Free fatty acid receptor 4-β-arrestin 2 pathway mediates the effects of different classes of unsaturated fatty acids in osteoclasts and osteoblasts

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Running title: FFAR4-βarr2 pathway mediate effects of UFAs in bone cells

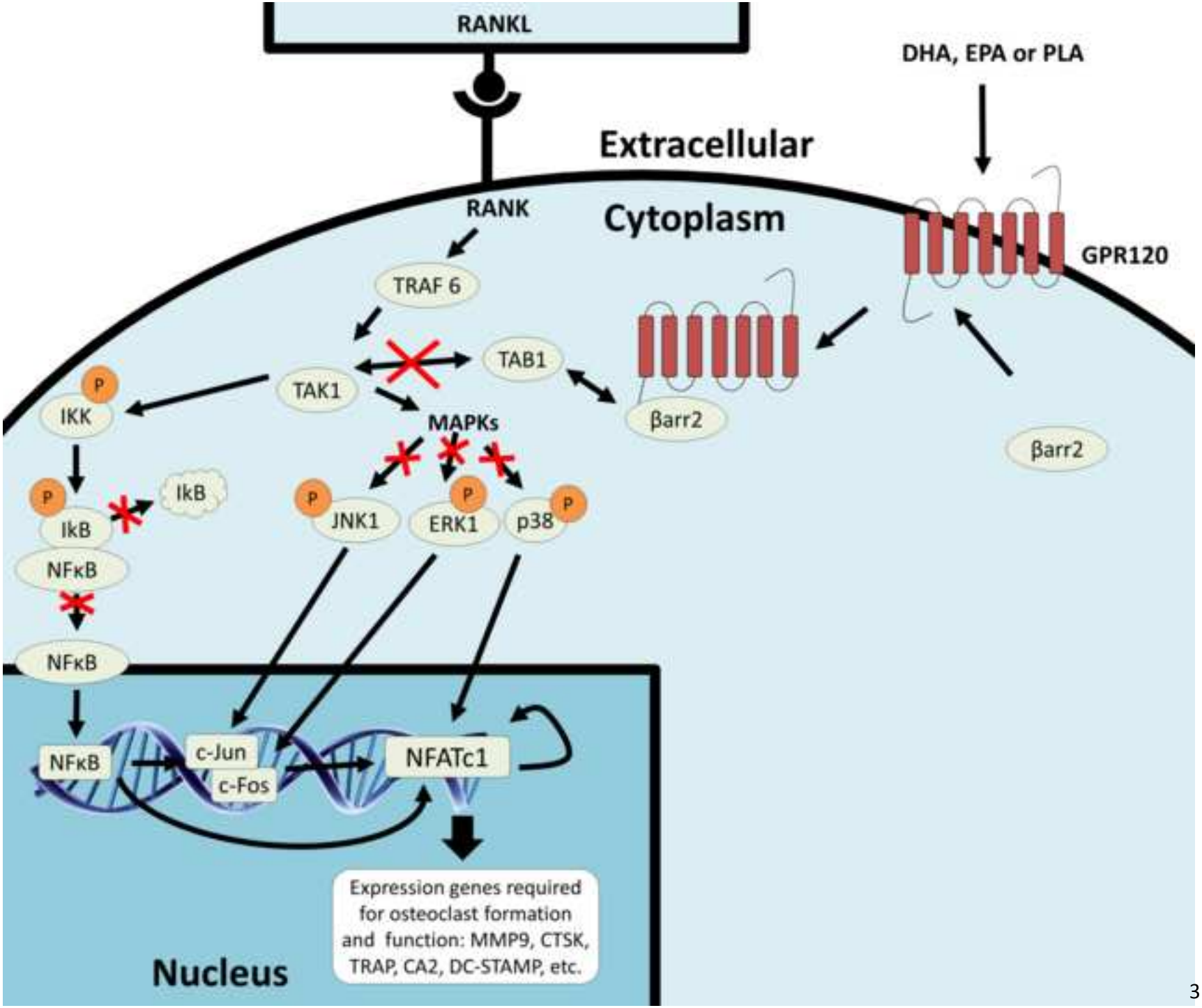
Funding: National Research Foundation of South Africa, Thuthuka Grant (Reference TTK160509164384)

Highlights

- DHA, EPA and PLA modulate osteoclastogenesis through FFAR-βarr2 signalling axis
- DHA, EPA, PLA and OA modulate osteoblastogenesis through FFAR-βarr2 signalling axis
- TUG891 modulates osteoclast activity through FFAR-βarr2 signalling axis
- TUG891 modulates osteoblast activity through FFAR-βarr2 signalling axis
- FFAR-βarr2 signalling may have potential bone therapeutic effects

Graphical Abstract

Potential mechanism of action of DHA, EPA and PLA on osteoclast signalling.



Abstract

Bone is a dynamic tissue that is constantly remodelled by bone resorbing osteoclasts and bone forming osteoblasts, respectively. A breakdown in the remodelling process underlies several bone diseases such as osteoporosis. Unsaturated fatty acids (UFAs) have been shown to have beneficial effects on bone health. However, the mechanism of action of UFAs in bone remains unclear. Free fatty acid receptor 4 (FFAR4) is expressed in bone cells and preferentially binds ω-3 and ω-7 UFAs. Therefore, we sought to determine if FFAR4 influenced the action of different classes of UFAs in bone cells. FFAR4 and potential signalling pathways, β-arrestin 2 (βarr2) and $G_{\alpha q}$, were silenced in RAW264.7 murine macrophages (pre-osteoclasts) and MC3T3-E1 murine pre-osteoblasts. Cell differentiation, activation of signalling pathways and expression of regulatory genes were evaluated. The ω-3 UFAs, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), and the ω-7 UFA, palmitoleic acid (PLA), were shown to require the FFAR4/βarr2 signalling pathway to inhibit osteoclast differentiation in RAW264.7 murine macrophages. The ω-6 UFA, arachidonic acid, and the ω-9 UFA, oleic acid (OA), were shown to inhibit osteoclast formation but did not use FFAR4. DHA, EPA, PLA and OA enhanced osteoblast signalling through the FFAR4/βarr2 signalling axis. This study reveals that FFAR4/βarr2 signalling may mediate the bone protective effects of different classes of UFAs in osteoclasts and osteoblasts.

Keywords: β-arrestin 2; free fatty acid receptor 4; osteoblast; osteoclast; unsaturated fatty acids

1. Introduction

Osteoclasts are large, multinuclear cells that are responsible for the resorption (breakdown) of bone [1]. Together with osteoblasts, the bone forming cell, they maintain the integrity of the skeleton through constant resorption and repair of bone. Osteoclast precursors of monocytic lineage fuse when exposed to the osteoblast derived factors, receptor activator of nuclear factor κB ligand (RANKL) and macrophage colony stimulating factor (M-CSF). RANKL binds to its receptor RANK on osteoclast precursors leading to the recruitment of tumor necrosis factor receptor associated factor 6 (TRAF6) and the formation of the transforming growth factor-β activated kinase 1 (TAK1)-TAK1 binding protein (TAB1)

complex [2]. This activates the mitogen activated protein kinase (MAPK) and NF-κB signalling pathways, resulting in the activation of nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1), the key regulator of osteoclastogenesis [3, 4]. MAPK signalling involves the phosphorylation of p38, JNK-1 and ERK-1 which activates nuclear targets leading to NFATc1 up-regulation.[4]NF-κB signalling begins with the phosphorylation of inhibitory kappa kinase (IKK), which leads to the phosphorylation and degradation of inhibitor of κB (IκB). This frees NF-κB to translocate into the nucleus and activate nuclear targets further amplifying NFATc1 expression.

Osteoblasts are mononuclear cells that originate from mesenchymal stems cells (MSCs) [5]. Osteoblasts are responsible for laying down new, mineralised bone after resorption. Runt-related transcription factor 2 (Runx2) expression is the earliest recognized event during osteoblast formation [6]. Runx2 up-regulates osteoblast specific genes such as alkaline phosphatase (ALP), collagen type 1 alpha 1 (COL1A1) and bone sialoprotein (BSP) [6]. Osteoblasts produce RANKL and M-CSF to regulate osteoclast differentiation. Osteoblasts further produce osteoprotegerin (OPG), which acts as a decoy receptor to RANKL and prevents RANKL binding to RANK [7]. In this way osteoblasts can control osteoclastic resorption and maintain the balance between resorption and formation. To ensure that neither resorption nor formation is excessive, the continuous activity of both osteoclasts and osteoblasts is tightly coupled in a process known as the bone remodelling cycle. Disruption of the bone remodelling cycle underlies several bone degenerative diseases such as osteoporosis.

For several years, unsaturated fatty acids (UFAs) have been studied for their beneficial effects on bone. Communities that consume high amounts of fish oils rich in ω-3 LCPUFAs have been shown to have lower incidences of osteoporosis [8-10]. However, much of the underlying mechanisms still remain unclear. We have previously reported that the ω-6 poly-UFA (PUFA), arachidonic acid (AA), the ω-3 PUFAs, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), and the ω-7 mono-UFA (MUFA), palmitoleic acid (PLA), can inhibit osteoclastogenesis and bone resorption, *in vitro* [11-13]. Drosatos-Tampakaki *et al*. have noted similar inhibitory effects of the ω-9 MUFA, oleic acid (OA), on osteoclast formation and function [14]. DHA, EPA and OA have further been shown to increase gene expression of osteoblast markers *in vitro* [15-17]. This may indicate a common mechanism

of action for these UFAs. Free fatty acid receptor 4 (FFAR4) is a G-protein coupled receptor (GPR) expressed throughout the body, including on osteoclasts and osteoblasts [18]. It is known to bind medium and long chain UFAs and therefore offers a potential as the mediator for the effects of UFAs in bone cells.

FFAR4 (also known as GPR120) activation can lead to either $G_{\alpha q}$ or β-arrestin 2 (βarr2) signalling. $G_{\alpha q}$ signalling results in an increase in intracellular calcium and promotes cell growth [19]. βarr2 signalling prevents the formation of the TAK1-TAB1 complex and thereby offers a promising mediator for the anti-osteoclastogenic effects of UFAs [20]. Taludukar *et al.* have shown that FFAR4 agonists are "functionally selective" and whether stimulation of FFAR4 will favour the $G_{\alpha q}$ or βarr2 pathway can be unique for different cell types [21]. Therefore, the aim of this study was to determine whether FFAR4 plays a role in the activity of different classes of UFAs on bone cells.

2. Materials and methods

2.1 Reagents and materials

DHA, AA, EPA, OA, antibiotic solution, and all other chemicals were supplied by Sigma-Aldrich (St Louis, MO, USA). DMEM and alpha-MEM without ascorbic acid were provided by GIBCO (Grand Island, NY, USA). Fetal bovine serum (FBS) and cell culture plates were provided by Capricorn Scientific (Ebsdorfergrund, Germany) and LASEC (Cape Town, South Africa), respectively. RANKL, TUG891 (FFAR4 agonist) and puromycin dihydrochloride were purchased from Research and Diagnostic Systems (Minneapolis, MN, USA). PLA, FFAR4 shRNA, control shRNA and $G_{\alpha q}$ and βarr2 siRNA were provided by Santa Cruz Biotech (Dallas, TX, USA). Primary antibodies were purchased from Sigma-Aldrich or Abcam (Cambridge, United Kingdom).

2.2 Preparation of fatty acids and FFAR4 agonist

AA, DHA, EPA PLA and OA were prepared in ethanol at a stock concentration of 100 mM while FFAR4 agonist (TUG891) was prepared in DMSO at a stock concentration of 100 mM. All compounds were aliquoted and stored at -70°C until required. Stock solutions were freshly diluted to working concentrations in culture medium just before the experiments. The concentrations of UFAs used in this study were based on previously reported

concentrations that have shown anti-osteoclastogenic effects. AA, DHA and EPA were used at a concentration of 40 μM [11], while PLA [13], OA [14] and TUG891 [22] were used at concentrations of 100 μM. The final concentration of DMSO or ethanol in the media did not exceed 0.1% and this was used as the vehicle control. Differentiation experiments showed no difference between osteoclast numbers or ALP activity, in RAW264.7 murine macrophages or MC3T3-E1 murine osteoblast-like cells respectively, when exposed to either DMSO or ethanol (data not shown). Therefore, the DMSO control was the only vehicle shown.

2.3 Cell culture

RAW264.7 murine macrophages (#TIB-71) and MC3T3-E1 murine osteoblast-like cells (#2593) were obtained from the American Tissue Culture Collection (ATCC, Rockville, MD, USA). Cells were incubated at 37°C in a humidified atmosphere with 95% air and 5% CO_2

RAW264.7 macrophages were maintained in complete DMEM (DMEM containing 10% heat inactivated FBS and 1% antibiotic solution (100 μg mL^{-1} streptomycin, 0.25 μg mL^{-1} fungizone and 100 μg mL^{-1} penicillin). Cells were scraped when needed and seeded after trypan blue exclusion.

MC3T3-E1 murine osteoblast-like cells were cultured in complete alpha-MEM (alpha-MEM without ascorbic acid containing 10% heat inactivated FBS and 1% antibiotic solution). Cells were trypsinized when needed and seeded after trypan blue exclusion. Cells were differentiated in freshly prepared osteogenic media (alpha-MEM with 50 μg ml^{-1} ascorbic acid and 10 mM β-glycerophosphate).

2.4 RNA interference

RAW264.7 murine macrophages or MC3T3-E1 murine osteoblast like cells were grown in 6-well plates until the cells reached 60-70% confluence. The cells were split the day before transfection to ensure they are in the logarithmic growth phase at the time of transfection.

For transfection with shRNA, on the day of transfection, 0.5 μg of control or FFAR4 shRNA plasmid were diluted in 100 μl of serum free media. Thereafter, GeneCellin Transfection Reagent (Biocell Challenge, Toulon, France) was added to the diluted plasmid according to

manufacturer's instructions. Transgene expression was analysed after 24-48 hrs by PCR. Stably transfected control shRNA and FFAR4 negative cells were generated by growing cells in selection media (growth media containing 10% heat inactivated FBS and 3% puromycin).

For transfection with siRNA, on the day of transfection, X-tremeGENE™ siRNA Transfection Reagent (Sigma-Aldrich) and siRNA were diluted in serum-free media according to the manufacturer's instructions. The cells were incubated for 48 hrs and transgene expression was determined via PCR. Transiently transfected control, $G_{\alpha q}$ and βarr2 negative cells were generated and used for downstream experiments.

2.5 Osteoclast experiments

2.5.1 Tartrate resistant acid phosphatase (TRAP) stain

Control or FFAR4 negative RAW264.7 murine macrophages were seeded in 96-well plates at $1x10^4$ cells cm^{-2} in complete DMEM in the presence of RANKL (15 ng ml^{-1}) and FFAR4 agonist, AA, DHA, EPA, PLA or OA. Medium and all factors were replaced on day 3. Experiments were terminated on day 5.

At the end of culture, cells were fixed with 3.7% formaldehyde in PBS and stained using a modified TRAP staining protocol [23]. TRAP is an enzyme highly expressed in mature osteoclasts.[24] Osteoclasts appear as large multinucleated cells staining pink. TRAP-positive stained cells with three or more nuclei per cell were counted as mature osteoclasts [25]. Photomicrographs were taken with an Olympus SC30 camera attached to an Olympus BH2 microscope (Olympus, Tokyo, Japan).

2.5.2 Western blot

Control, FFAR4 negative, βarr2 negative or $G_{\alpha q}$ negative RAW264.7 murine macrophages were seeded at a density of $1x10^5$ cells cm^{-2} in 6-well plates in complete DMEM and incubated at 37°C overnight. Cells were exposed to FFAR4 agonist or select UFAs for 4 hrs. RANKL (15 ng ml^{-1}) was then added and cells were incubated for 15 min.

At the end of culture, the cells were lysed with 100 µl lysis buffer (50 mM HEPES, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA and 100 mM NaF. pH 7.5) supplemented with 0.3 M PMSF and 5% protease inhibitor cocktail and phosphatase

inhibitor cocktail (Sigma-Aldrich). Lysates were prepared in Laemmli buffer containing 1% β-mercaptoethanol and separated on a 12% polyacrylamide gel. Proteins were electrotransferred onto a nitrocellulose membrane with Tris-glycine transfer buffer (192 mM glycine, 25 mM Tris, and 20% methanol) using a Bio-Rad transfer system (Bio-Rad, Hercules, CA, USA). The membranes were incubated with rabbit polyclonal primary antibodies against IκB, JNK, pJNK, ERK, pERK, p38 and pp38 (1:1 000) overnight at 4°C before probing with goat anti-rabbit IgG Antibody, HRP-conjugate (1:20 000) (Sigma-Aldrich). Membranes were then developed using a Clarity ECL Western Blotting Substrate (Bio-Rad) and visualised on a ChemiDoc MP (Bio-Rad) to obtain digital images of the blots. ImageJ software was used to quantify band densities [26].

2.5.3 Immunofluorescence

Control, FFAR4 negative or βarr2 negative RAW264.7 murine macrophages were seeded at a density of $1x10^4$ cells cm^{-2} in a 96-well plate in complete DMEM and incubated at 37°C overnight. Cells were exposed to FFAR4 agonist or select UFAs for 4 hrs. RANKL (15 ng ml^{-1}) was then added and cells were incubated for 30 min.

At the end of the culture period, cells were fixed with 3.7% (v/v) formaldehyde in PBS for 15 min. The cells were then permeabilised for 5 min with 0.1% Triton X-100 and then blocked with 5% BSA in PBS followed by incubation overnight at 4°C with anti-NF-κB antibody (1:400). The cells were then incubated at room temperature with CF™568-goat anti-rabbit IgG (1:1 000) (Sigma-Aldrich) for an hour. Nuclei were stained with 35 µg ml^{-1} Hoechst 33342 (Sigma-Aldrich). Visualisation was done by confocal laser scanning microscopy using a Zeiss Axiovert40 CFL microscope (Carl Zeiss AG, Oberkochen, Germany). Hoechst (Excitation: 352 nm, Emission: 455 nm); CF™568 IgG (Excitation: 562 nm, Emission: 583 nm)

2.5.4 Immunoprecipitation

Control, FFAR4 negative or βarr2 negative RAW264.7 murine macrophages were seeded at a density of $1x10^5$ cells cm^{-2} in a 60 mm petri dish in complete DMEM and incubated at 37°C overnight. Cells were exposed to 15 ng ml^{-1} RANKL in combination with FFAR4 agonist or select UFAs for 24 hrs.

At the end of culture, the cells were lysed with lysis buffer supplemented with 0.3 M PMSF and 5% protease inhibitor cocktail. Lysates were pre-cleared with protein-A agarose (Sigma-Aldrich) in TBS (1:1) before being incubated with anti-TAK1 antibody (1:50) on a rotator at 4°C overnight. The following day protein-A agarose in TBS (1:1) was added and the samples were incubated for 3 hrs on a rotator at 4°C before centrifugation for 5 min at 2 000 xg at 4°C. The supernatant was removed and saved. The remaining slurry was resuspended in sample buffer. Both fractions were then resolved on a 12% polyacrylamide gel and electrotransferred onto a nitrocellulose membrane as described previously. The membranes were probed with anti-TAK1 or anti-TAB1 antibodies (1:1 000) and visualised on a ChemiDoc MP (Bio-Rad). ImageJ software was used to quantify band densities [26].

2.6 Osteoblast experiments

2.6.1 Alkaline phosphatase (ALP) activity assay

Control or FFAR4 negative MC3T3-E1 murine pre-osteoblasts were seeded at a density of $5x10^3$ cells cm^{-2} in 48-well plates in osteogenic media and TUG891, AA, DHA, EPA, PLA or OA for 14 days. Media and all factors were changed every 3-4 days.

At the end of the culture period, cells were fixed with 3.7 % formaldehyde in PBS and ALP activity was determined as previously described.[27]Briefly, cells were incubated for 60 min with ALP assay buffer at 37°C. Thereafter, 100 µl of the reaction product was transferred to a 96-well plate and the absorbance was read at 405 nm with 650 nm as the reference using an Epoch Micro-plate Spectrophotometer (BioTek, Winooski, VT, USA) and results were expressed relative to the control.

2.6.2 Quantitative PCR

Control, FFAR4 negative, βarr2 negative or $G_{\alpha q}$ negative MC3T3-E1 murine pre-osteoblasts were seeded at a density of $5x10^3$ cells cm^{-2} in 24-well plates in osteogenic media and TUG891, AA, DHA, EPA, PLA or OA for 7-14 days. Media and all factors were changed every 3-4 days.

RNA was collected and reverse transcribed as previously described.[11]The Roche FastStart Essential DNA Green Master (Roche Diagnostics, Basel, Switzerland) or the SensiFAST SYBR

Table 1: PCR cycling protocol for quantitative PCR. Protocol for Roche FastStart (black) and SensiFAST protocol (grey).

Step	Temperature		Duration		Cycles	
Hold	95°C	95°C	10 min	2 min	1	1
3-step amplification	95°C 60°C 72°C	95°C 65°C 72°C	20 sec 20 sec 20 sec	5 sec 10 sec 10 sec	35	40
Melting	65°C 95°C		1 min 1 sec		1	

Table 2: Primers used in this study

Gene	Forward primer sequence (5' – 3')	Reverse primer sequence (5' – 3')	GenBank accession number
GAPDH	CCAGCTTAGGTTCATCAGGT	TTGATGGCAACAATCTCCAC	NM_001289726
FFAR4	ATCTTTGTCGTCTCACTGCT	GTAGTCTTGTTGGGACACTC	NM_181748.2
βarr2	ATCACTTGTTGAAAGTGGGC	GTCTCGTCTTCAAGGATTGG	NM_145429.5
$G_{\alpha q}$	AGCCAGTGTCTCAAAATGTC	TGAAACTAACGCCAGTGAAG	NM_008139.5
Runx2	GCACTACCCAGCCACCTTTA	AAGGGTCCACTCTGGCTTTG	NM_001146038.2
Cola1	CTGACTGGAAGAGCGGAGAG	GGGAATCCATCGGTCATGCT	NM_007742.4
BSP	AATGGAGACGGCGATAGTTCC	CGAGAGTGTGGAAAGTGTGGA	NM_008318.3
RANKL	CCTGTACTTTCGAGCGCAGA	CCACATCCAACCATGAGCCT	NM_011613.3
OPG	AGAAGCCACGCAAAAGTGTG	TTCACTTTGGTCCCAGGCAA	NM_008764.3

No-ROX kit (Bioline Reagents, London, UK) and a LightCycler Nano System (Roche Diagnostics) were used for detection. The PCR cycling protocols are shown in Table 1. The $2^{-\Delta\Delta CT}$ method was used to analyse relative gene expression levels and the results were normalized to the housekeeping gene (GAPDH). The primers used (Table 2) were synthesized by Inqaba Biotec (Pretoria, South Africa).

2.7 Data processing

Data was expressed as mean ± SD of three replicate experiments, unless stated otherwise, and expressed relative to the control. Data was analysed using an analysis of variance (ANOVA) followed by a Bonferroni post hoc test using GraphPad Prism software. A p value less than 0.05 was considered significant.

3. Results

3.1 DHA, EPA and PLA decrease osteoclast numbers through FFAR4

A TRAP cell count was done to determine if the UFAs affected osteoclast formation through FFAR4. Cells grown in the presence of RANKL formed large multinucleated TRAP positive cells whereas cells not exposed to RANKL showed no signs of differentiation (Figure 1A). TUG891 and all the UFAs tested reduced the number of osteoclasts formed compared to the RANKL+ control in the control plasmid cells (Figure 1B). In FFAR4 negative cells, the inhibitory effect of TUG891, DHA, EPA and PLA were abrogated (Figure 1B). However, AA and OA still caused significant decreases in osteoclast formation after FFAR4 knockdown. Successful knockdown of FFAR4, βarr2 and $G_{\alpha q}$ was determined by PCR (Figure 1C).

3.2 DHA, EPA and PLA modulate RANKL signalling through the FFAR4/βarr2 pathway

Addition of RANKL resulted in the degradation of IκB in control plasmid and $G_{\alpha q}$ negative cells (Figure 2A). TUG891, DHA, EPA and PLA prevented the degradation of IκB after exposure to RANKL in control plasmid cells and $G_{\alpha q}$ negative cells. However, when FFAR4 or βarr2 was silenced, these inhibitory effects were lost. Furthermore, the phosphorylation of the three MAPKs, p38, JNK and ERK was inhibited by TUG891, DHA EPA and PLA in control plasmid and $G_{\alpha q}$ negative cells. However, the FFAR4 agonist and the three UFAs failed to

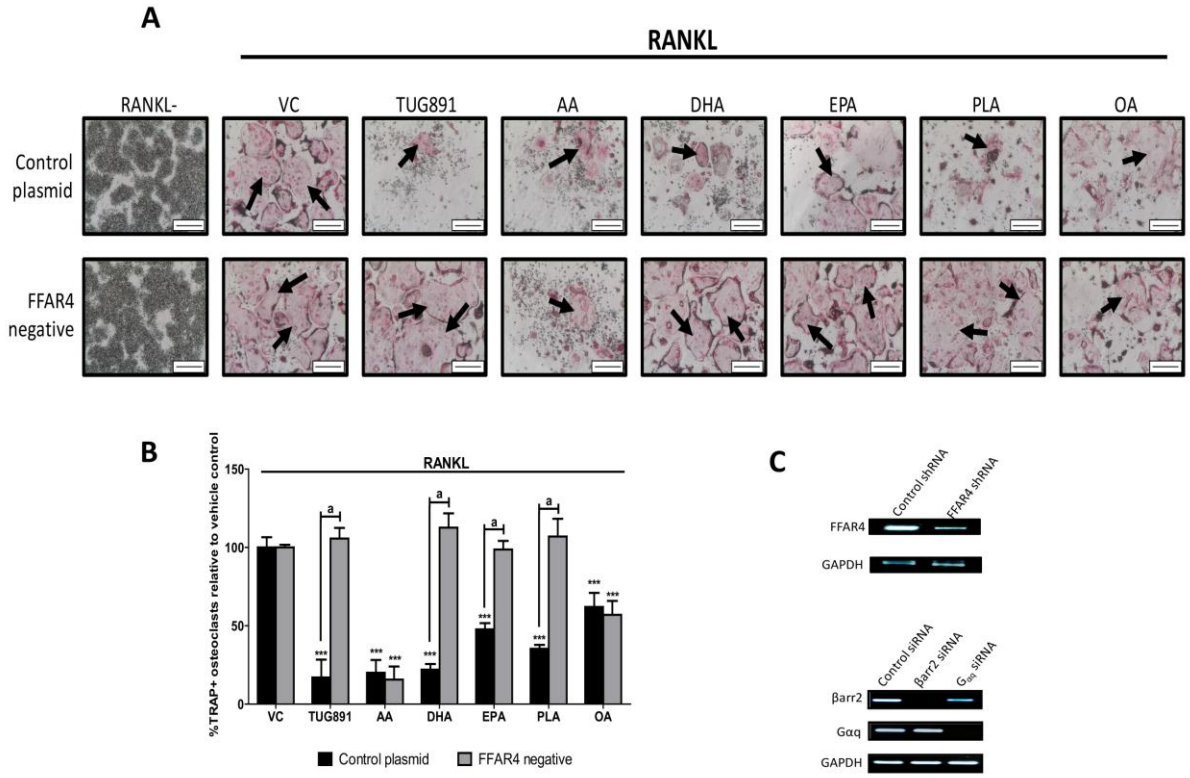


Figure 1: DHA, EPA and PLA inhibit osteoclast formation through FFAR4. A. Control plasmid and FFAR4 negative RAW264.7 murine macrophages were seeded in the presence of RANKL and TUG891, AA, DHA, EPA, PLA or OA for 5 days with medium changes on day 3. Cells were fixed and stained for TRAP (black arrows). Scale bar=500 µm. B. TRAP positive cells with 3 or more nuclei were counted as mature osteoclasts. ***p<0.001 vs control. C. Successful gene knockdown was determined by PCR after transfection with FFAR4 shRNA, βarr2 siRNA, Gαq siRNA or control plasmids. Experiments were repeated 3 times in tripicate. Data was analysed using an ANOVA followed by a Bonferroni post hoc test. VC: vehicle control. RANKL-: no RANKL added.

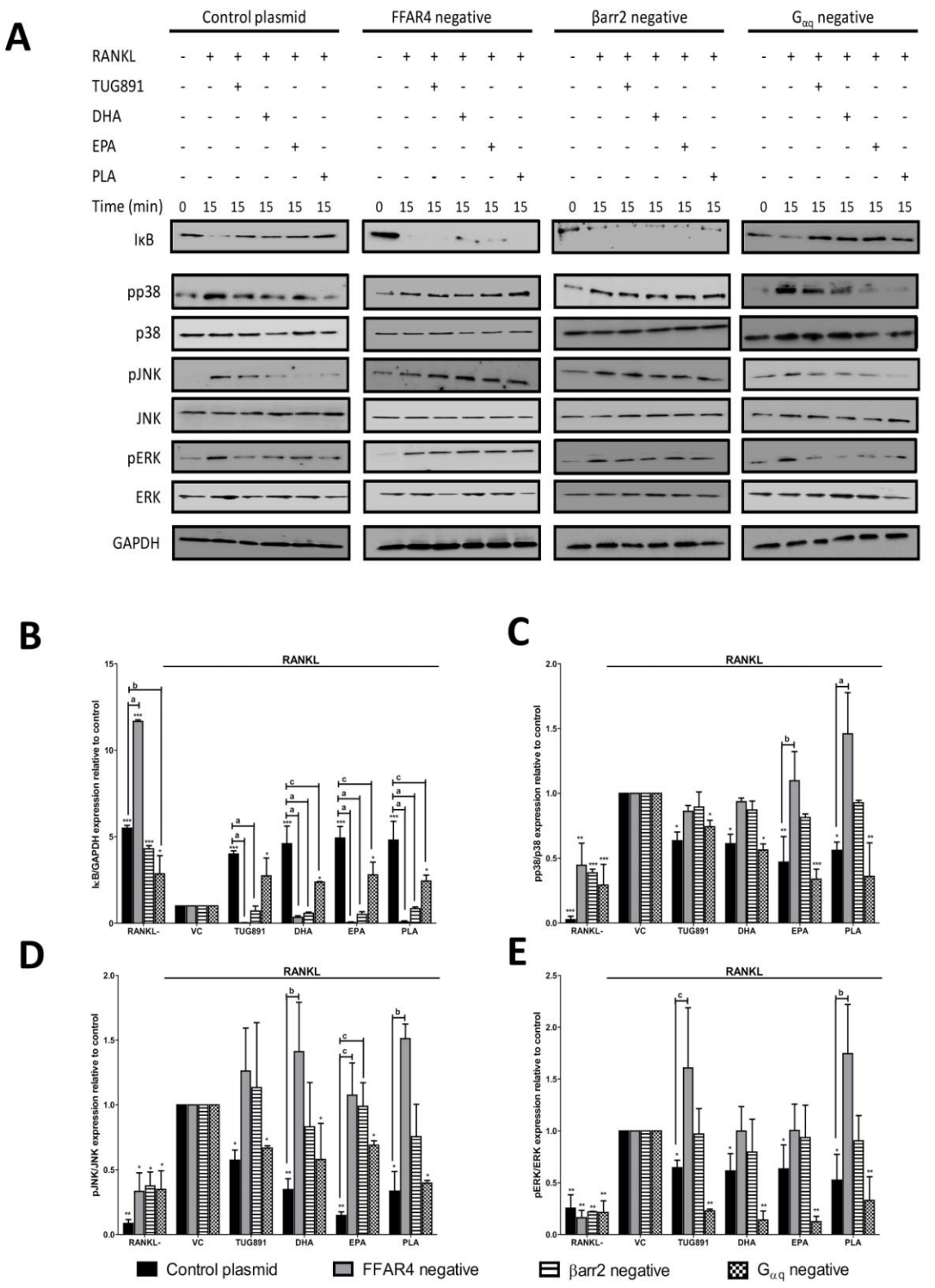


Figure 2: DHA, EPA and PLA modulate RANKL signaling through FFAR4-βarr2 signalling axis. A. RAW264.7 macrophages were exposed to TUG891, DHA, EPA or PLA for 4 hrs and then exposed to RANKL (15 ng ml^{-1}) for 15 min. Protein was isolated and the expression of IκB, pp38, p38, pJNK, JNK, pERK and ERK was determined by western blotting. GAPDH served as the loading control. B-E. Band densities were quantified using ImageJ software. Experiments were repeated twice in duplicate. Data was analysed using an ANOVA followed by a Bonferroni post hoc test. VC: vehicle control. RANKL-: no RANKL added. *p<0.05, **p<0.01, ***p<0.001 vs control. a=p<0.001, b=p<0.01, c=p<0.05 vs vehicle control plasmid cells exposed to same compound.

inhibit MAPK phosphorylation when FFAR4 or βarr2 was silenced (Figure 2A). Quantification of band densities revealed that all changes were statistically significant (Figure 2B-E).

After IκB is degraded it frees up NF-κB to translocate to the nucleus to activate DNA binding sites. The translocation of NF-κB to the nucleus was determined using immunofluorescence. Without the addition of RANKL, NF-κB was shown to remain in the cytoplasm in control plasmid, FFAR4 negative and βarr2 negative cells (Figure 3A). The addition of RANKL resulted in translocation of NF-κB to the nucleus. DHA, EPA, PLA and TUG891 prevented the nuclear translocation of NF-κB in control plasmid cells. However, in FFAR4 negative and βarr2 negative cells, TUG891, DHA, EPA or PLA were no longer able to prevent the translocation of NF-κB to the nucleus.

RANKL further caused an increase in the formation of the TAK1-TAB1 complex in control plasmid cells (Figure 3B). TUG891, DHA, EPA and PLA all inhibited the formation of the TAK1-TAB1 complex after exposure to RANKL. βarr2 silencing prevented the inhibitory effects of TUG891, DHA, EPA and PLA on the formation of the TAK1-TAB1 complex (Figure 3C).

3.3 FFAR4 agonist increases ALP activity in MC3T3-E1 murine pre-osteoblasts

In the absence of osteogenic media (OM-), ALP activity was significantly lower in both control plasmid and FFAR4 negative cells (Figure 4A). In control plasmid cells, TUG891 caused a significant increase in ALP activity relative to the control. However, when FFAR4 expression was silenced, TUG891 no longer showed any effect on ALP activity. AA significantly reduced ALP activity in control plasmid and FFAR4 negative cells. However, when exposed to AA, FFAR4 negative cells showed a significantly reduced effect on ALP activity compared to the control. None of the other UFAs affected ALP activity in control plasmid or FFAR4 negative cells. Successful knockdown of FFAR4, βarr2 and $G_{\alpha q}$ was determined by PCR (Figure 4B).

3.4 DHA, EPA, PLA and OA modulate osteoblast specific gene expression through FFAR4/βarr2

Runx2, COL1A1 and BSP expression were all significantly higher in control cells grown in osteogenic media compared to cells grown in the absence of osteogenic media (Figure 4C). TUG891, DHA, EPA, PLA and OA caused a significant increase in Runx2, COL1A1 and BSP

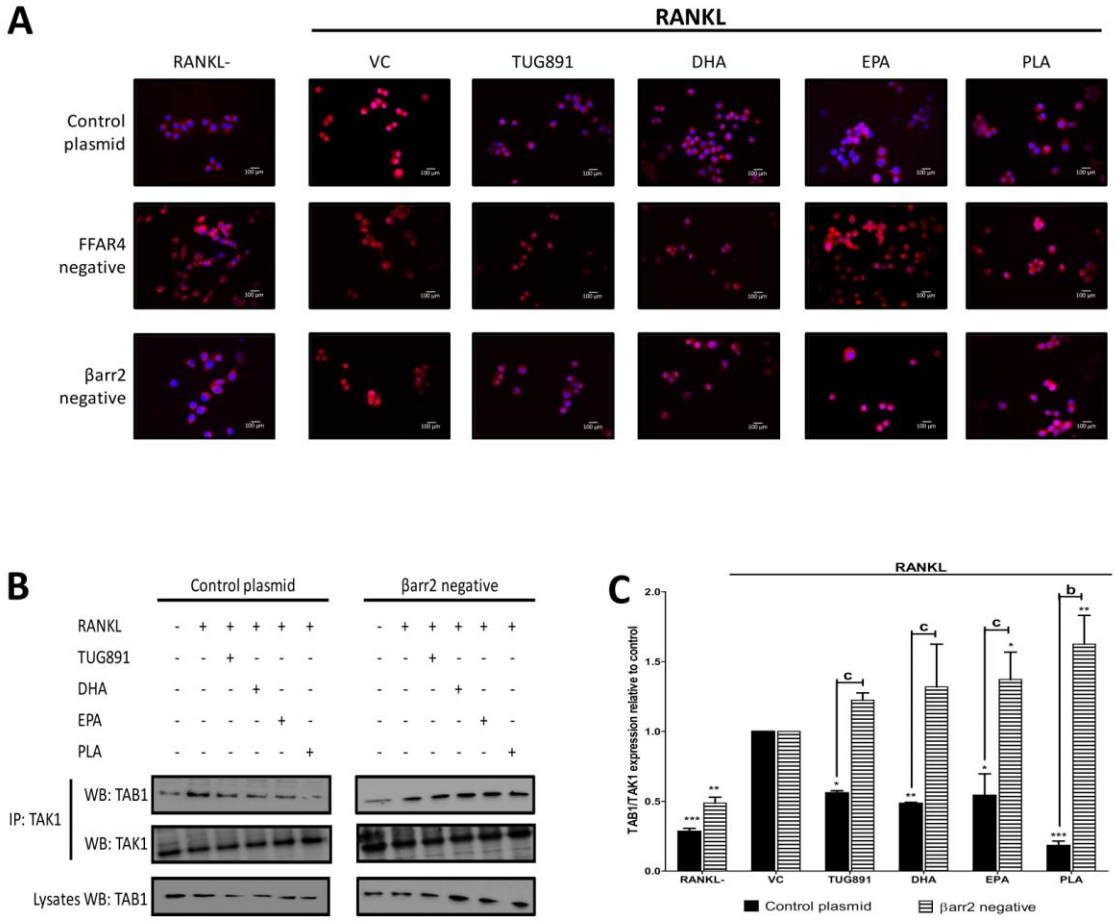


Figure 3: FFAR4-βarr2 signalling axis mediates effects of DHA, EPA and PLA in osteoclasts. A. RAW264.7 macrophages were exposed to TUG891, DHA, EPA or PLA for 4 hrs and then exposed to RANKL (15 ng ml^{-1}) for 30 min. The cells were probed for NF-κB (red) and visualized using a fluorescent secondary antibody and the nuclei was stained with Hoechst (blue). The pink stain indicates where the NF-κB has translocated to the nucleus. Scale bar=100 µm. B. The cells were treated with TUG891, DHA, EPA or PLA and RANKL (15 ng ml-1 for 24 hrs. Protein was isolated and TAK1 was immunoprecipitated before the products were probed for TAB1 and then visualized by western blotting. IP: Immunoprecipitate. WB: Western blot. VC: vehicle control. RANKL-: no RANKL added. C. Band densities were quantified using ImageJ software. Experiments were repeated twice in duplicate. Data was analysed using an ANOVA followed by a Bonferroni post hoc test. *p<0.05, **p<0.01, ***p<0.001 vs vehicle control. b=p<0.01, c=p<0.05 vs control plasmid cells exposed to same compound.

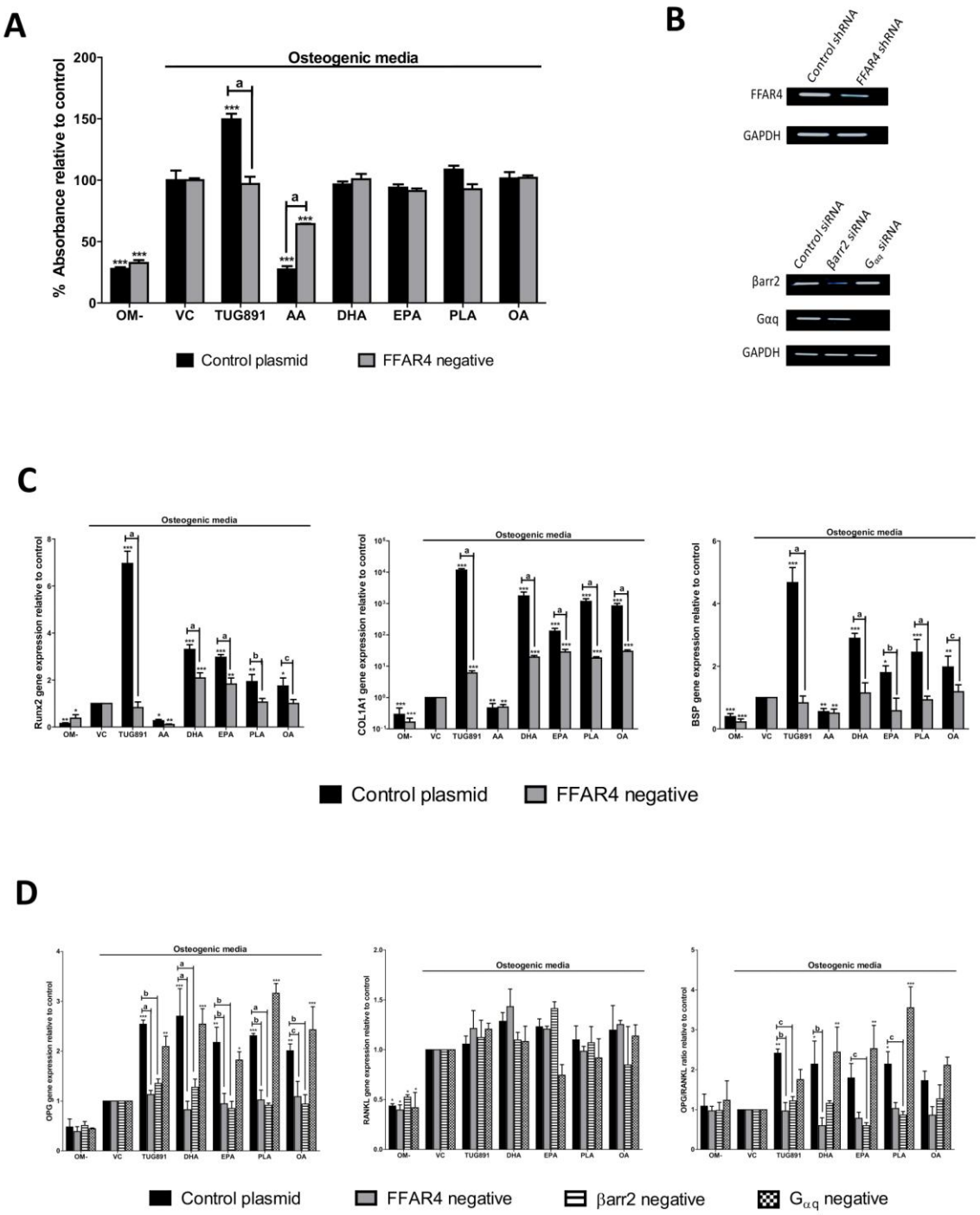


Figure 4: FFAR4/βarr2 signalling axis mediates effects of DHA, EPA, PLA and OA in osteoblasts. A. MC3T3-E1 murine pre-osteoblasts were seeded in the presence of osteogenic media and TUG891, AA, DHA, EPA, PLA or OA. ALP activity was determined via ALP assay. B. Successful gene knockdown was determined by PCR after transfection with FFAR4 shRNA, βarr2 siRNA, Gαq siRNA or control plasmids. C. Cells were then exposed to TUG891, AA, DHA, EPA, PLA or OA in the presence of osteogenic media. Expression of Runx2, COL1A1 and BSP was determined by PCR. D. Expression of OPG and RANKL was determined by PCR and used to determine the OPG/RANKL ratio. Experiments were repeated 3 times in tripicate. Data was analysed using an ANOVA followed by a Bonferroni post hoc test. OM-: no osteogenic media. VC: vehicle control. *p>0.05, **p>0.01, ***p<0.001 vs vehicle control. a=p<0.001, b=p<0.01, c=p<0.05 vs control plasmid cells exposed to same compound.

expression in control plasmid cells. FFAR4 silencing significantly reduced the effect of TUG891, DHA, EPA, PLA and OA on Runx2 expression. FFAR4 negative cells exposed to DHA or EPA still had significantly higher levels of Runx2 than the osteogenic media positive control. However, this was significantly lower than the control plasmid cells exposed to the same UFA. Furthermore, in FFAR4 negative cells the effect of TUG891, DHA, EPA, PLA and OA on COL1A1 and BSP expression was significantly lower than in control plasmid cells exposed to the same UFA (Figure 4C). However, in FFAR4 negative cells exposed to TUG891, DHA, EPA, PLA or OA the expression of COL1A1 was significantly higher than the OM positive control, but significantly lower than the control plasmid cells exposed to the same UFA. AA significantly reduced Runx2, COL1A1 and BSP expression in control plasmid and FFAR4 negative cells.

The expression of OPG and RANKL was determined by PCR and used to determine the OPG/RANKL ratio (Figure 4D). TUG891, DHA, EPA, PLA and OA all greatly increased OPG expression compared to control grown in osteogenic media in control plasmid and $G_{\alpha q}$ negative cells. However, in FFAR4 negative cells this effect was abrogated. Similarly, when βarr2 was silenced TUG891, DHA, EPA, PLA and OA did not affect OPG expression compared to the osteogenic media positive control.

A significant increase in RANKL expression was observed in cells grown in osteogenic media compared to cells grown in the absence of osteogenic media. Neither TUG891 nor any of the UFAs affected RANKL expression in control plasmid cells. FFAR4, βarr2 or $G_{\alpha q}$ silencing did not alter the effect TUG891 or the UFAs on RANKL expression.

TUG891, DHA, and PLA significantly increased the OPG/RANKL ratio in control plasmid and $G_{\alpha q}$ negative cells. The effect of TUG891, DHA and PLA on the OPG/RANKL ratio was neutralised by FFAR4 and βarr2 silencing. $G_{\alpha q}$ knockout did not disrupt the effects of TUG891, DHA and PLA on the OPG/RANKL ratio. In the absence of $G_{\alpha q}$, EPA significantly increased the OPG/RANKL ratio. OA showed no significant effect on the OPG/RANKL ratio in control plasmid, FFAR4 negative, βarr2 negative or $G_{\alpha q}$ negative cells.

4. Discussion

FFAR4 is a G-protein coupled free fatty acid receptor that has been reported to be expressed in osteoclasts and osteoblasts [18]. In this study the role of FFAR4 on the effects of different classes of UFAs, the ω-6 PUFA, AA, the ω-3 PUFAs, DHA and EPA, the ω-7 and ω-9 MUFAs, PLA and OA respectively, were investigated on osteoclast and osteoblast cell lines. Osteoclast and osteoblast formation was evaluated as well as the activity of several signalling pathways. The purpose of this study was to elucidate the role of FFAR4 in the effects of different classes of fatty acids in bone cells.

Similar to Oh *et al*., [28] we report that RAW264.7 murine macrophages express high levels of FFAR4, βarr2 and $G_{\alpha q}$ (Figure 1C). Previous studies have shown that FFAR4 activation can inhibit osteoclast formation through inhibition of NF-κB and MAPK signalling pathways [29, 30]. *In vivo* studies further revealed that in the presence of high levels of ω-3 fatty acids, FFAR4 activation stimulated bone formation while supressing bone resorption [31]. TUG891, a synthetic FFAR4 agonist, and all the UFAs used in this study inhibited RANKL-induced osteoclast formation in RAW264.7 murine macrophages (Figure 1A). However, in the absence of FFAR4, TUG891, DHA, EPA and PLA did not reduce osteoclast formation (Figure 1A). Silencing of the FFAR4 or βarr2 prevented the inhibitory effects of TUG891, DHA, EPA and PLA on the NF-κB and MAPK signalling pathways (Figure 2 and 3) as well their inhibitory effects on the formation of the TAK1-TAB1 complex (Figure 3). These results indicate that TUG891, DHA, EPA and PLA may require the FFAR4-βarr2 pathway to exert their anti-osteoclastogenic effects. It may be suggested that UFAs that are naturally present in the FBS may contribute to the effects described. In this present study, all data was normalized to the vehicle control. However, when looking at the absolute values for the cell count (data not shown), there was no difference in cell numbers between control shRNA and FFAR4 shRNA cells in the vehicle treated cells. Therefore, any effect of the UFAs present in the FBS was deemed to be negligible.

Oh *et al*. reported that the FFAR4-βarr2 signalling pathway was crucial to the anti-inflammatory effects of DHA in RAW264.7 macrophages [28]. Interestingly, these researchers also noted that DHA, EPA, PLA and OA could activate FFAR4 while AA and saturated fatty acids could not [28]. In the present study, FFAR4 silencing did not prevent

the anti-osteoclastogenic effects of AA, and similarly did not prevent the anti-osteoclastogenic effects of OA, a known activator of the FFAR4 (Figure 1A). However, this may indicate that AA and OA may use alternative pathways to influence osteoclast formation. Similar to FFAR4, FFAR1 preferentially binds medium to long chain UFAs and AA and OA has been shown to activate FFAR1 in rat islet beta cells [18, 32, 33]. Furthermore, RAW264.7 murine macrophages were shown to express FFAR1 and activation of this receptor inhibited RANKL induced osteoclast formation [34]. Further studies are needed to investigate whether or not FFAR1 may mediate the anti-osteoclastogenic effects of AA and OA.

We further report that MC3T3-E1 murine pre-osteoblasts were also shown to express FFAR4, βarr2 and $G_{\alpha q}$ (Figure 4B). Gao *et al*. have reported that activation of FFAR4 can promote mineralization and osteoblast gene expression in bone marrow mesenchymal stem cells [22]. In the present study, TUG891, DHA, EPA, PLA and OA were shown to promote the expression of pro-osteoblast genes in MC3T3-E1 pre-osteoblasts through FFAR4 (Figure 4). OPG acts as a decoy receptor for RANKL thereby inhibiting osteoclast differentiation [1]. A low OPG/RANKL ratio will result in increased osteoclast formation and resorption whereas a high OPG/RANKL would decrease osteoclasts and resorption. The expression of OPG was increased by TUG891, DHA, EPA, PLA and OA in control and $G_{\alpha q}$ negative MC3T3-E1 cells. However, these effects were lost in FFAR4 or βarr2 silenced MC3T3-E1 cells (Figure 4). RANKL expression remained unchanged when exposed to any of the compounds in control plasmid, FFAR4 negative, βarr2 negative or $G_{\alpha q}$ negative cells. These results are similar to Casado-Dìaz *et al*. who reported that, after 7 days, DHA and EPA increased OPG expression while RANKL expression was unchanged in mesenchymal stem cells stimulated with osteogenic media [17]. This led to an increase in the OPG/RANKL ratio. In the present study, we reported increases in the OPG/RANKL ratio in control plasmid cells when exposed to TUG891, DHA and PLA (Figure 4). However, when FFAR4 or βarr2 were silenced, TUG891, DHA and PLA showed no effect on the OPG/RANKL ratio.

Interestingly, we have shown that OA required FFAR4 to induce osteoblast gene expression in MC3T3-E1 pre-osteoblasts, but not to inhibit osteoclastogenesis in RAW264.7 murine macrophages. As previously suggested, FFAR1 may mediate the effects of OA in osteoclasts. However, FFAR1 has also been shown to be expressed in MC3T3-E1 pre-osteoblasts and

FFAR1 activation was shown to promote early stage mineralization but inhibit late stage osteoblast mineralization [35]. These results may indicate that activation of FFAR1 or FFAR4 by OA in bone cells may be cell specific. Further studies are needed to elucidate the importance of FFAR1 in mediating the bone protective effects of OA.

Abdelmagid *et al*. have reported that the total plasma free fatty acid concentration in young Canadian adults was 474.6 ±251.7 μM [36]. The total plasma concentrations were reported for AA (393 ±119.1 μM), DHA (88.8 ±36.8 μM), EPA (40.3 ±28.3 μM), PLA (133 ±67.2 μM) and OA (1285.5 ±416.7 μM) [36]. In the present study we made use of AA, DHA and EPA at 40 μM and PLA and OA at 100 μM, indicating that our concentrations may be achievable in the human body. Results from the present study reveal that, at physiologically relevant concentrations, the FFAR4-βarr2 pathway may be crucial for the anti-osteoclastogenic effects of DHA, EPA and PLA and for the pro-osteoblast effects of DHA, EPA, PLA and OA. Contrasting to the anti-osteoclastogenic effects of UFAs that we report, Yuan *et al*. have reported that at lower concentrations (10 μM) AA and EPA enhanced osteoclast formation in murine bone marrow macrophages [37]. This may indicate that activation of FFAR4-βarr2 signalling could be dose dependant. High doses of UFAs could be required to stimulate the bone beneficial effects of UFAs through FFAR4 *in vivo*.

In vivo studies on the role of FFAR4 in mediating the effects of UFAs has delivered conflicting results. Some studies have shown that FFAR4 is crucial for the effects of ω-3 LCPUFAs [28], while others have shown it is not [38]. These conflicting results underlie the importance of further studying the role of this fatty acid receptor in mediating the effects of UFAs in bone. However, this present study reveals that activation of the FFAR4-βarr2 signalling pathway may offer potential as a drug target for bone degenerative diseases by promoting osteoblast differentiation while inhibiting osteoclast formation.

5. Conclusion

In this study it was shown for the first time that different classes of UFAs can modulate osteoclast and osteoblast activity through FFAR4 signalling pathways. TUG891 was shown to inhibit osteoclast activity and promote osteoblast activity through the FFAR4-βarr2 signalling axis and may have potential to be developed as a treatment for osteoporosis. DHA, EPA and PLA were also shown to use the FFAR4-βarr2 signalling axis to modulate

osteoclast activity while DHA, EPA, PLA and OA used this same signalling pathway in osteoblasts. Taken together, these results indicate that the FFAR4-βarr2 pathway may be crucial to the bone protective effects of certain UFAs.

6. Funding

This study was supported by the National Research Foundation of South Africa, Thuthuka Grant (Reference TTK160509164384)

7. Conflict of interest

The authors declare no conflict of interest.

8. References

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Supplementary data

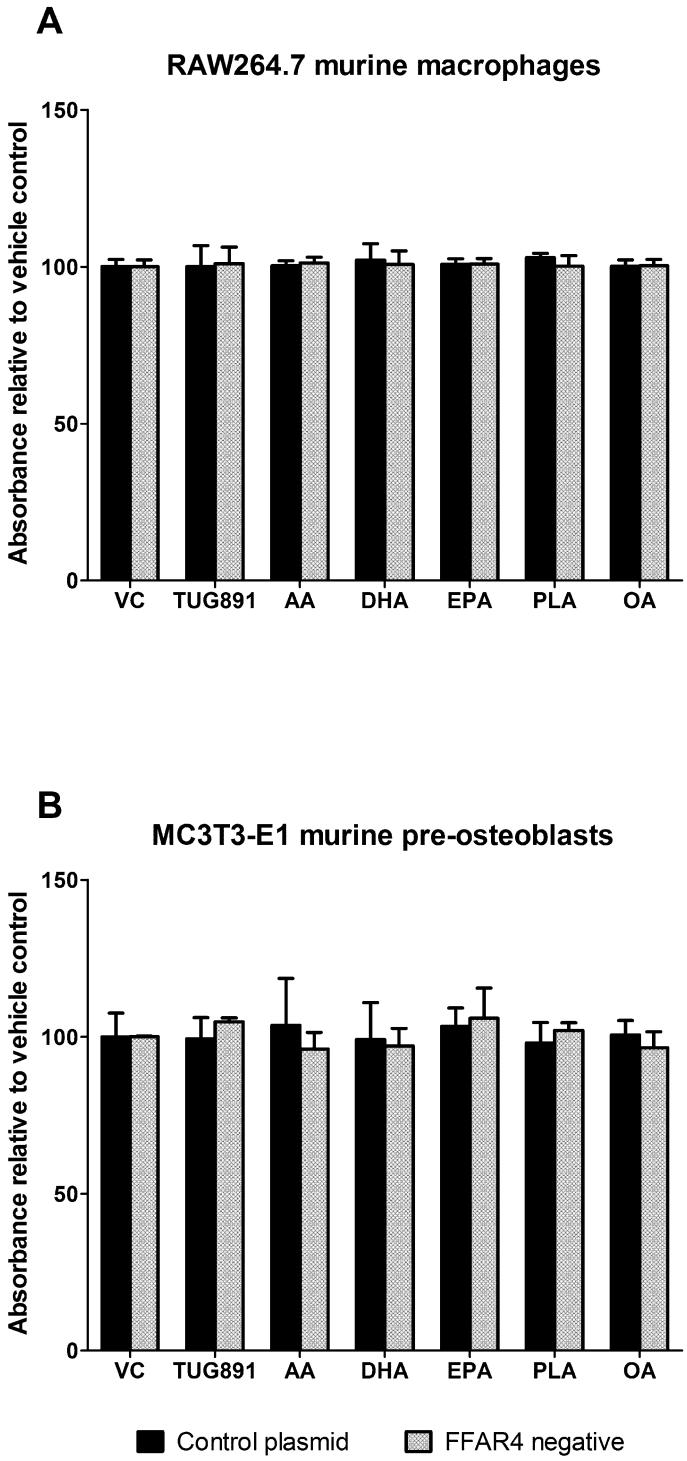

Figure S1: Resazurin assay. A resazurin assay was conducted to determine whether the TUG 891 or UFAs had cytotoxic effects on RAW264.7 murine macrophages or MC3T3-E1 murine pre-osteoblasts. Control plasmid and FFAR4 negative RAW264.7 macrophages (A) and MC3T3-E1 pre-osteoblasts (B) were exposed to AA (40 µM), DHA (40 µM), EPA (40 µM), PLA (100 µM), OA (100 µM) or TUG891 (100 µM) for 48 hrs. At the end of the culture period, cell viability was determined by adding 0.02% resazurin to each well and then incubating the plates at 37°C for 4 hrs. Absorbance was then read at 570 nm using 600 nm as a reference. Experiments were repeated 3 times in tripicate. Data was analysed using an ANOVA followed by a Bonferroni post hoc test.

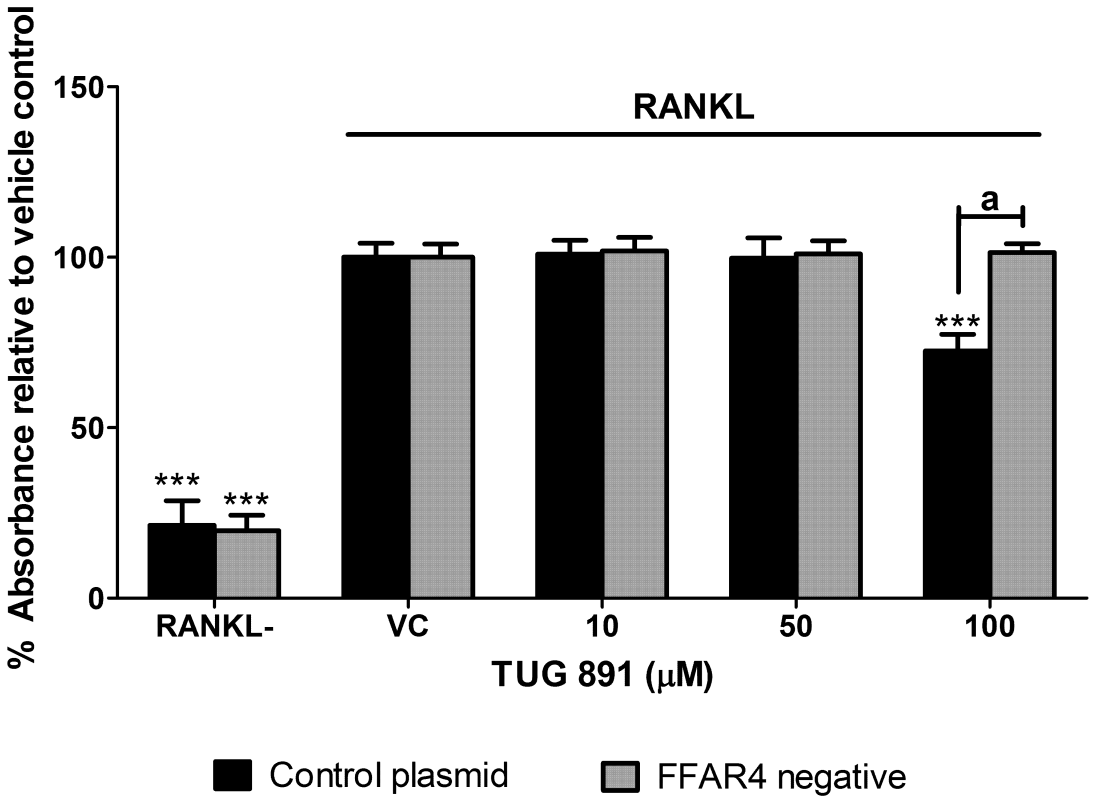

Figure S2: Effect of TUG891 on TRAP activity. Control plasmid and FFAR4 negative RAW264.7 murine macrophages were seeded into 96-well plates in the presence of RANKL and TUG891 for 5 days with medium changes on day 3. TRAP activity was determined from the conditioned media using pNPP as a substrate. TUG891 caused significant decrease in TRAP activity at 100 μM in control shRNA transfected cells. This effect was reversed in FFAR4 shRNA transfected cells demonstrating the sensitivity of TUG891 for FFAR4. VC: vehicle control. RANKL-: no RANKL added. ***$p<0.001$ vs vehicle control. a=$p<0.001$ vs control plasmid cells exposed to same concentration of TUG891. Experiments were repeated 3 times in tripicate. Data was analysed using an ANOVA followed by a Bonferroni post hoc test.