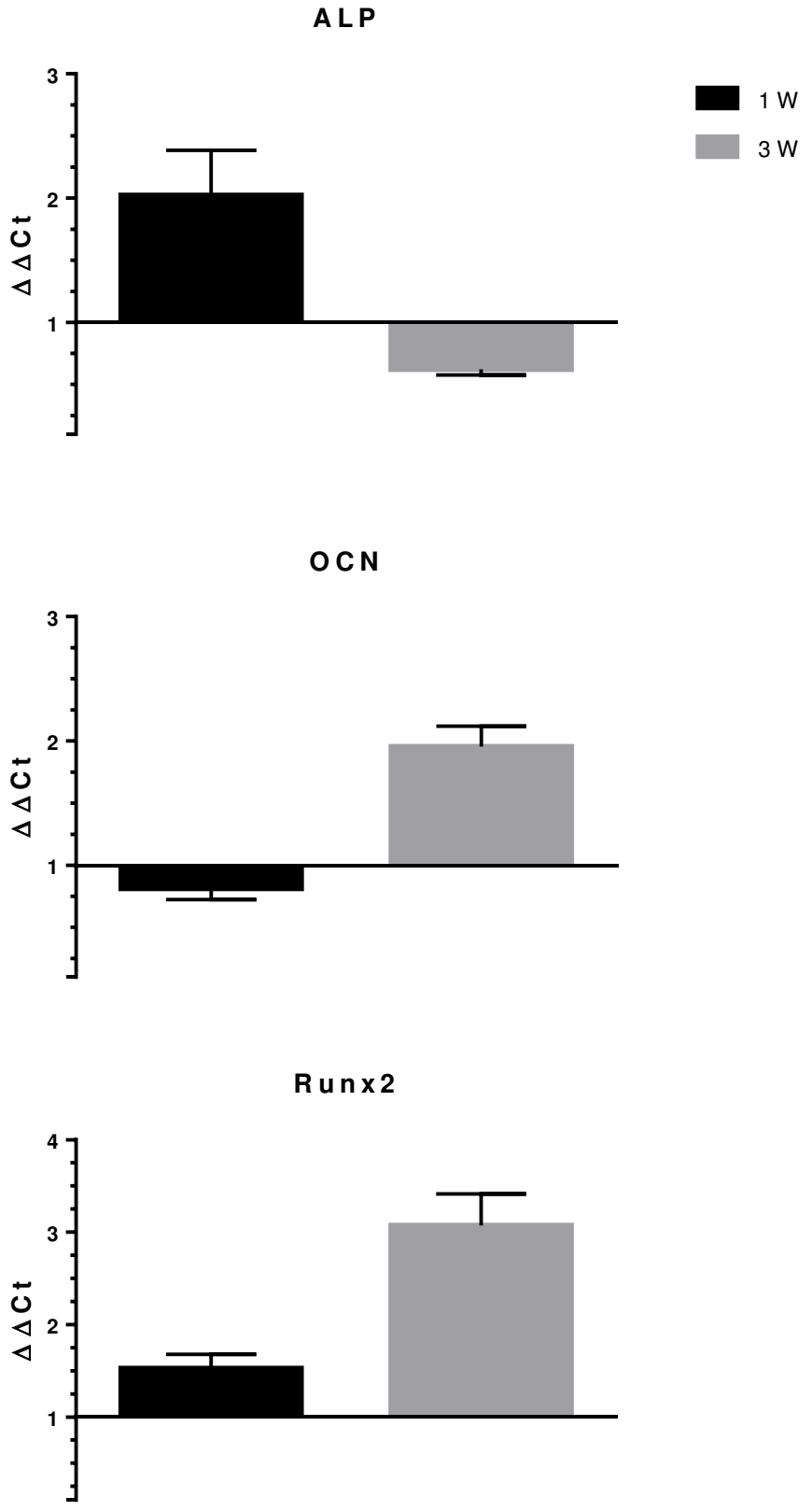
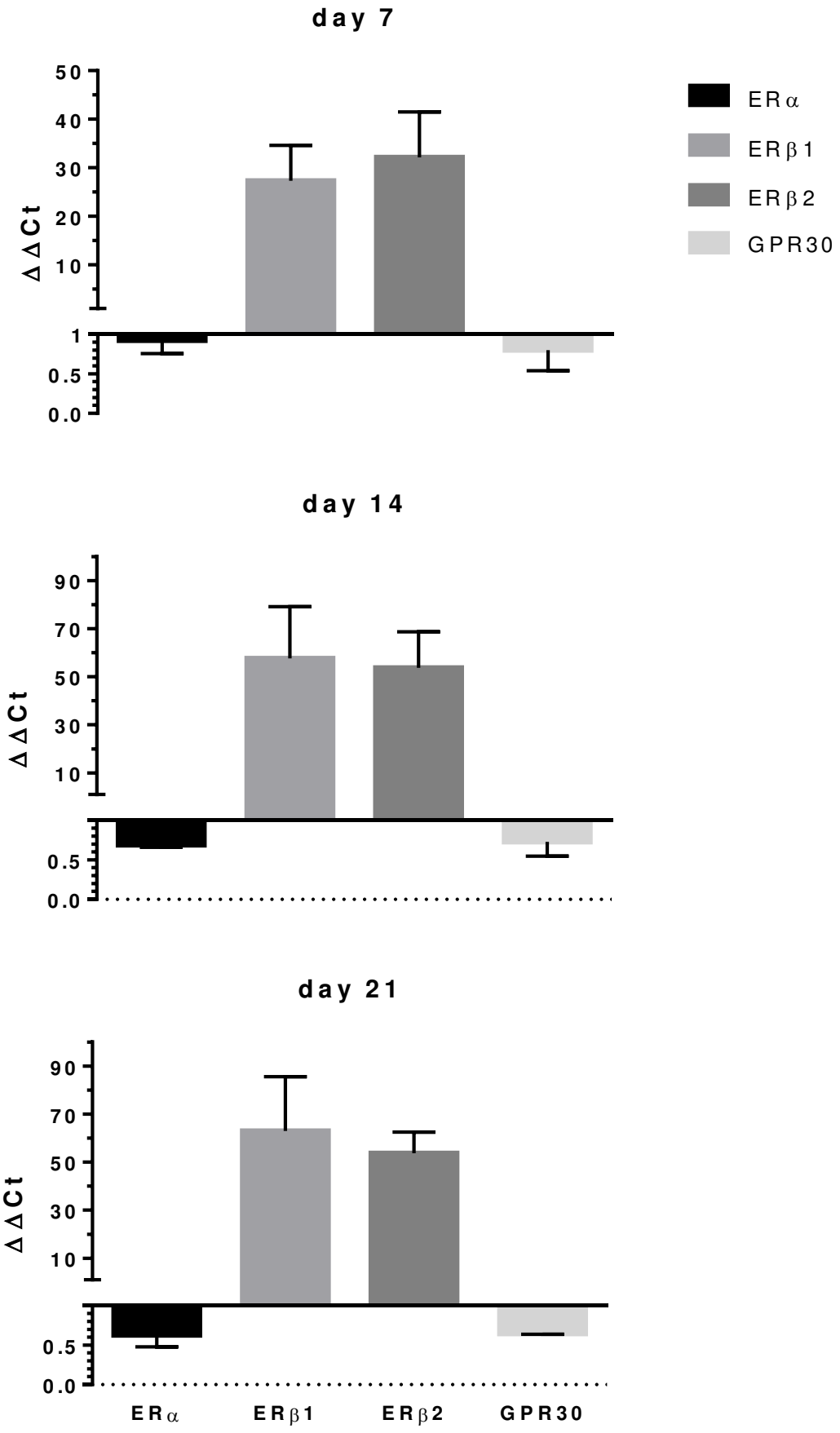


Figure 2


day 7
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40
30
20
10
1
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0.0
ΔΔCt
ERα
ERβ1
ERβ2
GPR30
day 14
90
70
50
30
10
0.5
0.0
ΔΔCt
day 21
90
70
50
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ΔΔCt
ERα
ERβ1
ERβ2
GPR30

Figure 3a

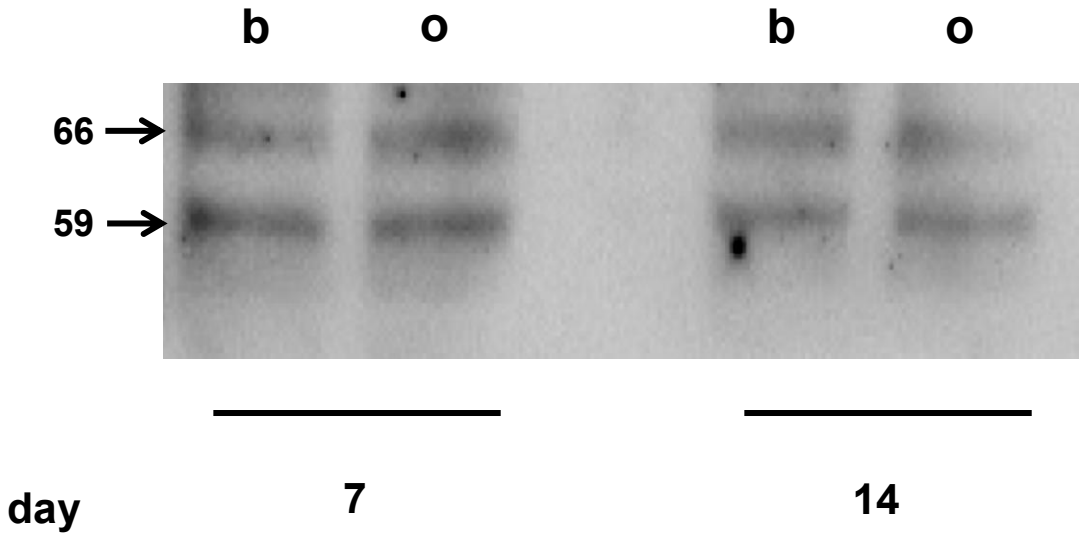


Figure 3b

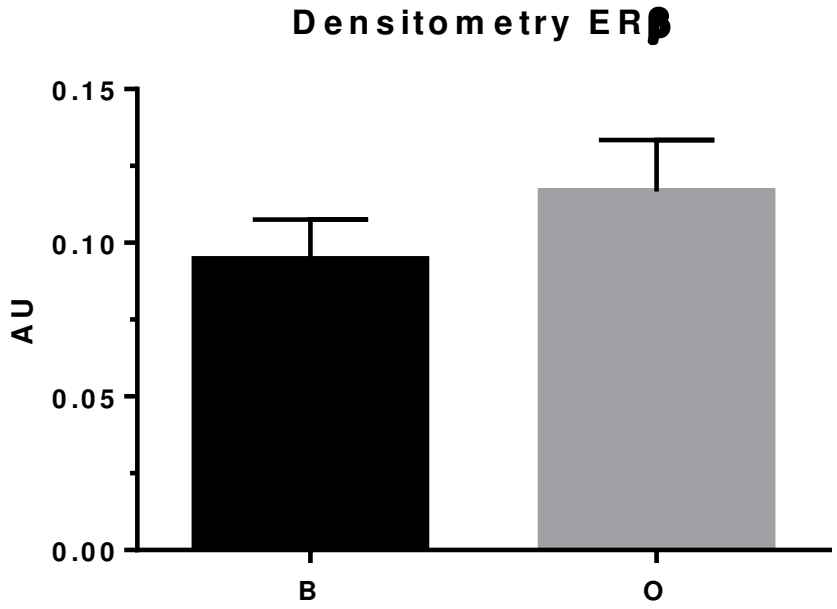


Figure 4

DAPI

ERβ

MERGE

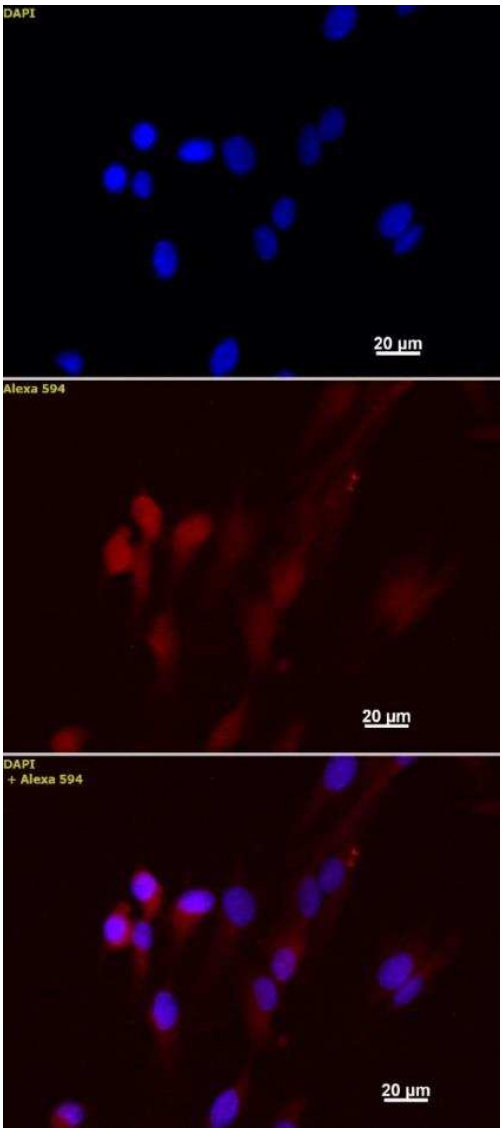


Figure 5

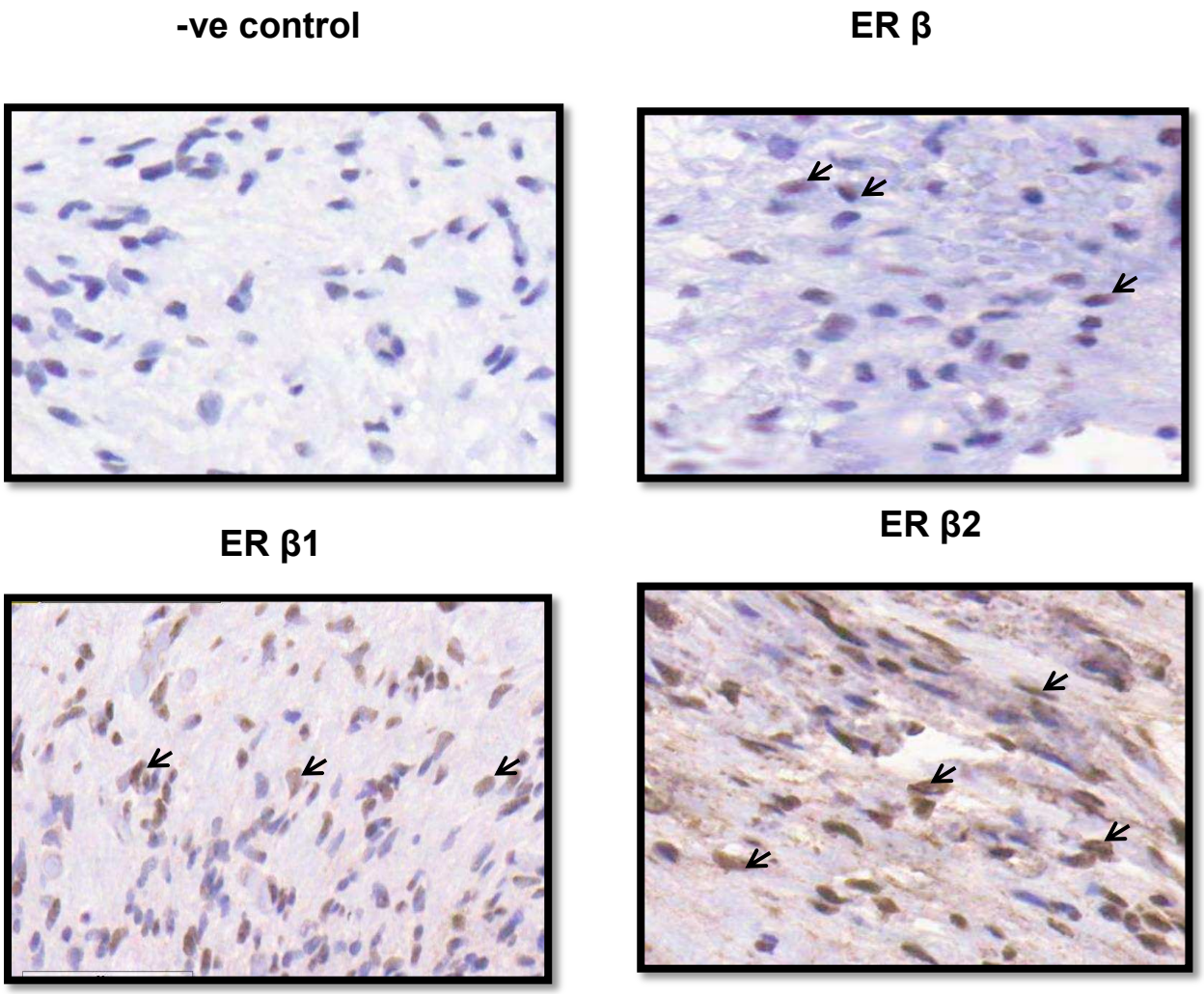


Figure 6

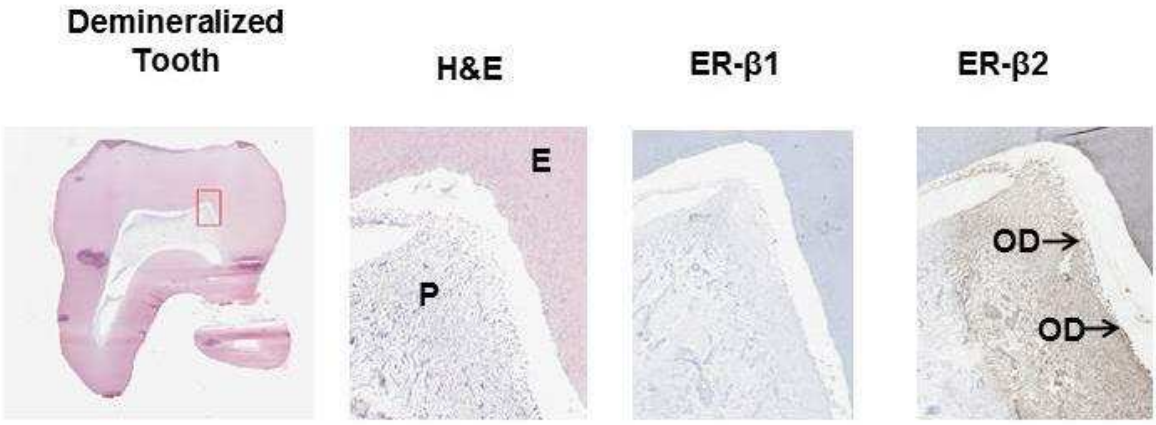
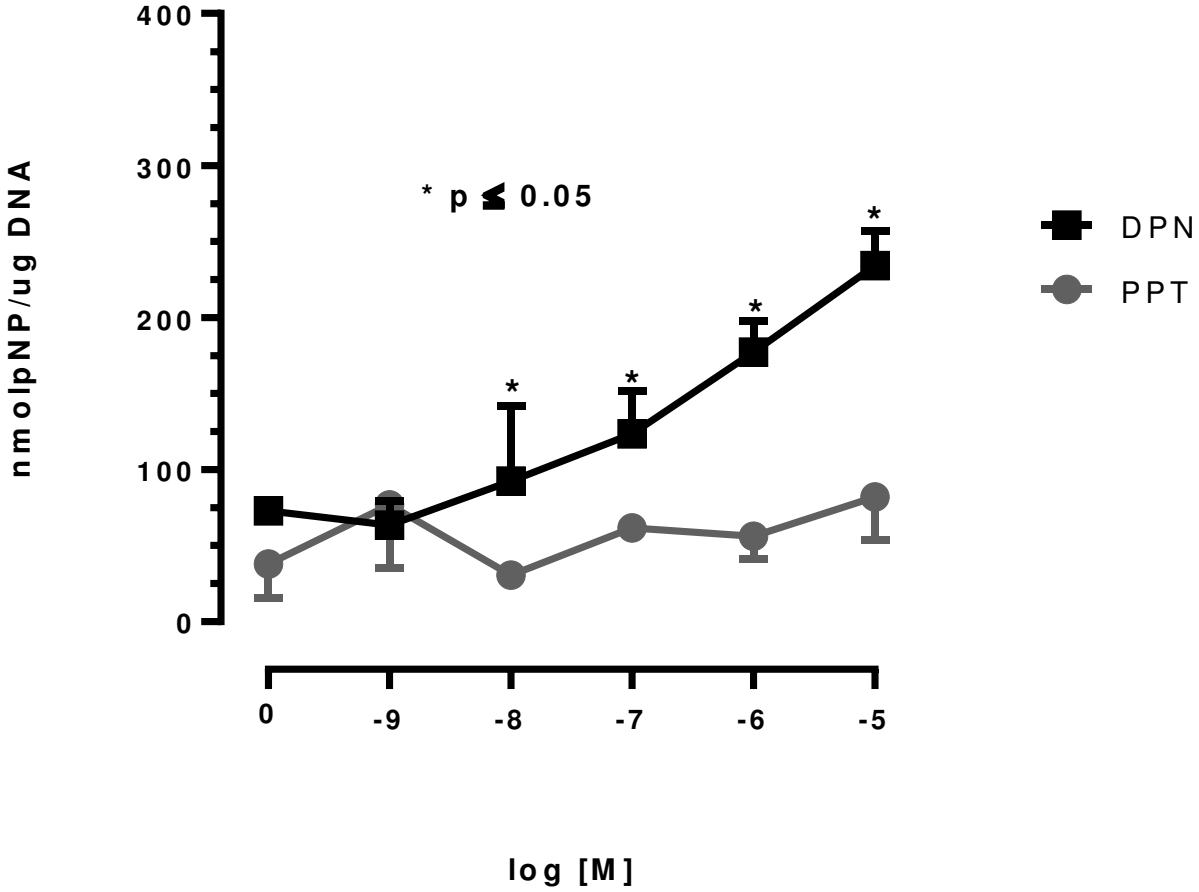


Figure 7



Supplementary Table 1

	Supplier	Host	Epitope	Application
ab 78946	Abcam	rabbit polyclonal	peptide (S105)	IF
ab 288	Abcam	mouse monoclonal	ERβ 1-153	WB
MC10	Wu et al [31]	mouse monoclonal	ERβ 1-140	WB
PPG5/10	Thermofisher	mouse monoclonal	C-terminal (β1)	IHC
57/3	GeneTex	mouse monoclonal	C-terminal (β2)	IHC

IF immunofluorescence; WB Western blot; IHC immunohistochemistry

Supplementary Table 2

Gene name	Taqman ® gene expression assay number
GAPDH	Hs99999905_m1
HGPRT	Hs03929098_m1
ALP	Hs01029144_m1
Runx2	Hs00231692_m1
OCN	Hs00609452_g1
ERα	Hs00174860_m1
ERβ1	Hs01100359_m1
ERβ2	Hs01105520_m1
GPR30	Hs01116133_m1