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Jonathan Park April 11, 2017

Hydroxyapatite suppression of osteoblast genes requires FGF receptor signaling and phosphate transporters.

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Abstract

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Hydroxyapatite (HAp) is the main mineral component of the skeleton and is naturally produced by osteoblasts during bone formation and bone remodeling. Due to its role in skeletal biology, the material is currently being investigated as a therapeutic biomaterial for orthopedic and dental use. Although HAp is a key product of the mineralizing osteoblasts, recent studies have suggested that extracellular HAp is not simply an inert biomaterial, but can suppress osteoblast lineage commitment through regulation of gene expression involved in early osteoblast differentiation. However, the cellular and molecular mechanisms by which HAp regulates osteoblast gene expression are not well delineated. In this study we investigated the mechanism(s) by which HAp suppresses gene expression in an *in vitro* osteoblast cell model. The study revealed that suppression of key genes, such as alkaline phosphatase, osterix and RUNX2, involved in osteoblast differentiation occurs within the first few hours after osteoblasts are exposed to extracellular HAp. HAp-effects are mediated through both the Fibroblast Growth Factor (FGF) receptor and the Sodium-dependent phosphate (P_i) transporters, but not the calcium-sensing receptor (CaSR). Interestingly, the key osteoblastic transcription factors RUNX2 and osterix—although suppressed by HAp—do not appear to regulate the early osteoblastic differentiation gene alkaline phosphatase. The study identifies previously unknown signaling pathways involved in osteoblast responses to extracellular HAp that down-regulate osteoblast gene expression. These data on the effects of HAp on osteoblast function may have importance in understanding HAp-feedback regulation of physiological mineralization, pathological calcification, and potential biomaterial development.

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Hydroxyapatite suppression of osteoblast genes requires FGF receptor signaling and phosphate transporters.

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INTRODUCTION

A healthy skeleton requires lifelong renewal and regeneration, a process known as bone remodeling. When the skeleton does not rejuvenate properly, bone-related diseases such as osteoporosis occur, which can lead to fracture. Bone remodeling replaces the entire skeleton approximately every 7-10 years. The remodeling process requires constant interaction and balanced activity between key cell types: bone forming osteoblasts, bone resorbing osteoclasts, and osteocytes that sense bone condition and promote local bone remodeling at sites of weak or damage bone.

Osteoblasts are mononucleated cells responsible for the formation of the extracellular bone matrix. Originating from the mesenchyme lineage, osteoblasts are derived from multipotent bone marrow stromal cells[1] and differentiate into mineralizing osteoblasts, and ultimately, mature osteocytes[2]. The osteoblast differentiation process is divided into three distinct stages that are defined by: 1) proliferation, 2) matrix maturation, and 3) mineralization[3, 4]. The differentiation process of osteoblasts requires several key transcription factors, including runt-related transcription factor 2 (RUNX2) and osterix (OSX). Mice without either of these transcription factors show a complete lack of osteoblasts, and mineralization of the skeleton does not occur in these mice[5]. As key transcription factors for osteoblast differentiation, RUNX2 and osterix gene expression levels also serve as useful early markers for progenitor commitment to the osteoblast lineage. The purpose of osteoblasts is to form a mineralized matrix in the form of collagen and hydroxyapatite (HAp), the main component of the skeleton.

Hydroxyapatite is a naturally synthesized mineral produced by osteoblasts in the skeleton and ameloblasts in tooth enamel. It is comprised of calcium and phosphate at a ratio of 1.67, has a chemical formula of $Ca_5(PO_4)_3OH$ or $Ca_{10}(PO_4)_6(OH)_2$, and is crystalline in form. HAp is

produced endogenously by osteoblasts during bone formation as well as during pathological calcification of cartilage and vasculature[6-14] where it is deposited in soft tissues in the form of dystrophic and metastatic calcifications[15, 16]. HAp is produced during mineralization of bone, a highly controlled process in differentiating osteoblasts[17]. Osteoblasts concentrate and sequester calcium and phosphate in the form of skeletal HAp on collagen matrices. The mechanism is thought to occur through the formation and secretion of matrix vesicles. Matrix vesicles are made up of lipid membrane vesicles, of about 50-300nm in size, with nano-sized HAp enclosed within the vesicles. The secreted matrix vesicles are then deposited onto the collagen matrix, and this is thought to further promote more substantial HAp growth[17, 18].

With the advent of nanochemistry, nano-sized CaP products such as HAp have begun to be synthesized chemically and investigated for their effects on the osteoblast lineage. Although results have varied—likely due to size, shape and method of synthesis—it has become clear that HAp not only is a structural element of bone but also regulates cell function. However, the cellular and molecular regulatory mechanisms of HAp on osteoblasts are still mostly unknown. Understanding the mechanisms by which HAp regulates osteoblast function has biomedical implication related to basic functional mineralization, as well as pathological calcification often associated with cardiovascular and kidney disease. Further, in light of the fact that HAp is currently being investigated as a potential biomaterial in orthopedic repair and for tissue engineering, results might lead to novel designs with the specific intent to target genes for therapeutic benefits[19, 20].

A key gene in the early stages of osteoblast differentiation is alkaline phosphatase (ALP). ALP (gene name:*Alpl*) is a metalloenzyme that catalyzes the hydrolysis of phosphomonoesters ($R\text{-}O\text{-}PO_3$). It is an ectozyme bound to the outer face of the plasma membrane through a

phosphatidyl inositol-glycophospholipid (GPI) anchor attached to the C-terminus of the enzyme. It is positioned so that its enzymatic subunit is extracellular. ALP is a critical gene for bone formation and physiological mineralization. Mutations and deletion of the gene results in bone defects in humans and mice[21-23] such as hypophosphatasia. Studies have suggested that ALP functions by cleaving inorganic pyrophosphate (PPi), a mineralization inhibitor, and by generating high local levels of inorganic phosphate through the cleavage of organic phosphate containing compounds[23, 24]. Inorganic pyrophosphate (PPi) blocks the mineralization process by impairing the growth of hydroxyapatite crystals, and cleavage by ALP releases this inhibition[24]. Although other mechanisms have been suggested for the function of ALP, its importance in osteoblast mineralization is undisputed, and its expression serves as a great early marker for osteoblasts.

Previous studies have indicated that exposure of osteoblast lineage cells to increasing concentrations of HAp altered the RNA levels of several genes. One of the most notable genes was ALP, which showed decreased total RNA levels (expression)[25]. However, the upstream mechanism(s) by which exposure to HAp regulates gene expression remained largely unknown.

In this study we investigated the mechanism(s) by which HAp down-regulates gene expression in the osteoblast lineage, with an emphasis on the early osteoblast marker ALP. A pre-osteoblast cell line (MC3T3-E1) model was used to test the effect of synthesized 10 x 100nm HAp on osteoblast signaling pathways leading to changes in gene expression, using RNA quantification, pharmacologic inhibition of signaling and membrane regulated events, and protein analyses of intracellular signaling proteins. A previous study had determined that HAp, at concentrations up to 100μg/mL, demonstrated no significant toxicity in multiple osteoblast lineage cell types, and 25μg/mL was the optimal dose for RNA quantification [25].

In the present study we showed a specific requirement of specific membrane proteins including the Fibroblast Growth Factor Receptors (FGFr) and sodium-dependent phosphate co-transporters (P_i-transporter; P_iT) for HAp activity on osteoblasts, whereas the Calcium-Sensing Receptors were not required. Pharmacologic inhibition of either receptor/transporter partially relieved the block of HAp-regulated ALP suppression whereas simultaneous inhibition of both membrane proteins fully relieved HAp-regulated ALP suppression. Our investigations into the specific genes/proteins involved further identified FGFr2 as the likely FGF receptor family member and either SLC20A1 or SLC20A2 as the relevant phosphate transporter/s. In addition to ALP, other genes were demonstrated to be suppressed by HAp including the key osteoblast transcription factors OSX and RUNX2. However, investigation of the temporal expression of protein levels and down-regulation of ALP suggested that changes in these transcription factors are not likely responsible for ALP suppression. Therefore, an alternative mechanism is likely responsible, potentially ALP methylation[25].

MATERIALS AND METHODS

Synthesis and characterization of nano-sized hydroxyapatite (HAp).

Pure phase hydroxyapatite (HAp: $Ca_{10}(PO_4)_6(OH)_2$) was previously synthesized using a sonochemistry based precipitation method and reported in [25]. HAp was acquired by filter press and lyophilization. To confirm that stoichiometric HAp was synthesized, HAp was previously characterized by powder X-ray diffractometry (XRD), Fourier Transfer Infrared spectroscopy (FT-IR), and TEM. TEM analysis identified the nano-sized HAp particles as rod-like in shape, and approximately 10 nm in width and 100 nm in length. Characterization of HAp by Zeta potential for surface charge and size revealed a nearly neutral particle and size of just greater than 1 μm, suggesting a degree of aggregation[25].

Cell Culture

MC3T3-E1 cells were originally chosen from eight MC3T3 cell lines, developed from C57BL/6 mouse calvaria[26]. MC3T3-E1 cells are undifferentiated, but committed pre-osteoblast cell lines, and have the capacity to differentiate into osteoblasts, produce collagen and deposit mineral in vitro[27]. A bone marrow stromal cell (BMSC) line with osteoblastic potential was developed from repeated sub-culturing of primary mouse BMSCs obtained from red marrow by centrifugation of the femur as described in [28]. These BMSCs are characterized as pre-osteoblast cells based on their ability to mineralize, induce expression to alkaline phosphatase and express osteoblastic marker genes in a time dependent factor when stimulated to differentiate. Cells were cultured in α-MEM at 37°C in 5% CO_2 and supplemented with 50U/mL penicillin, 50mg/mL streptomycin, and 2mM L-glutamine (Thermo) and 10% fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA) [25, 27, 29].

Pharmacologic inhibitors

SSR128129E (FGF receptor 1 inhibitor), BLU9931(FGF receptor 4 inhibitor) and PD173074 (pan-FGF receptor inhibitor) were purchased from SelleckChem (Houston, TX), sodium phosphonoformate (Foscarnet; P_i-transporter inhibitor) and NPS2143 hydrochloride (Calcium Sensing receptor inhibitor) from Sigma-Aldrich (St. Louis, MO).

Total RNA isolation, cDNA synthesis and qRT-PCR

Total RNA was extracted from culture cells using TRIzol reagent following the manufacturer's protocol (Thermo). After extraction, RNA concentration was measured by using a Nanodrop spectrophotometer (Thermo). Complementary DNA (cDNA) was synthesized using QuantiTech Reverse Transcription kit (Qiagen, Valencia, CA). qRT-PCR was performed using VeriQuest SYBR Green qPCR master mix (Affymetrix, Santa Clara, CA) on a StepOnePlus thermocycler (Applied Biosystems, NY). Primers were designed using qPrimer Depot software (http://mouseprimerdepot.nci.nih.gov/) and synthesized by Integrated DNA Technologies, Inc. (Coralville, IA) **(Table 1)**. Fold changes were calculated using the $2^{-\Delta\Delta}$CT method[30].

Western Blot Analysis

The HAp treated and untreated (control) cells were rinsed with PBS. Total cell lysate was obtained by lysing in p300 lysis buffer (250mM NaCl, 0.1% NP-40, 20mM sodium phosphate, 30mM sodium pyrophosphate, 5mM EDTA, and 10mM sodium fluoride (Sigma), adjusted at pH 7.0) supplemented with Halt Protease & Phosphatase single-Use Inhibitor Cocktail (Thermo). The total cell lysate (35µg) was separated by polyacrylamide gel electrophoresis (8-10%) and electro-transferred to PVDF membrane Hybond-P (GE Health Sciences, Piscataway, NJ). Initial

blocking was done in 1x Tris-buffered saline/Tween 20 (TBST; 20mM Tris, 150mM NaCl, pH 7.5) with 3% non-fat dry milk (NFDM). Membranes were probed with antibodies in 3% NFDM in 1x TBST. For phospho-specific antibodies, membranes were probed in 3% Bovine Serum Albumin (Sigma) in 1x TBST. The blots were visualized by chemiluminescence development using a Western Blotting Detection System (Thermo).

Immunoprecipitation

The HAp treated and untreated (control) cells were rinsed with PBS and total cell lysate was obtained for Western blotting. The lysate (500μg) was incubated with primary antibody and Protein A/G PLUS-Agarose (Santa-Cruz) overnight with rotation. The immunoprecipitates were spun at 2,000g and washed with IP lysis buffer, and analyzed by SDS-PAGE (8%) and Western blotting.

Antibodies

Phospho-FGFR1/2 (Tyr463, Tyr466) was purchased from Invitrogen (Carlsbad, CA), Phospho-Tyrosine (P-Tyr-100), Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204), p44/42 MAPK (Erk1/2) from Cell Signaling Technology (Danvers, MA), Osterix (F-3), Actin (I-19), Flg/FGFR1 (C-15), Bek/FGFR2 (C-17), FGFR-3 (C-15), FGFR4 (H-121) from Santa-Cruz (Dallas, TX), and RUNX2/CBFA1 from R&D systems (Minneapolis, MN).

Statistical Analysis

For multiple comparisons a one-way ANOVA was performed with Tukey's multiple comparison *post hoc* test on normally distributed data (normality determined by Shapiro-Wilk test) **(Fig. 1,2,**

4A). For simple comparisons a standard two-tailed unpaired Student's t-test analysis was performed on the qRT-PCR data of other experiments **(Fig. 3, 4B/C & 7)**. $P \leq 0.05$ was considered statistically significant.

RESULTS

HAp-induced changes in gene expression occur within hours. In order to define the approximate time point of the *Alpl* gene down-regulation in osteoblasts and, therefore, provide a general time frame to study the upstream mechanisms required for regulation, a time course was performed. MC3T3-E1 cells were treated with 25µg/mL of HAp in growth medium for 0 (control), 2, 4, 8, and 24 hours and harvested for RNA analysis. The treated and untreated control (0 hours) cells were harvested at the same time. Results showed a substantial and statistically significant down-regulation of *Alpl* gene expression by 4 hours, and a sustained down-regulation of *Alpl* gene expression thereafter for at least 24 hours (**Fig. 1**). We also analyzed the key osteoblast transcription factors Osterix (*sp7*) and RUNX2 (*cbfa1*). Quantification of these genes also showed a similar trend, with down-regulation of gene expression occurring between the 2 hour and 4 hour time point **(Fig. 1)**.

Fibroblast Growth Factor Receptor (FGFr) mediates the inhibitory effect of HAp on ALP gene expression. Previous studies on the role of inorganic phosphate in regulating osteoblast gene expression had suggested the requirement of Fibroblast Growth Factor receptor (FGFr) [28]. In order to test whether HAp requires FGFr signaling, MC3T3-E1 were pre-treated with pharmacologic FGFr inhibitor (iFGFr; PD 173074) at a concentration of 10nM, 30nM, 100nM, and 300nM for 30 minutes prior to addition of HAp for 24 hours (25µg/mL). Quantification of *Alpl* expression showed that the inhibition of the FGF receptors relieved, in part, the HAp-induced suppression of *Alpl* expression **(Fig. 2A)**. With increasing concentrations of the FGFr inhibitor, there was a progressive increase in restoration of gene expression demonstrating a dose-response **(Fig. 2A)**.

Sodium-dependent phosphate transporter is required for HAp regulation of ALP gene expression. The main components of HAp are calcium and phosphate. Phosphate is recognized by cells through a family of sodium-dependent phosphate transporters [31, 32]. To determine if phosphate transport is necessary for HAp-stimulated signaling, cells were pretreated with a pharmacologic inhibitor phosphonoformic acid (iP_iT; Foscarnet) at a concentration of 300μM and 1mM 30 minutes prior to HAp treatment of cells (25μg/mL). Quantification of *Alpl* expression showed that pan-inhibition of phosphate transporters resulted in a partial blockage of the HAp-stimulated response **(Fig. 2B)**.

Calcium-sensing receptor is not required for HAp-stimulated gene regulation. Calcium is recognized by cells through a calcium-sensing receptor (CaSR) [33]. To determine if CaSR is necessary for HAp-stimulated signaling, cells were pretreated with a pharmacologic CaSR inhibitor (iCaSR) NPS2143 at a concentration 100nM for 30 minutes prior to 24 hours of HAp treatment (25μg/mL). Quantification of *Alpl* gene expression showed that inhibition of the CaSR had no noticeable effect on HAp-induced suppression **(Fig. 2C)**.

Dual inhibition of FGFr and Pi-transporters fully block HAp-regulated ALP suppression. MC3T3-E1 cells were pre-treated with iFGFr (PD173074), iP_iT (Foscarnet), or both iFGFr and iP_iT for 30 minutes prior to a 24-hour treatment with HAp (25μg/mL). Results showed that iFGFr and iP_iT individually only provided a partial block of the HAp signaling response while iFGFr and iP_iT produced complete inhibition of ALP suppression **(Fig. 3A)**. Quantification of osterix and RUNX2 gene expression showed a similar trend **(Fig. 3B & 3C).**

FGFr2 expression is down-regulated in response to HAp stimulation. The FGFr family consists of four receptors (FGFr1, 2, 3, 4)[34] and PD173074 is considered a pan-inhibitor. In order to identify the specific FGF receptor mediating HAp signaling, two different inhibitors—FGFr1 (SSR128129E) inhibitor and FGFr4 inhibitor (BLU9931)—were used. MC3T3-E1 cells were pretreated for 30 minutes with FGFr1 inhibitor (iFGFr1) at a concentration of 0.3μM, 1μM and 3μM, and the FGFr4 inhibitor (iFGFr4) at a concentration of 100nM followed by 24 hours of HAp treatment (25μg/mL). Quantification of *Alpl* gene expression showed that inhibition of FGFr1 and FGFr4 did not change levels of gene expression **(Fig. 4A and 4B)**. Specific inhibitors for FGFr2 and FGFr3 are not available, and therefore we sought to quantify expression of the FGFr family for clues. Cells were treated with HAp (25μg/mL) for 24 hours and mRNA samples of untreated control, and HAp-treated cells were quantified for the gene expression levels of each family of FGF receptors (FGFr1-4). Results identified FGFr2 as the only family member of the four FGF receptor proteins to be regulated at the level of gene expression **(Fig. 4C)**.

The signaling kinases ERK1/2 are activated within 15 minutes of HAp treatment. Activation of growth factor receptors, including the FGF receptor family, results in activation of downstream signaling pathways often involving one or more of the three main mitogen activated protein kinases, ERK1/2 (Extracellular signal-regulated kinase), p38, and JNK (Jun-terminal kinase) [35]. Previous studies have identified ERK1/2 as a key signaling protein in the osteoblast response to elevated phosphate[36]. To determine if ERK1/2 is activated by HAp and if this could provide a time frame to assess FGFr activation, a time course experiment was performed. MC3T3-E1 cells were treated with HAp for minutes to hours, and cell lysates were analyzed by Western blotting. Results identified a stimulation of ERK1/2 phosphorylation (indicative of

activation) within 15 minutes of HAp treatment **(Fig.5)**. Stimulation of ERK1/2 is known to be a transient event, and the results suggest, in fact, that the signal is rapidly decreased. These results identify ERK1/2 as a signaling pathway activated by HAp within 15 minutes.

FGFr2 is phosphorylated in response to HAp stimulation. Activation of FGF receptors results in autophosphorylation. Phosphorylation is a common indication of protein activity in cells. In order to identify whether FGFr2 was phosphorylated, and thus, activated in response to HAp, bone marrow stromal cells (BMSC) were treated with HAp at a concentration of 25μg/mL for 15 minutes. Untreated control and treated cells were harvested at the same time. Each FGF receptor protein (FGFr1, 2, 3, 4) was analyzed separately via immunoprecipitation followed by Western blotting. An antibody to phospho-FGFr1/2 suggested an increase in phospho-FGFr2 (predicted size 92kDa) in response to HAp with little change in FGFr1 **(Fig.6A)**. The autophosphorylation of FGF receptors occurs on tyrosine residues. Because specific phospho-antibodies to FGFr3 and FGFr4 are not commercially available, we also probed with a pan phospho-tyrosine antibody. Interestingly, in addition to the activation of FGFr2, we also detected a strong decrease in phospho-bands in the FGFr4 immunoprecipitation (predicted size of FGFr4 is 88kDa) **(Fig 6B)**.

SLC20A1 & SLC20A2 are up-regulated in response to HAp stimulation. In order to attempt to identify the specific inorganic phosphate transporter involved in the HAp-stimulated signaling pathway, MC3T3-E1 cells were treated with HAp for 24 hours. Untreated and treated samples were quantified for gene expression levels of SLC20A1, SLC20A2, SLC34A1, SLC34A2 and SLC34A3. Results showed that SLC20A1 and SLC20A2 are up-regulated in response to HAp stimulation, while SLC34A1-3 does not show any substantial change in its gene expression

levels are were only minimally detected **(Fig. 7)**. Although a change in RNA levels do not necessitate functional requirement, it does suggest involvement in the response, and the inability to detect SLC34A strongly suggests that SLC34A1-3 are not involved.

RUNX2 and osterix protein levels do not decrease within the first hour of HAp stimulation. Osterix and RUNX2 are key transcription factors that regulate many osteoblast related genes. To determine whether RUNX2 and osterix protein levels decreased within the first hour of HAp stimulation, MC3T3-E1 cells were treated with 25μg/mL of HAp for 0 hours (untreated), 15 minutes, 30 minutes, 45 minutes, 1 hours, 2 hours, 4 hours, 8 hours and 24 hours. Cells were harvested at the same time, and Western blotting was performed. Results showed that protein levels of RUNX2 increased within the first hour, then decreased back to baseline after the first hour. Although faintly detected, osterix similarly did not show a decrease in protein expression within the first hours of treatment. However, osterix was minimally detected, and a nuclear fractionation would provide a more conclusive result. Actin is shown as a loading control **(Fig. 8)**.

DISCUSSION

Physiological importance of Hydroxyapatite (HAp).

HAp was utilized in the current study to investigate the relevance of matrix mineralization to physiologically important osteoblast signaling pathways. Hydroxyapatite (HAp) is naturally produced by osteoblasts of the skeleton and ameloblasts of dentition, and the HAp synthesized and used in this study closely approximates natural nano-sized HAp that mineralizing cells would be exposed to under normal physiological conditions. Nano-sized HAp is generated endogenously by osteoblast and secreted in the form of matrix vesicles, and osteoblast precursors near the site of mineralization are therefore exposed to nano-sized HAp[17, 18, 37]. Dental enamel is also comprised of crystalline HAp, which is constructed of bundled parallel crystallites of about 26nm x 68nm. Pathological calcification, mainly vascular calcification, has also shown the involvement of nano-sized HAp[6, 7]. Results herein suggest that the generation of nano-sized HAp—either during physiological mineralization or possible pathological calcification—has the potential to strongly influence cell behavior through specific changes in cell signaling and gene expression.

Sodium-dependent Phosphate Co-Transporters; SLC20A1 & SLC20A2.

The upstream mechanism of HAp stimulation was investigated, and we identified that one or more inorganic phosphate transporters is a required membrane protein for the signal transduction events causing changes in gene expression. Phosphate transporters are classified into three families or types [32]. The type 1 transporters, SLC17A1-7, are thought to be not phosphate specific and may serve as general anion channels. [38] The type 2 transporters, SLC34A1-3, are thought to be mainly responsible for absorption in the intestine and resorption in the kidney [39].

The type 3 transporters, SLC20A1&2, are expressed more ubiquitously [40]. Previous studies have suggested that type 3 transporters may be involved in physiological mineralization [41], enamel formation[42] and pathological calcification[43-45]. In addition, SLC20A1 has been suggested to activate the NF-κB and MAPK signaling pathways[46], and may serve as an originating point of signal transduction. Finally, a recent study has identified the requirement of SLC20A1 for the promotion of osteogenic differentiation[47]. Results from this study revealed that SLC20A1 and SLC20A2 are both up-regulated in response to HAp, while SLC34A1-3 are not **(Fig. 7)**, and suggested that type 3 transporters (SLC20A1 and SLC20A2) may be the inorganic phosphate transporter required for the HAp-stimulated signaling cascade. Future studies to determine the specific transporter using siRNA knockdown are underway.

Fibroblast Growth Factor Receptor (FGFr) signaling and FGFr2 requirement.

Previous studies have indicated that elevated levels of inorganic phosphate require FGF receptor signaling to induce changes in gene expression[28]. Similarly, we determined that HAp stimulation requires FGF receptor signaling to induce changes in ALP gene expression **(Fig. 2)**. The FGF receptor is a family of membrane bound tyrosine kinase receptors that recognize fibroblast growth factors (FGF). The FGF receptors are mainly classified into four different family members (FGFr1, FGFr2, FGFr3 & FGFr4) with a number of splice variants[34]. Pharmacologic inhibition of FGFr1 and FGFr4 suggested that of these receptors, FGFr2 and FGFr3 were the only FGF receptors with changed gene expression levels **(Fig. 4C**). However, FGFr3 only presented a 1.3 fold change of mRNA and despite a statistical significance level of *$p<0.05$, due to the inherent variability and high coefficient of variation of the qRT-PCR methodology, the FGFr3 results may be physiologically irrelevant and likely do not represent a

real biological event leading us to thus focus on FGFr2. Further, immunopreciptation and Western blotting showed an increase in phosphorylation levels of the FGFr2 protein in the HAp-treated sample, suggesting its requirement for HAp-stimulated signaling response after 15 minutes of HAp exposure. The activation of growth factor receptors is often followed by a feedback loop that shuts down this signaling pathway, which may explain the decrease in gene expression at 24 hours. However, additional studies are necessary to prove this hypothesis. Interestingly, a number of phospho-tyrosine positive bands were detected in the FGFr4 IP lane. One band correlates with the predicted 88kDa size of FGFr4, and the other prominent band was approximately 150kDa. This size is almost identical to the size of SOS and intracellular adapter protein known to bind to the intracellular side of FGF receptors in a complex with other kinases such as FRS2 and GRB2. Additional studies using FGFr4 and SOS specific antibodies should be able to determine if this complex is negatively regulated by HAp.

Both the FGF receptor and Phosphate transporter are required for HAp down-regulation of gene expression.

Interestingly, inhibition of either the FGF receptor or phosphate transporter was not sufficient to completely inhibit the HAp response **(Fig. 2 & 3)**. However, simultaneous inhibition of both proteins provides full blockage of the HAp-stimulated gene suppression **(Fig. 3)**. This generates a number of interesting hypotheses including the possibility that a single, common downstream protein requires activation by both membrane proteins or that two downstream pathways are involved and converge at the gene promoter to regulate expression (**Fig. 9A,B**). These ideas could be tested using siRNA to knockdown one or the other membrane proteins, in combination with immunoprecipitations of the membrane proteins to identify downstream signaling

complexes. In regards to FGFr, many, if not most, of the signaling proteins are known (such as FRS2, Grb2, SOS, etc.), whereas little is known about P_i-transporter initiated signaling. In this case, the immunoprecipitate could be analyzed by separation on a poly-acrylamide gel followed by silver staining to identify interacting proteins. These proteins could ultimately be identified by mass spectrometry. The mechanism(s) by which HAp works through these membrane proteins to regulate intracellular signaling is not known. As the FGF receptors are known to be activated by ligand binding, it would seem unlikely that there is a physical interaction with HAp. Certain FGFs are known to be membrane tethered through proteoglycans, and it is interesting to speculate that HAp might activate an enzyme at the cell surface releasing an FGF and thereby activating the FGF receptor **(Fig. 9)**. Much less is known about the P_iTs and how they might influence intracellular signaling. The fact that P_iTs do transport phosphate may suggest a form of direct physical interaction, however, this has yet to be confirmed.

RUNX2 and osterix do not likely regulate alkaline phosphatase gene expression in HAp-stimulated response.

The two main hypotheses for the downstream mechanism by which HAp regulates gene expression at the transcriptional level are (1) the increased activity of a transcriptional repressor, such as the family of DNA methyltransferases that shuts down gene expression or (2) the activation of the signaling pathways leads to the down-regulation of transcriptional activators (**Fig. 9 C, D**). The two most likely candidates to fulfill hypothesis (2) would be RUNX2 and/or Osterix. This study examined whether the decrease in RUNX2 and/or osterix RNA levels resulted in decreased protein levels and the subsequent decrease in the ALP gene expression. Results suggested that this was not the case, as RUNX2 and osterix protein levels actually

increased slightly **(Fig. 8)** in response to HAp stimulation within the first hour. The results therefore favor the hypothesis that HAp results in the activation of a transcriptional repressor **(Fig. 9A,B)**.

CONCLUSION

The studies presented here examine the mechanism of HAp-induced signaling pathways required to suppress early osteoblastic differentiation. Our results provide insight into the upstream mechanism of the signal transduction pathway, identifying FGF receptor and phosphate-transporter as required. Furthermore, the studies suggested the involvement of FGF receptor 2 and SLC20A1 and/or SLC20A2 as the specific family members required, whereas the calcium-sensing receptor was not required in the response. The study also revealed that key osteoblastic transcription factors RUNX2 and osterix do not likely regulate the HAp response but are nonetheless regulated by HAp. Our study adds to the understanding of the biological mechanism(s) and physiological response generated during physiological mineralization as well as the need for caution in using HAp as a biomaterial in tissue engineering and as an orthopedic therapeutic.

FIGURE LEGENDS

Figure 1: Time course of HAp-regulated gene expression. Pre-osteoblasts (MC3T3-E1) were cultured in growth medium. HAp was added to cells at a concentration of 25µg/mL for 0 (untreated control), 2, 4, 8 and 24 hours in reverse sequence so that all samples were harvested at the same time. RNA was isolated and gene expression quantified by qRT-PCR; **(A)** alkaline phosphatase (ALP), **(B)** osterix (OSX) and **(C)** RUNX2. The results were normalized to 18S and are expressed as fold changes. **p<0.01, ***p<0.001 and ****p<0.0001 compared to untreated control (one-way ANOVA) with Tukey's multiple comparison.

Figure 2: Inhibition of FGF receptor and Sodium-dependent Phosphate Co-Transporter provides partial blockage of HAp-induced changes in alkaline phosphatase expression. Pre-osteoblasts (MC3T3-E1) were cultured in growth medium. Cells were pretreated with inhibitors (PD173074 as FGFr inhibitor; Foscarnet as Pi-transport inhibitor; NPS2143 as CaSR inhibitor) as indicated for 30 minutes prior to HAp treatment (25µg/mL) for 24 hours. **(A)** displays iFGFr at different doses (10nM, 30nM, 100nM and 300nM) with untreated and HAp treated controls; **(B)** displays iP_iT at different doses (0.3mM and 1mM) with untreated and HAp-treated controls; **(C)** displays iCaSR at different doses (100nM and 300nM) with untreated and HAp-treated controls. RNA levels were quantified by qRT-PCR for *Alpl* expression. The results were normalized to 18S and are expressed as fold changes. ****p<0.0001 compared to untreated control; ns = not statistically significant, $^{\#}$p<0.05 and $^{\#\#}$p<0.01 compared to the HAp-treated (one-way ANOVA) with Tukey's multiple comparison.

Figure 3: Simultaneous inhibition of FGF receptor and Sodium-dependent Phosphate Co-Transporter provides full blockage of HAp-induced changes in down-regulated gene expression. Pre-osteoblasts (MC3T3-E1) were cultured in growth medium. Cells were pretreated with inhibitors (FGFr inhibitor (iFGFr); Foscarnet as Pi-transport inhibitor (PiT)) as indicated for 30 minutes prior to HAp treatment (25µg/mL) for 24 hours. RNA levels were quantified by qRT-PCR for **(A)** ALP, **(B)** OSX and **(C)** RUNX2 gene expression. The results were normalized to 18S and are expressed as fold changes. **p<0.01, ****p<0.0001 compared to untreated control; $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001, $^{\#\#\#\#}$p<0.0001 compared to the HAp-treated (Student's t-test).

Figure 4: HAp stimulates the down-regulation of FGF receptor 2 gene expression. **(A)** Pre-osteoblasts (MC3T3-E1) were cultured in growth medium. Cells were pretreated with a FGFr 1 inhibitor (SSR128129E) 30 minutes prior to treatment with HAp at a concentration of 25µg/mL for 24 hours. RNA samples were quantified by qRT-PCR for *Alpl* expression. The results were normalized to 18s and are expressed as fold changes. ****p<0.0001 compared to untreated control; ns = not statistically significant compared to HAp-treated (one-way ANOVA) with Tukey's multiple comparison. **(B)** Pre-osteoblasts (MC3T3-E1) were cultured in growth medium. Cells were pre-treated with a ubiquitous FGF receptor inhibitor (PD173084; iFGFr) or an FGFr 4 inhibitor (BLU9931; 100nM) 30 minutes prior to treatment with HAp at a concentration of 25µg/mL for 24 hours. RNA samples were quantified by qRT-PCR for *Alpl* expression. The results were normalized to 18s and are expressed as fold changes. ****p<0.0001 compared to untreated control; ns = not statistically significant and $^{\#\#\#}$p<0.001 compared to HAp-treated (Student's t-test). **(C)** Pre-osteoblasts (MC3T3-E1) were cultured in growth medium. Cells were treated with HAp at a concentration of 25µg/mL for 24 hours. RNA samples

were quantified by qRT-PCR for FGFr1, FGFr2, FGFr3 and FGFr4 gene expression. The results were normalized to 18s and are expressed as fold changes. *$p<0.05$ and ***$p<0.001$ compared to respective untreated control (Student's t-test).

Figure 5: HAp rapidly stimulates the signaling proteins ERK1/2. MC3T3-E1 cells were treated with HAp (25µg/mL) for indicated times and samples analyzed by Western blotting. **(A)** phospho-ERK1/2 and **(B)** total ERK1/2.

Figure 6: HAp stimulates the phosphorylation of FGF receptor 2 proteins. Bone marrow stromal cells (BMSC) were cultured in growth medium. Cells were treated with HAp at a concentration of 25µg/mL for 15 minutes. Immunoprecipitation was performed for FGFr1, FGFr2, FGFr3 and FGFr4. Western blotting was performed for **(A)** phospho-FGFr1/2, **(B)** Total FGFr2 and **(C)** phospho-Tyrosine.

Figure 7: HAp stimulates the up-regulation of Sodium-dependent Phosphate Co-Transporter family SLC20A1 & SLC20A2. Pre-osteoblasts (MC3T3-E1) were cultured in growth medium. Cells were treated with HAp at a concentration of 25µg/mL for 24 hours. RNA samples were quantified by qRT-PCR for SLC34A1/2/3 & SLC20A1/2 expression. The results were normalized to 18s and are expressed as fold changes (comparative to control of SLC20A1). ***$p<0.001$ compared to respective untreated control (Student's t-test).

Figure 8: Western blot of osteoblast transcription factors. Pre-osteoblasts (E1) were cultured in growth medium. Cells were treated with HAp at concentration of 25µg/mL for 0 hours

(untreated), 15 minutes, 30 minutes, 45 minutes, 1 hour, 2 hours, 4 hours, 8 hours and 24 hours in reverse sequence so that all samples were harvested at the same time. Western blotting was performed for **(A)** RUNX2, **(B)** osterix (OSX) and **(C)** Actin for loading control.

Figure 9: Schematic of potential mechanism by which HAp regulates gene expression.

A schematic of potential downstream mechanisms by which HAp regulates gene expression has been depicted. Although the mechanisms remain unclear, a working model is that HAp could directly interact with the phosphate transporter (Pi-T) while activation of the FGF receptor (FGFr) could occur through HAp activation of a membrane bound enzyme, which releases proteoglycan tethered FGFs. Following membrane protein activation, signal transduction and gene regulation could occur through the following mechanisms: **(A)** Activation of the two membrane proteins identified herein as an FGFr and P_iT family member initiate two distinct downstream pathways (represented by 1 and 2) that converge at the gene promoter (ALP, Runx2, Osx, etc.) to activate a transcriptional repressor and **(B)** Activation of the membrane proteins converge on a single, common downstream protein target (1), which then activates a transcriptional repressor. **(C)** The activation of the two membrane proteins activates two distinct downstream pathways (1 and 2) that converge at the gene promoter to stimulate a transcriptional activator and **(D)** a single, common downstream protein target (1) stimulates a transcriptional activator.

Table 1: Primer

Common Name	Forward (5'-3')	Reverse (5'-3')
18S	TTGACGGAAGGGCACCACCAG	GCACCACCACCCACGGAATCG
ALP	ACAGACCCTCCCCACGAGT	TGTACCCTGAGATTCGTCCC
FGFr1	TCACAGCCACTCTCTGCACT	GTGGACCAGGAGAGACTCCA
FGFr2	ACCACACCTACCACCTCGAT	GACAAACTCCACATCCCCTC
FGFr3	TCGTGGCTGGAGCTACTTC	CTCCTGCTGGCTAGGTTCAG
FGFr4	CAGAGGCCTTTGGTATGGAT	CAGGTCTGCCAAATCCTTGT
OSX	CTCTCCATCTGCCTGACTC	CCAAATTTGCTGCAGGCT
RUNX2	TATGGCGTCAAACAGCCTCT	GCTCACGTCGCTCATCTTG
SLC20A1	ATTCTTCCTTGTTCGTGCGT	TGGAAAAGAGGTTGATTCCG
SLC20A2	GACCTCGCCTTCGTCACTT	CCATAGTTTTTCTCCCAGGC
SLC34A1	GGGATGAGTCCCTGAGGAAT	ATGGCCTCTACCCTGGACAT
SLC34A2	CTCCTGCTGTCCCTTACCTG	CGATGAATTTACCGGGGTT
SLC34A3	CAGCCCTGCAGACATGTTAAT	AGACAGGCACCAGGTACCAC

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Figure 1

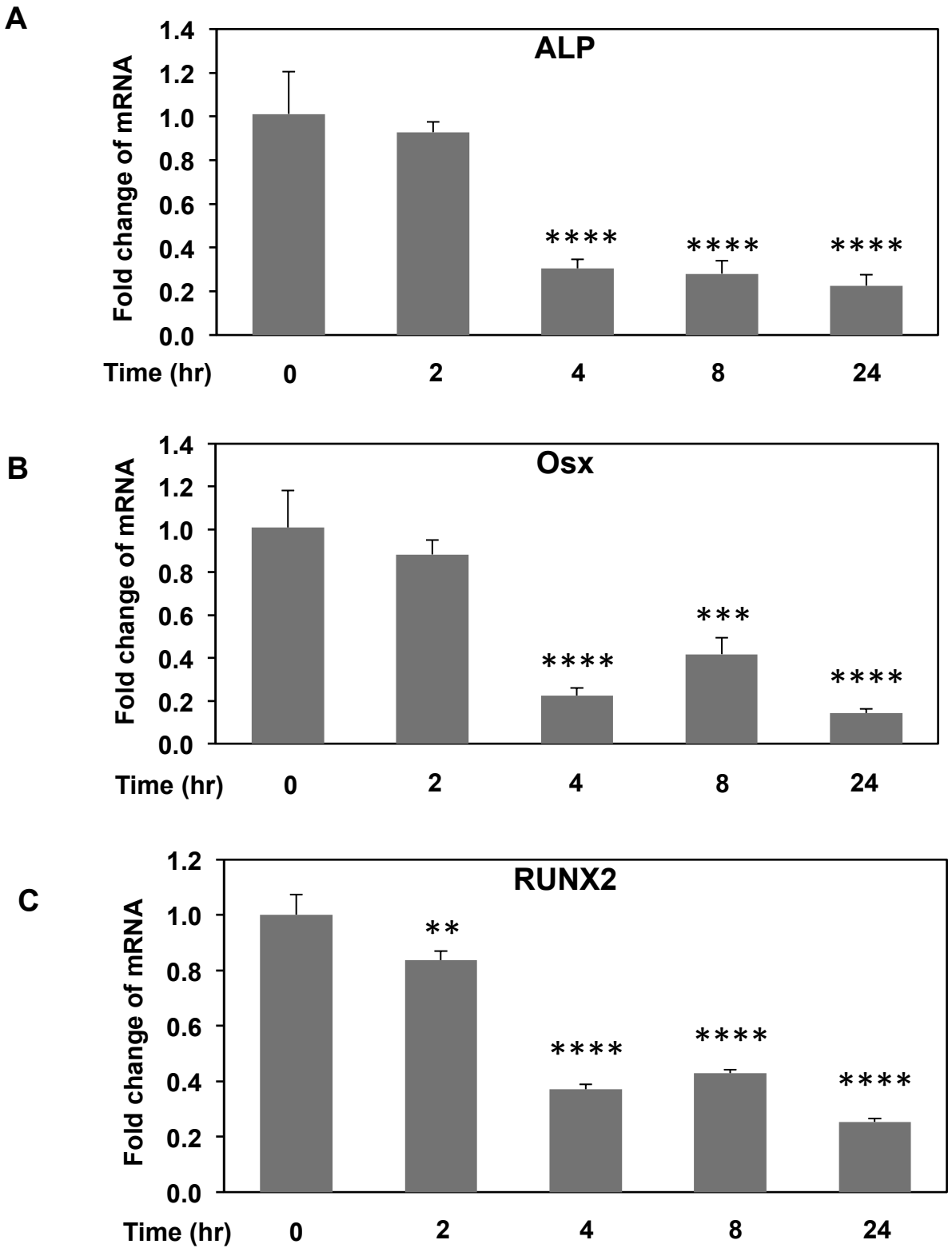
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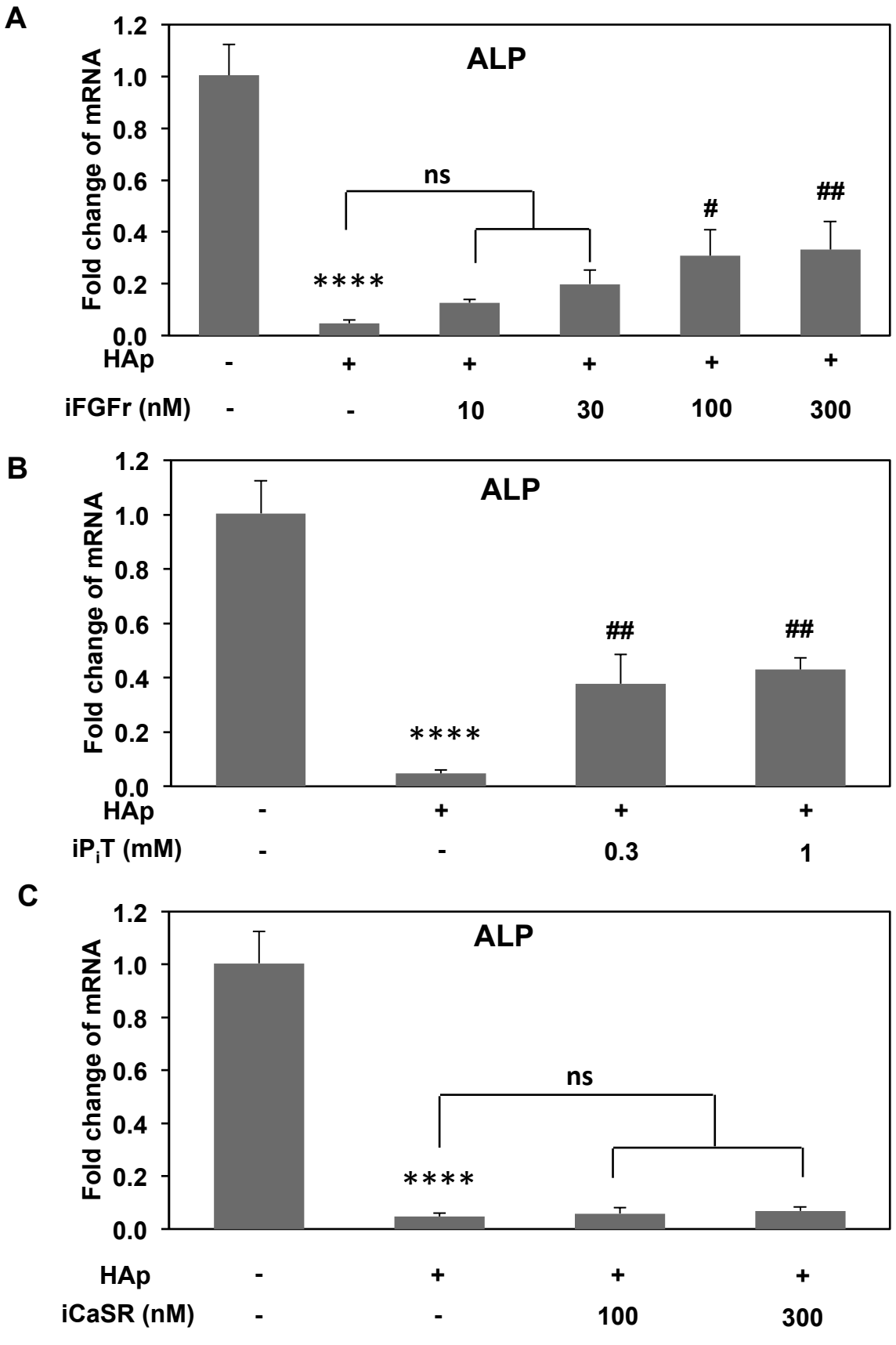
Figure 2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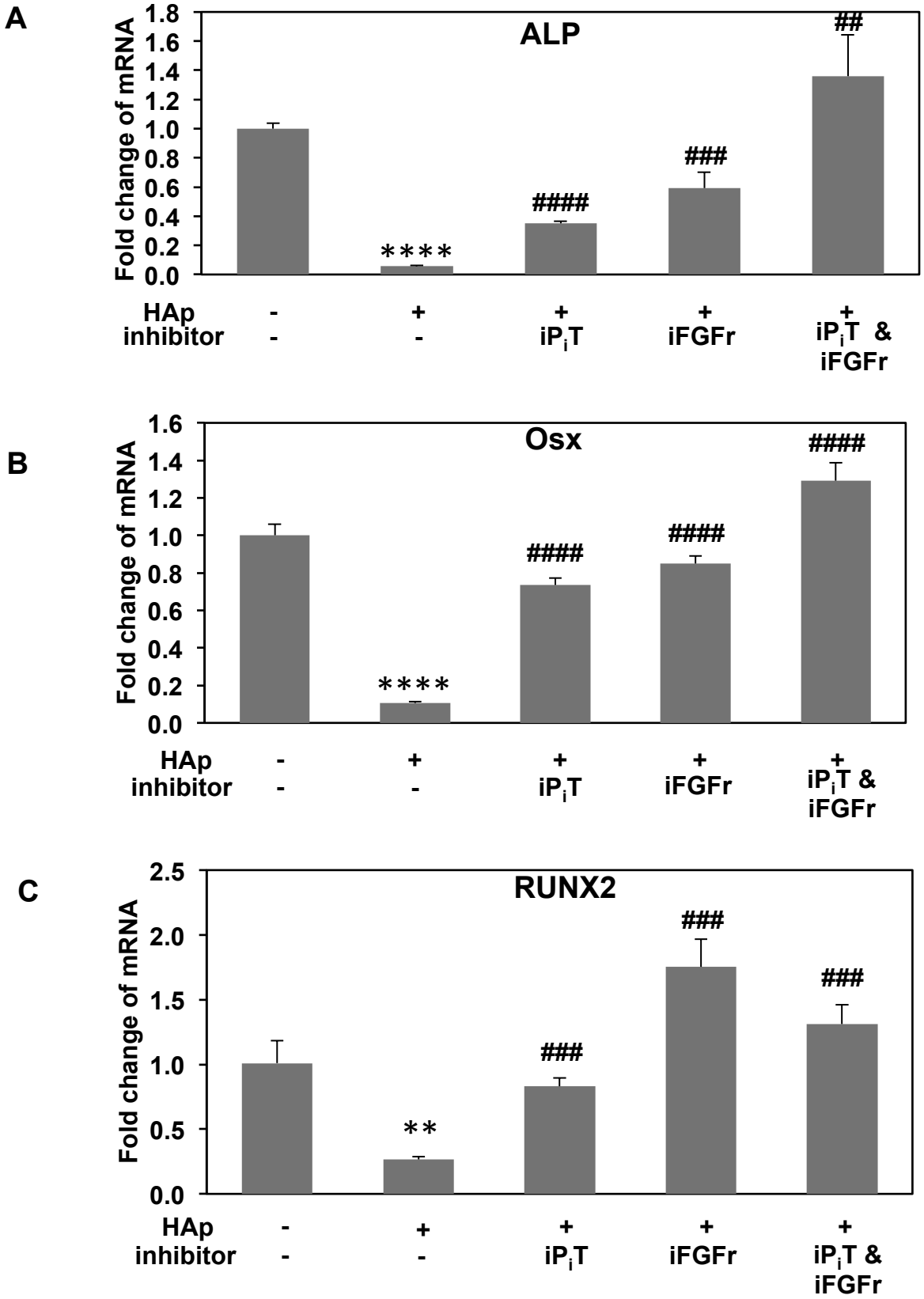
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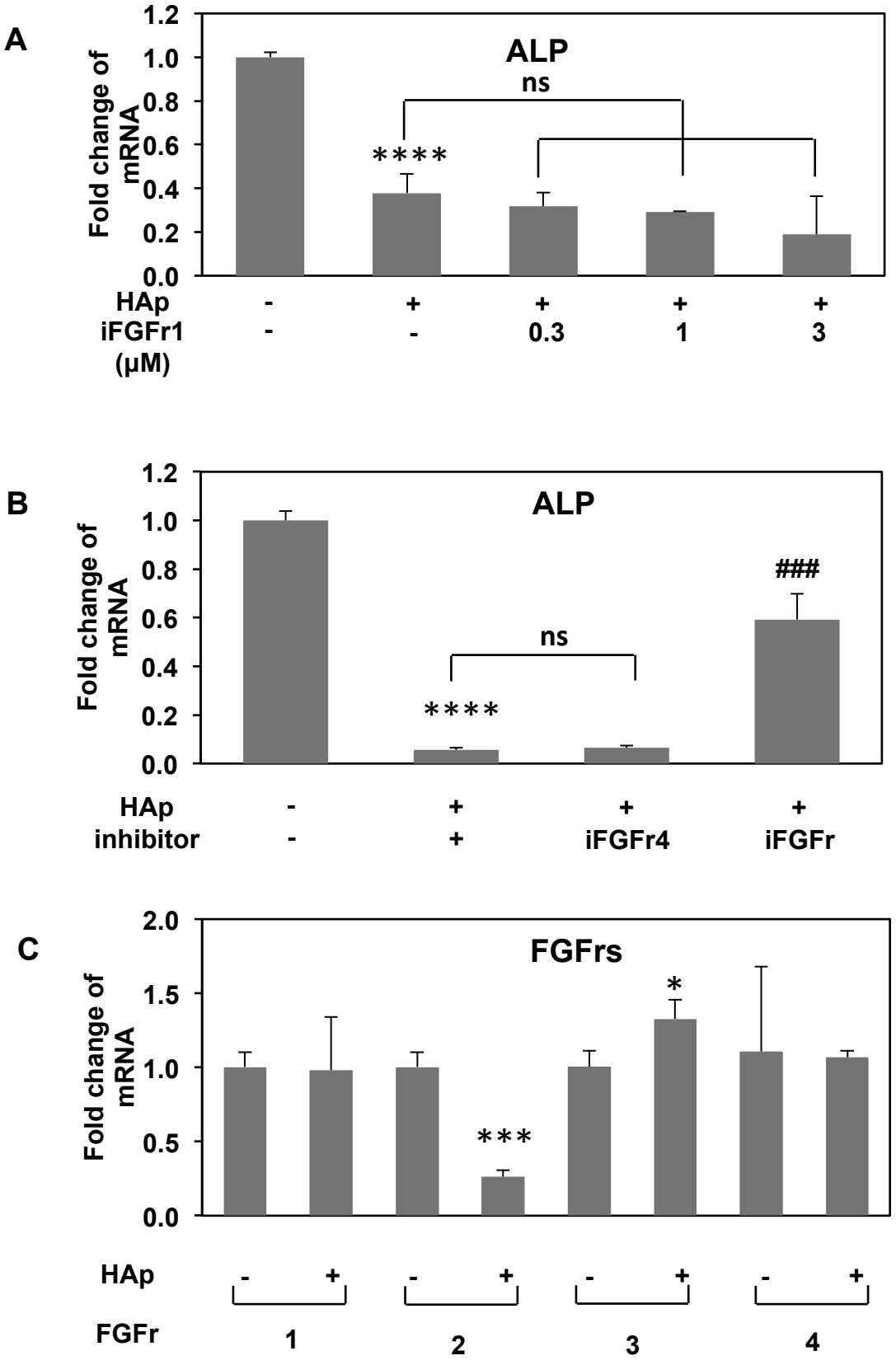
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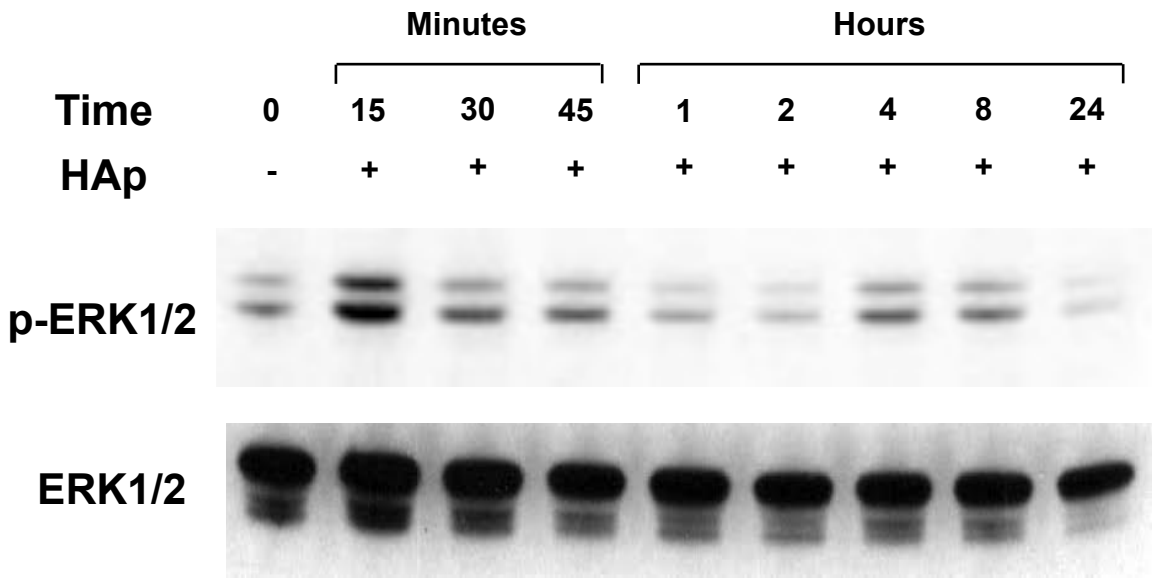

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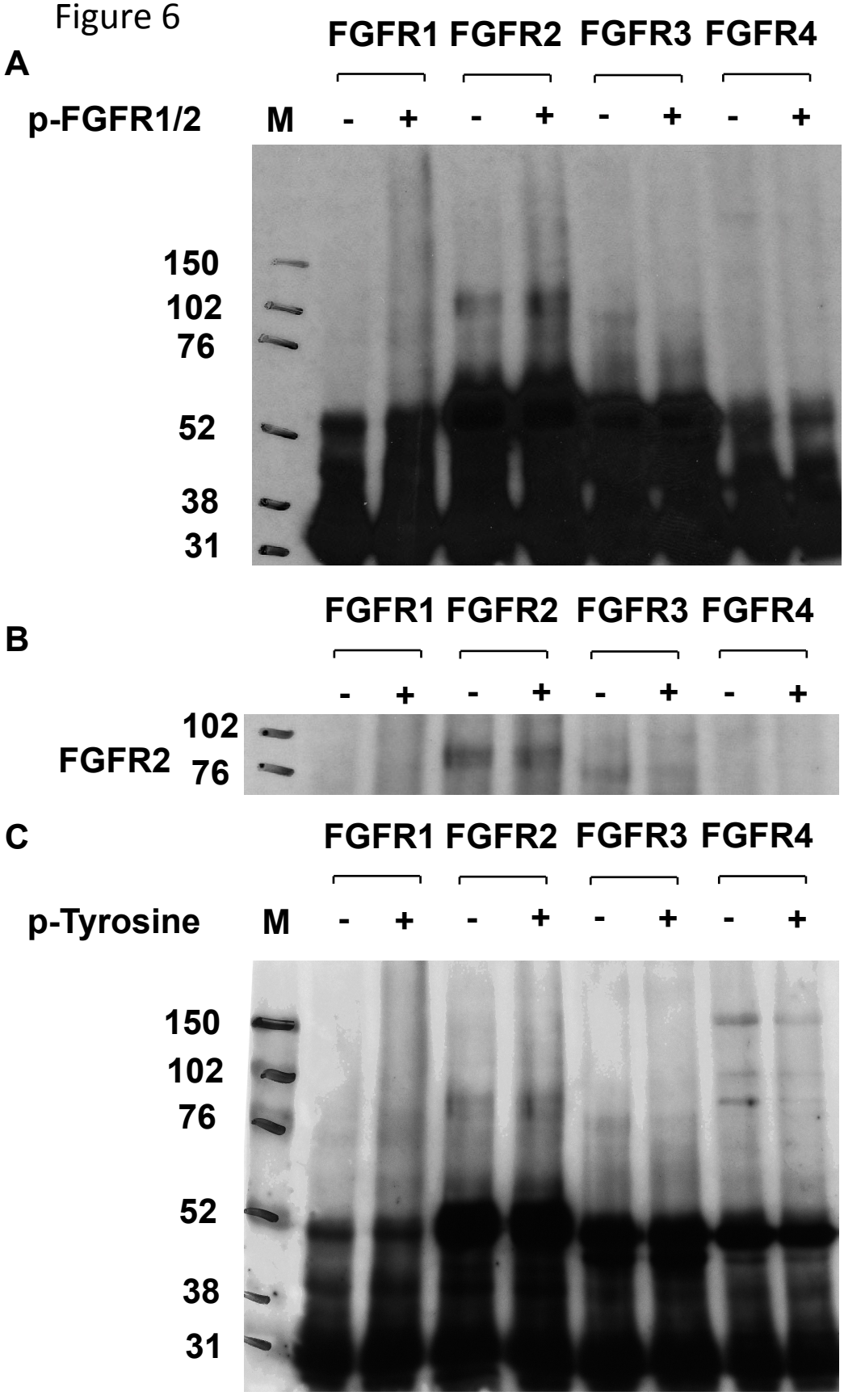
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Figure 7

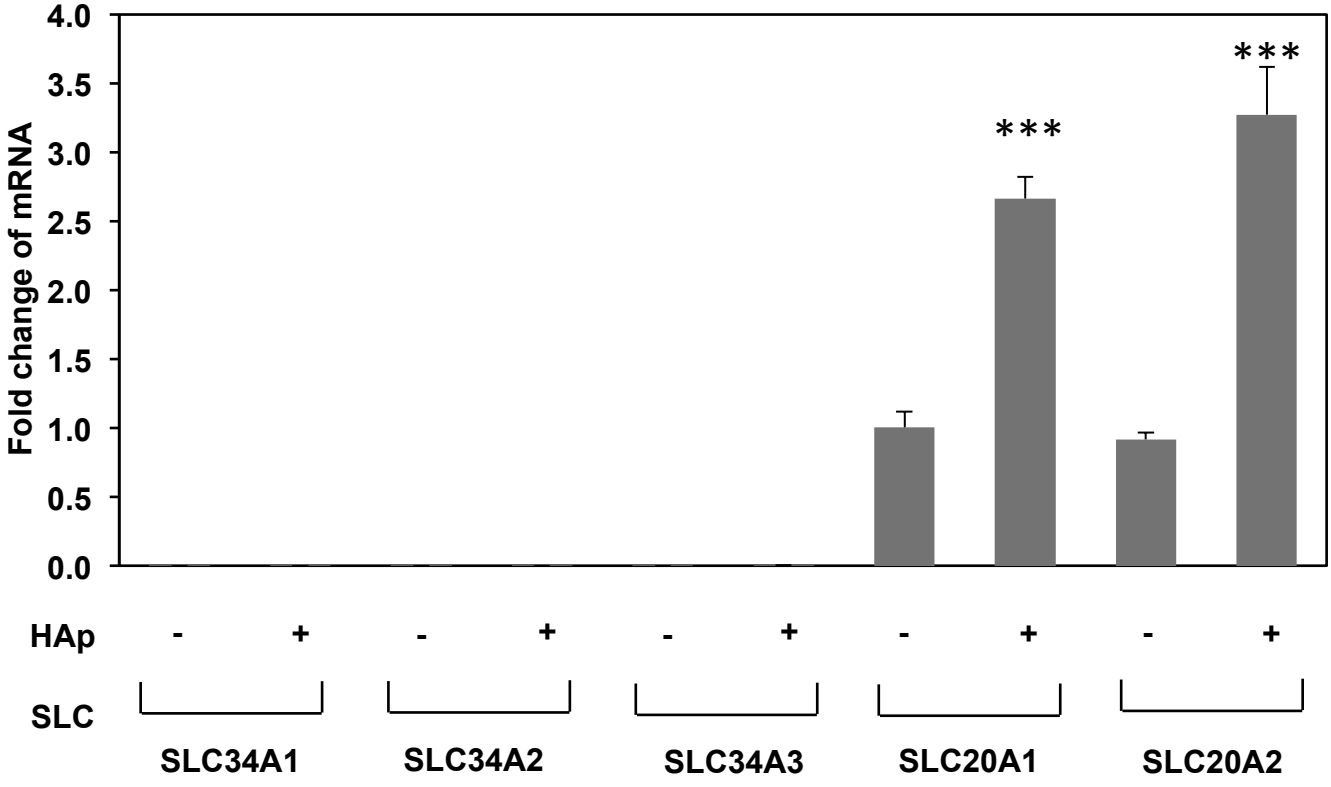
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Figure 8

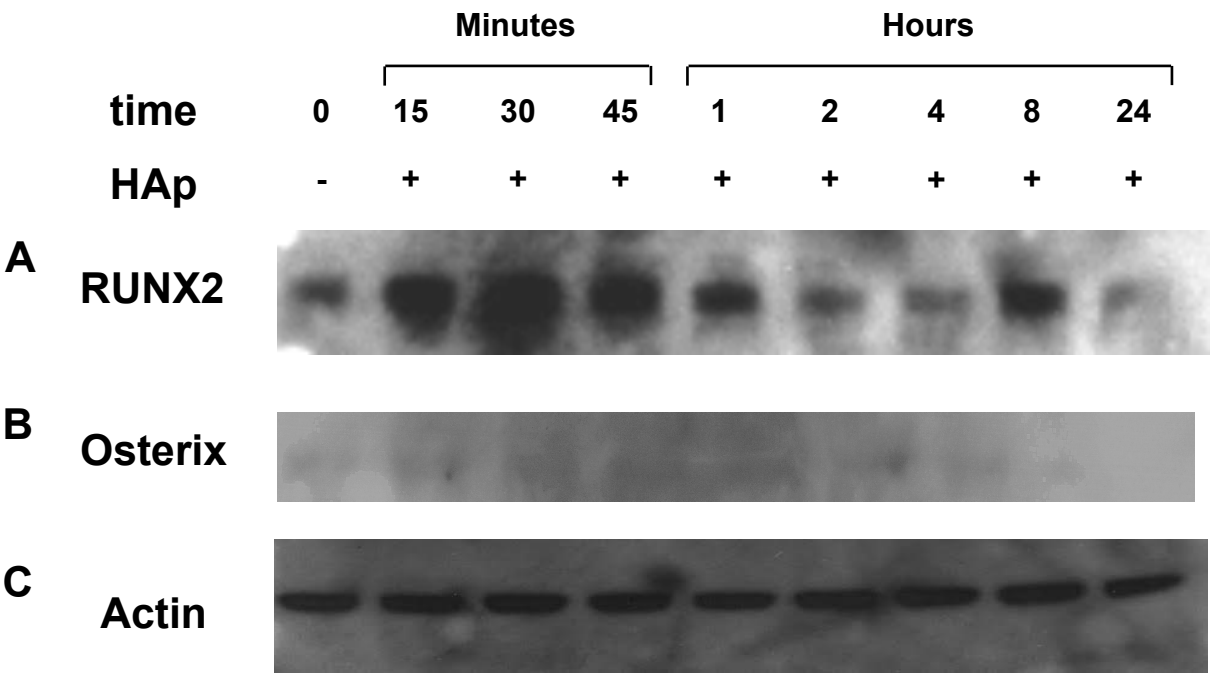
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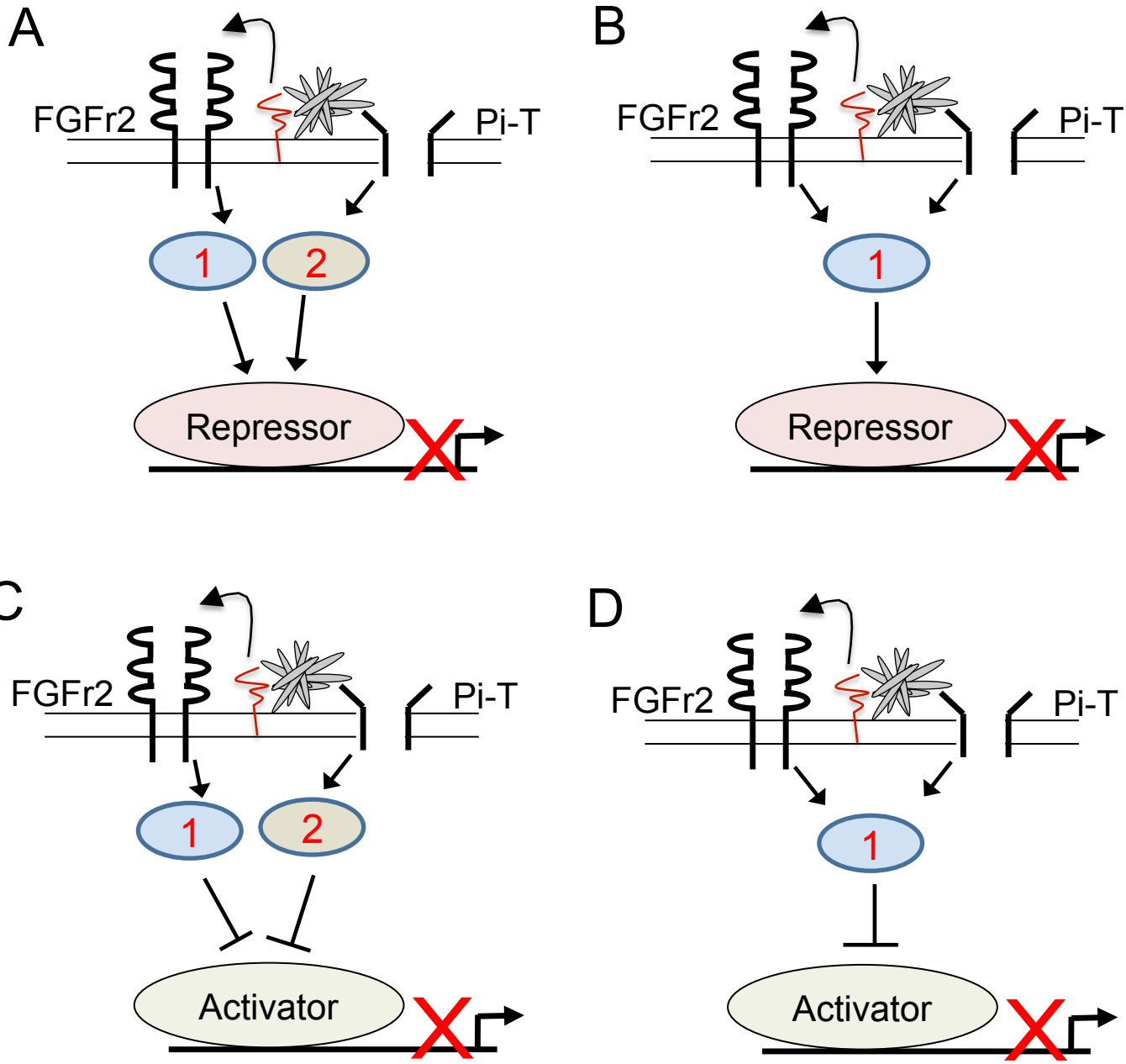
Figure 9



represents HAp