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Ca2+-Independent Phospholipase A2 beta-derived Lipid Signals and Osteogenesis

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Ca^{2+}-INDEPENDENT PHOSPHOLIPASE A_2 BETA-DERIVED LIPID SIGNALS AND OSTEOGENESIS

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ABSTRACT

Bone modeling can be modulated by lipid signals and arachidonic acid (AA); the derivative cyclo-oxygenase 2 (COX2) metabolite, prostaglandin E_2 (PGE_2), is an important mediator of optimal bone formation. Hydrolysis of AA from membrane glycerophospholipids is catalyzed by phospholipases A_2 (PLA_2s). We reported that mice deficient in the Ca^{2+}-independent PLA_2beta ($iPLA_2\beta$), encoded by *PLA2G6*, have decreased bone formation, relative to wild type (WT) mouse bones. Here, we examined at the mechanistic and molecular levels the role of $iPLA_2\beta$ in bone formation using bone marrow stromal cells and calvarial osteoblasts from WT and $iPLA_2\beta$-deficient mice and MC3T3-E1 osteoblast precursor cell line. Our data reveal that osteogenesis and osteogenic factors (BMP2, alkaline phosphatase, and Runx2) are decreased with $iPLA_2\beta$-deficiency. These results are corroborated and recapitulated in WT cells treated with a selective inhibitor of $iPLA_2\beta$, and rescued in $iPLA_2\beta$-deficient preparations by additions of AA and more prominently by PGE_2. Further, under osteogenic conditions we find an association of Runx2 with its promoter region, enhanced *PLA2G6* transcriptional activity, and a surprisingly strong association of $iPLA_2\beta$ with both *Runx2* and *PLA2G6* promoter regions when transcriptional activity is high. These findings reveal a strong link between osteogenesis and $iPLA_2\beta$-derived lipids and raise the intriguing possibility that $iPLA_2\beta$ itself participates in transcriptional regulation of *Runx2* and *PLA2G6*. We postulate that

targeting the link between iPLA$_2$β and Runx2 may be a means to decrease bone loss associated with disease states.

Keywords: iPLA$_2$β, Runx2, Osteogenesis, Phospholipase

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INTRODUCTION

The Cells Of Skeletogenesis

Comprised of 206 individual bones, the vertebrate skeleton is a complex organ. Each bone provides an environment for various cells; especially immune cells, adipocytes, fibroblasts, myocytes and the variety of cells important to bone formation and maintenance. The principle cells of bone formation are chondrocytes and osteoblasts. Maintenance of bone relies additionally on osteoclasts, and osteocytes (1-3).

All connective tissues are derived from the embryonic mesenchyme. Bone, cartilage, blood, and lymph all spring from this origin. The construction of the skeleton falls to the osteoblast; frequently following a template laid by chondrocytes, these cells are central to skeletogenesis and bone maintenance. The fate of the majority of osteoblasts is to undergo apoptosis; more rarely osteoblasts can persist as a flattened, quiescent bone-lining osteoblast or become entombed in bone as an osteocyte (4).

The destruction of mineralized bone is the purview of the osteoclast. This necessary function is essential to the remodeling and repair of bone. Osteoclasts are a massive, multinucleated conglomeration of macrophages fused to form a tent-like structure that can adhere to bone. Under this tent, resorption of bone is achieved through secretion of acid and collagenase by osteoclasts for remodeling or calcium homeostasis (6).

Within mature bone, the three cell types involved in maintenance vary in abundance. Osteocytes represent more than 90% of the cells present; osteoblasts make up 5% and 1% are osteoclasts. While important to bone formation, chondrocytes are not among mature bone cells; these cells are only transiently present in the development of long bones as a template. The permanent home for chondrocytes is in service to protecting joints, connecting bone to muscle, and supporting elastic structures (7).

Skeletogenesis

The majority of bones are formed by endochondral ossification; few others, such as the flat bones of the clavicle and the skull develop by intramembranous ossification. Condensation of mesenchymal cells forming cartilaginous templates produces endochondral bone formations; these will be eventually remodeled into bones. Cells within the condensation center undergo differentiation into cartilaginous cells, surrounding cells differentiate into the perichondrium, later giving rise to the periosteum. The periosteum is the location for osteoblast precursor development. The chondrocytes within the cartilage template undergo further maturation and become hypertrophic; involving the end of proliferation and a stop in cell cycling. Hypertrophic chondrocytes further mature to produce a mineralized matrix; this remains after the cells have undergone apoptosis to be used as a template for trabecular bone formation by osteoblasts (Figure 1a), adapted from (8).

Chondrocyte maturation couples with osteoblast differentiation within endochondral bone formation via Indian hedgehog (Ihh) signaling. Ihh derived from

prehypertrophic chondrocytes precipitated the differentiation of osteoblasts in neighboring perichondrial cells. Transcription factors that control chondrocyte differentiation and maturation indirectly also affect bone formation. In a stark contrast, no signals from cartilage are required for intramembranous bone development. Cells within the intramembranous mesenchymal condensations differentiate osteoblasts directly (Figure 1b); thusly, transcription factors unique to chondrocyte differentiation do not effect membranous bone formation (8).

Figure 1a: Endochondral Ossification

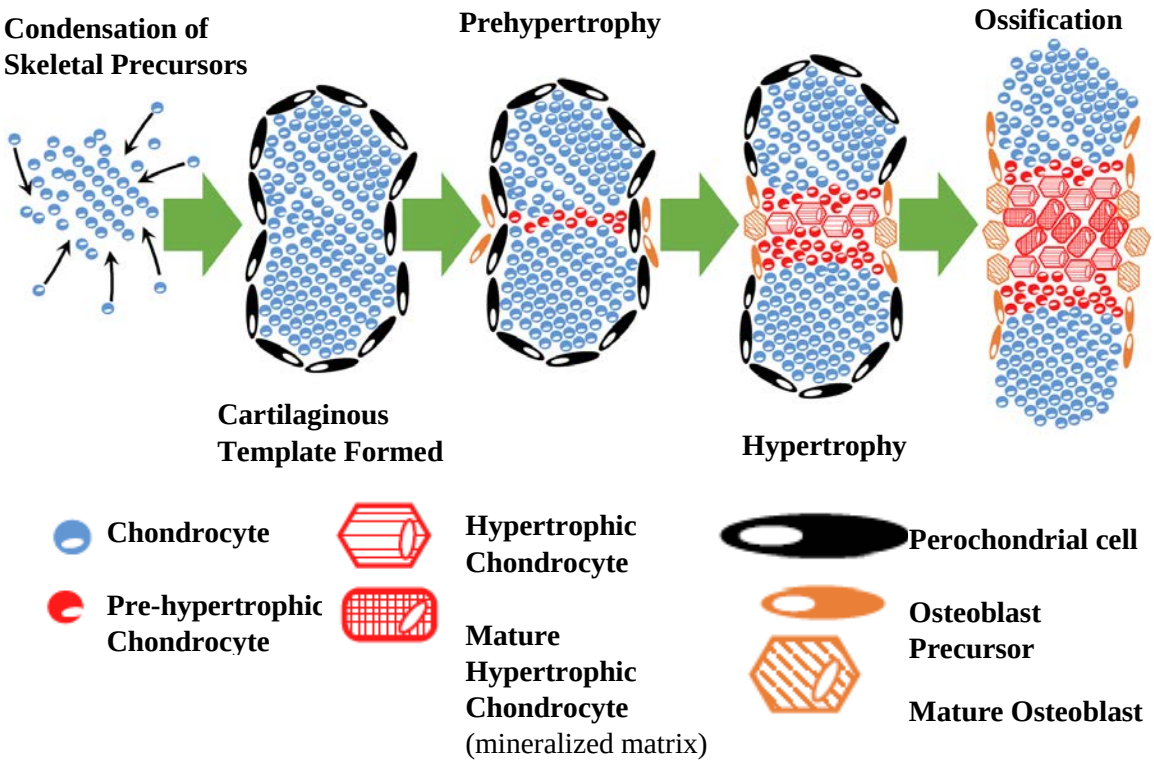
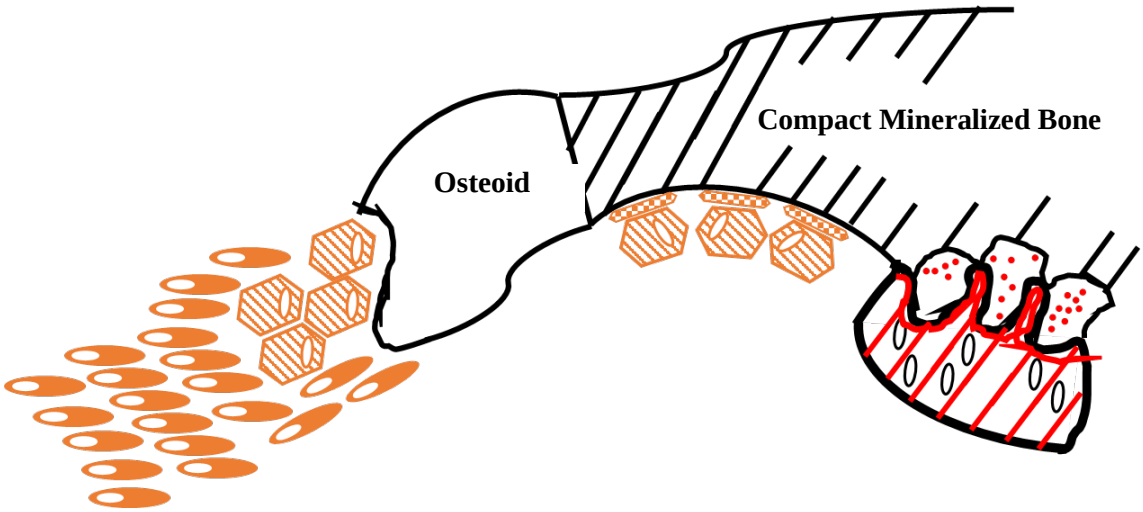


Figure 1. Diagram of the two processes of bone formation. (a) Endochondral bone formation as in long bones formation, skeletal precursors condense in to a nucleation of bone, this proceeds to formation of the cartilaginous template for bone formation. Following this, prehypertrophic cells differentiate at the center of the cartilaginous template; these secrete indian hedgehog homolog, Ihh, inducing differentiation of pre-osteoblasts located in the perichondrium nearby. Following

this, hypertrophic chondrocytes appear and continue to differentiate into mature hypertrophic chondrocytes. These produce a mineralized matrix that osteoblasts will enter and start to make trabecular bone. (b) Membranous bone formation occurring in the flat bones: osteoblast progenitors differentiate directly from condensed mesenchyme. These eventually differentiate into mature osteoblasts producing osteoid. Adapted from (8)

Figure 1b: Membranous Ossification



Osteoblast Differentiation Transcription Factors

The maturation of osteoblasts is governed by transcription factors regulating lineage commitment, differentiation, function, and fate. The expression of phenotypic genes provides the hallmarks for these stages. Principle among these, Runx2 has been identified as central to osteoblast commitment and differentiation (9). The expression of Runt family of transcription factors, of which Runx2 is a member, in mesenchymal stem cells begins with the onset of skeletal development persisting throughout osteoblast differentiation. While Runx2 is the most prominent factor in osteoblast differentiation, Runx3 is also present in osteoblast lineage cells (9). Runx2 controls bone formation during both developing bone and adult bone maintenance (10).

Genetic manipulation and molecular studies of Runx2 *in vivo* revealed that Runx2 is necessary for mesenchymal stem cell differentiation in to osteoblasts (Figure 2), adapted from (11).

Figure 2: Transcriptional Regulation of Mesenchymal Fate

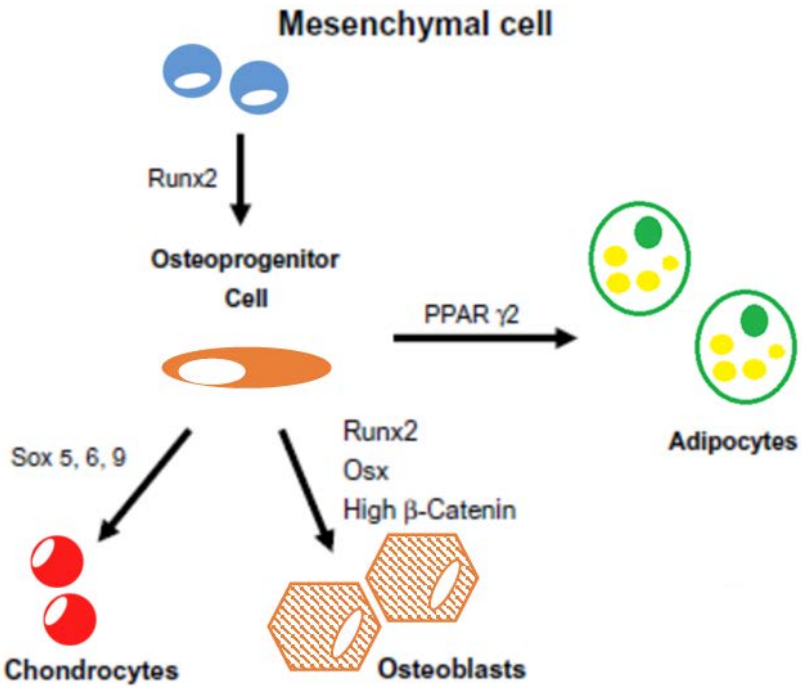


Figure 2. Prominent transcription factors in lineage determination of mesenchymal stem cells. Among the potential fates of mesenchymal stem cells are chondrocytes, osteoblasts, and adipocytes; these fates are most relevant to bone tissue. Mesenchymal stem cells can undergo mitotic division and maintain differentiation potential. Osteoprogenitor cells localize to the inner cell later of the periosteum, and endosteum; they are activated during growth and fracture repair.

Chondrocytes provide the cartilaginous template for ossification. Osteoid producing osteoblasts localize to the surface of bone tissue and produce mineralized matrix. Adipocytes accumulate in the marrow space with age; potentially as a balance in fates with osteoblasts. Adapted from (3)

Blocking Runx2 inactivation stops the differentiation of mesenchymal stem cells to the osteoblast lineage (12,13). In humans, cleidocranial dysplasia results from Runx2 haploinsufficiency. Cleidocranial dysplasia is a disease characterized by defective bone and tooth formation; especially in the collarbone (14,15). The molecular mechanism of Runx2 activity is mediated by binding to the Runx consensus motif (5-

((T/A/C)G(T/A/C)GG(T/G)); a sequence attracting Runx1 and Runx3 binding as well (16). Expression of Runx1 and Runx3 overlap in expression with Runx2 in some osteoblasts (16). The consensus motif can be found in the promoter of major osteoblast genes; influencing expression of genes like osteopontin, bone sialoprotein, type I collagen chain, and osteocalcin. This expression profile establishes the osteoblast phenotype.

The potency of Runx2 activity also requires regulation at the level of post-translational modifications. Runx2 can undergo acetylation, phosphorylation, sumoylation, or ubiquitination (17). Typically inside the nucleus, Runx2 can be phosphorylated via the mitogen-activated protein kinase pathway; this can be induced by integrin binding of type I collagen at the osteoblast surface (18). The mitogen-activated protein kinase pathway can also activate Runx2 as a consequence of stimuli from bone morphogenic protiens, fibroblast growth factor2, protein kinase A, mechanical loading of bones, and hormones such as parathyroid hormone (18,19).

As an extension of Runx2 control over osteoblasts, Runx2 can halt proliferation by acting on the cell cycle in G1 transition (20). By manipulating the expression of kinases such as p85 PI3K, Runx2 controls osteoblast differentiation and subsequent survival (21). Runx2 can control osteoblastogenesis, osteoblast proliferation, and osteoblast lifetime through multiple mechanisms. Interestingly, loss of Runx2 expression in committed osteoblasts is not essential for embryonic skeletogenesis (22); but it does impair postnatal skeletogenesis (23).

As to be expected from the pivotal placement of Runx2 in osteoblastogenesis, both expression and activity of Runx2 are strongly controlled by transcription factors,

protein–DNA and protein–protein interactions (Figure 2). Runx2 is manipulated by Hoax2; is a Hox homeodomain family member of transcription factors that control skeletal patterning. Hoxa2 inhibits Runx2 expression and thereby bone formation (24). Transcriptional activity can be reduced by the transcription factors Stat1 and Sox9 (25-27). Sox8 suppresses Runx2 expression and negatively regulates osteoblast differentiation (27). Aj18, myeloid Elf-1 factor and Nrf2 exhibit repression of Runx2 activity (28-30). The Yes-associated protein (YAP) interacts with the native Runx2 protein directly, suppressing Runx2 transcriptional activity (31). Also, tumor suppressor protein p53 negatively regulates Runx2; consistently, blocking p53 in mice activates osteoblast differentiation (32,33).

Runx2 activity is positively influenced by transcriptional activators such as Rb, TAZ, HOXA10 or BAPX-1 (34-37). The WW domain is a motif containing two tryptophan residues is among the smallest protein modules; it mediates specific protein-protein interactions with short proline-rich or proline-containing motifs. The WW domain containing TAZ directly interacts with Runx2; it co-activates some Runx2-dependent gene transcriptions (37). Among the principle co-activators of Runx2 is CBFb1, which is necessary for Runx2-dependent bone formation (38,39).

It is difficult to discuss Runx2 without the pivotal regulatory interaction with the extracellular-signal transducers of transforming growth factor beta superfamily receptors, Smads. Runx2 works in synergy with Smad1 and Smad5 to control bone-specific genes (40,41). The Runx2-Smad complex has been characterized *in vivo* as driving osteoblastogenesis (42). Additional transcriptional co-activators of Runx2 have been identified; such as C/EBPb, C/EBPd and Menin (43).

Figure 3: Expanded Transcriptional Regulation of Osteogenesis

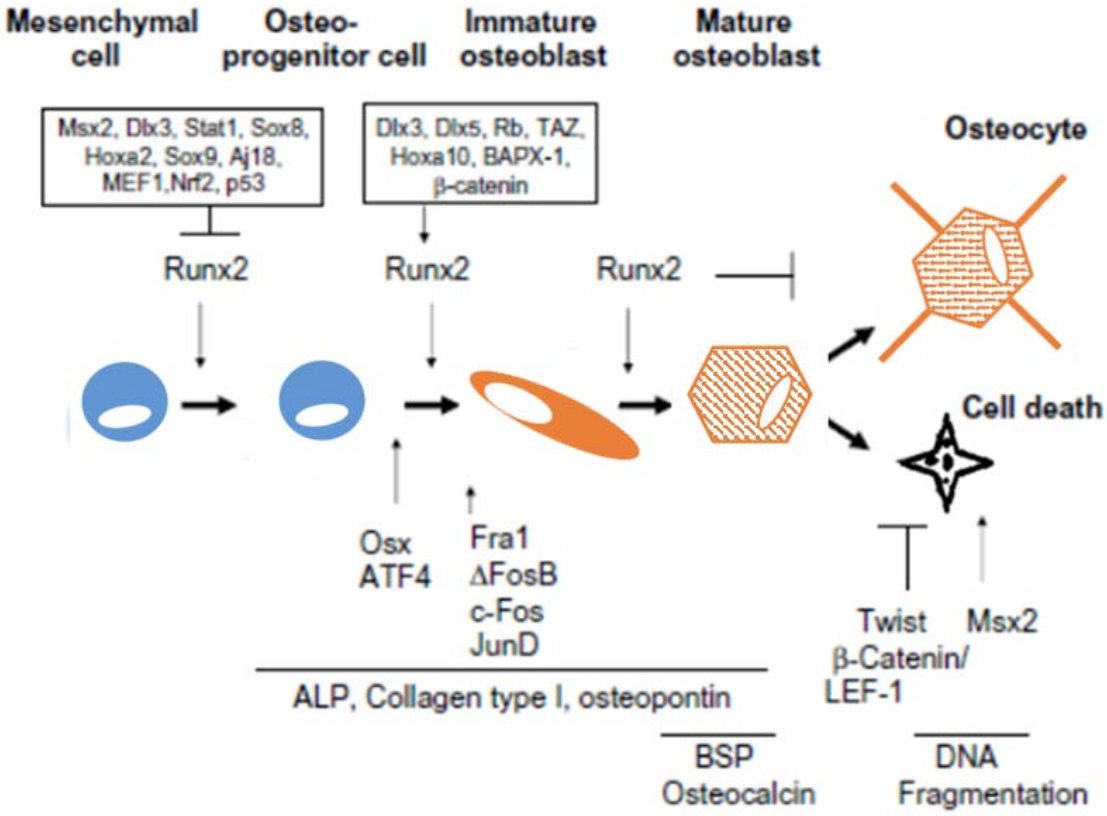


Figure 3. Osteoblast differentiation is under transcription factor control. Differentiation of osteoblasts begins at commitment of a progenitor cell from the mesenchymal stem cell population. The differentiation of osteoprogenitors into immature osteoblasts denotes a lineage commitment that is irreversible. Maturation of the osteoblast is denoted by expression of phenotypic genes and secretion of mineralized matrix. Important transcription factors for each state are denoted. Interestingly, some transcriptional regulation is positive and negative, depending on time and location. Dlx3, in particular, represents this dual role of control. In turn, however, these transcriptional regulators are tightly controlled themselves; being modulated further upstream signals, as indicated. (3)

Within the nucleus, histone acetyl transferase activity has been affected by Runx2 interactions with factors such as E1A binding protein p300, facilitating control of transcriptional machinery access to DNA by a balancing of acetylation and deacetylation of the chromatin (43,44). Interestingly, Runx2 and histone deacetylases (HDACs) interact, with HDAC/Runx2 activity negatively regulating osteoblast differentiation (45-

47). Another common form of regulation is protein stability; with Runx2 regulation via the ubiquitin ligase Smurf1.

Attacking the WW binding-motif on the C terminus of Runx2, Smurf1 promotes degradation of Runx2 by the proteasome. Other proteins, like Shn3, control Runx2 protein levels by promoting its degradation through recruitment of the E3 ubiquitin ligases to Runx2 (1,48). These regulators attacking Runx2 through the WW binding-motif to control Runx2 activity or reduce Runx2 protein lifetime illustrate the importance of the role this molecular interaction can offer to regulate osteoblast differentiation. Within the larger picture, Runx2 is subject to tight control via an interesting array of interactions at various levels; this ensures timely and effective expression, function, and cessation of Runx2 activity, thereby control osteoblast differentiation (Figure 3), adapted from (3).

Phospholipases

Phospholipases are a group of enzymes that catalyze the cleavage of membrane glycerophospholipids. Some phospholipases exhibit substrate specificity for individual phospholipid species; some catalyze the cleavage of other lipophilic molecules, such as triglycerides, in addition to phospholipids (49,50). These enzymes exhibit a diversity in their tissue distribution, subcellular localization, and substrate specificity. As well as this diversity, functional importance varies widely in the phospholipid metabolites. Phospholipids do not simply serve as structural components of cellular membranes; enzymatic processing of phospholipids by phospholipases converts this repository of potent molecules into lipid mediators or second messengers. Arachidonic acid,

phosphatidate and diacylglycerol are among the powerful metabolites released by phospholipase activity; these play pivotal roles in membrane trafficking, signal transduction, cell proliferation and apoptosis. These roles exhibit phospholipase impacts in lipid metabolism, and disease progression (49).

Phospholipids consist of a glycerol-3-phosphate molecule esterified at its carbon 1 (*sn*-1) and carbon 2 (*sn*-2) positions to nonpolar fatty acids and at its phosphoryl group to a polar head group (Figure 5). Phospholipids are structurally complex as a product of the variation in combinations of polar head groups and fatty acyl chains. Typically, *sn*-1 positions of a phospholipid is occupied by saturated fatty acids, conversely the *sn*-2 position can be filled by unsaturated fatty acids (51).

Figure 4: Map of Phospholipase Activity Sites

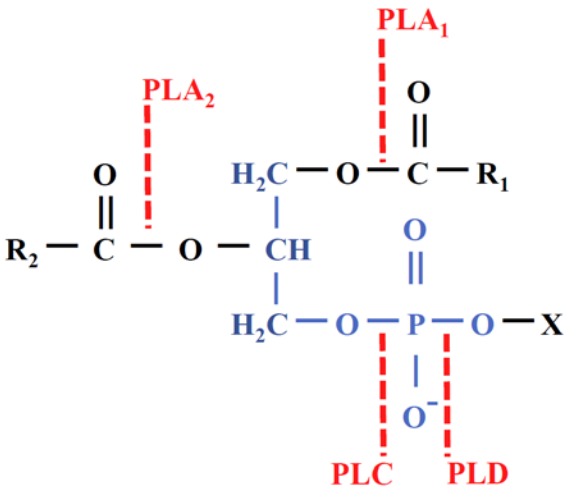


Figure 4. Phospholipid structure with the sites of action of phospholipases. Phospholipid molecules consists of a glycerol-3-phosphate (blue) esterified at its *sn*-1 and *sn*-2 positions to nonpolar fatty acids (R1 and R2) and a phosphoryl group to a polar head group, X. Phospholipase A1 and phospholipase A2 cleave the acyl ester bonds at *sn*-1 and *sn*-2, respectively. Phospholipase C cleaves the glycerophosphate bond whereas phospholipase D removes the head group (5).

Based on the ester bond that is cleaved within the glycerophospholipid molecule, phospholipases are grouped into families, namely A, B, C and D. Phospholipase A enzymes cleave the acyl ester bond at either the *sn*-1 (A_1) or *sn*-2 (A_2) position (Figure 4). The term phospholipase B is given to phospholipases that hydrolyze acyl ester bonds

at both *sn*-1 and *sn*-2 positions. Enzymes grouped under phospholipase C cleave the glycerophosphate bond, while phospholipase D enzymes remove the polar head group (Figure 5). The phospholipase A, phospholipase C, and phospholipase D enzyme families are further classified into subgroups with unique properties and characteristics (5).

Phospholipases A_2 (PLA_2s) specifically hydrolyze the *sn*-2 ester bond of glycerophospholipids; these enzymes show greater activity in lipids occupying lamellar and micellar aggregates in membranes and at other lipid-water interfaces (52-54). These enzymes are widely distributed in nature and were initially classified as 'intracellular' or 'extracellular'. Intracellular PLAs are involved in phospholipid metabolism and signal transduction (55). Extracellular PLAs are abundant in mammalian pancreatic juices; these enzymes also appear in the venoms of snakes and insects displaying diverse roles, including blood platelet aggregation (56). Often, their enzymatic activity results in the release of arachidonic acid, an eicosanoid precursor, known to trigger inflammatory reactions (57,58).

Exhibiting functional complexity, phospholipases draw upon the phospholipid bilayer to produce potent bioactive lipids. Broad implications of these bioactive lipids are borne out in unique spectrum of intracellular and extracellular functions. Phospholipase products react as integral components of bewildering array of signaling, metabolic, inflammatory, and regulatory pathways. As the understanding of this chemical space of phospholipase family members expands, the potential of these potent enzymes comes to greater and greater light as therapeutic targets for prevention and treatment of disease.

Ca^{2+}Independent Phospholipase A_2s

The calcium independent phospholipases A_2 ($iPLA_2$s) are enzymes ubiquitously expressed, and contrasting with secretory PLA_2s ($sPLA_2$s) and cytosolic PLA_2s ($cPLA_2$s), do not require Ca^{2+} for translocation or activity. First observations of $iPLA_2$ activity were made in the 1980s with the isolation of a plasmalogen-selective PLA_2 in the cytosol of canine myocardium (59,60). Analogous activity was later found in insulinoma cells and in renal proximal tubules (59,61). Subsequently, $iPLA_2$ enzymes have been located and cloned from rat, mouse, and hamster cells (61-63). These species homologs are 85 kDa proteins containing 752 amino acids with 95% homology across species. Each homologus enzyme contains a lipase motif and eight stretches of the repeating motif homologous to the integral membrane protein-binding domain of Ankyrin (61,63,64). Inhibition of the enzymes can be achieved by bromoenol lactone (BEL); this is a potent and selective inhibitor that does not act to inhibit $sPLA_2$s or $cPLA_2$s (65,66). Additional $iPLA_2$s have been discovered; including: $iPLA_2\beta$, $iPLA_2\gamma$, $iPLA_2\delta$, $iPLA_2\varepsilon$, $iPLA_2\zeta$, and $iPLA_2\eta$ (67). $iPLA_2$s are found ubiquitously; each has multiple splice variations.

The cytosolic $iPLA_2\beta$ and membrane-associated $iPLA_2\gamma$ show expression in many tissues; within individual tissues, each exhibits specific activity (67). $iPLA_2\delta$ shows subcellular localization to the ER and Golgi apparatus in neurons. Within neurons, $iPLA_2\delta$ is involved in basal neuronal function; deletion or inhibition has serious pathologic effects including lethality (67). $iPLA_2\varepsilon$ and iPLA2ζ show expression in adipocytes (67). $iPLA_2\varepsilon$ activity has been observed in liver dysfunction, as well as obesity. Absence of iPLA2ζ has been implicated in triacylglycerol accumulation as well

as lipid storage diseases. Increased risk of T2D has also been associated with iPLA$_2$ζ (67). iPLA$_2$η has been associated with normal function in adipocytes (68).

iPLA$_2$β

iPLA$_2$β, as an A$_2$ phospholipase, hydrolyzes glycerophospholipids at the *sn*-2 position; this yields a free fatty acid and a lysophospholipid (69). Participation of iPLA$_2$β has been implicated in bone formation, skeletal muscle fatty-acid oxidation, muscle contraction, muscle degeneration, schizophrenia, nerve degeneration, and pancreatic islet β-cell function (70-75). iPLA$_2$β contains a lipase motif preceded by eight N-terminal ankyrin-repeats (69). iPLA$_2$β includes a caspase-3 cleavage site, an ATP-binding domain, a serine lipase consensus sequence, a putative bipartite nuclear localization sequence, and a C-terminal calmodulin-binding motif (69). The iPLA$_2$β gene exhibits alternatively splicing; this yields variants that display differences in intracellular location, catalytic activity, and likely cellular function (76-78).

Human cells exhibit five splice variants identified so far. The short form, termed GVIA-1, is trimmed to a 752-amino acid sequence; the resulting molecular mass of 84 kDa (64). The long form variant is termed GVIA-2; this unique isoform is more analogous to the predominant form in mice and rats; it bears an 806-amino acid sequence yielding a molecular mass of 88 kDa. The product of an exon-skipping mechanism, a 54-amino acid sequence is added; this is absent in the short form (78,79). Each of these isoforms show biological activity, participating in multiple enzymatic events (79,80). A third variant, a product of alternate splicing designated GVIA-3, has a molecular mass of

70 kDa (81). Two other isoforms bear the ankyrin repeats of the iPLA$_2$β gene, but do not contain the iPLA$_2$β active site; GVIA Ankyrin-1 and GVIA Ankyrin-2 (78). These inactive isoforms inhibit iPLA$_2$β activity by competing for docking to the binding sites in the cell; preventing docking of the catalytically active iPLA$_2$β. Alternatively, these isoforms may disturb the quaternary structure of iPLA$_2$β; altering enzymatic activity (78). The induction and control of these splice variants is under investigation. The mechanisms responsible for generating iPLA$_2$β splice variants would be of interest in studying the function of iPLA$_2$β within its ubiquitous expression in mammalian tissues. This could be of particular interest in tissues exhibiting higher expression, such as the brain, heart, testes, pancreatic β-cells and macrophages (64,82-85). iPLA$_2$β predominately localizes to the cytosol, this strikes a contrast with other PLA$_2$s; such as the membrane-associated iPLA2γ (86).

Widely reported, data show iPLA$_2$β is prominently present in a variety of important roles; functioning in phospholipid remodeling, liberation of eicosanoid precursors, maintenance of the plasma membrane, and signal transduction (75,87-106). iPLA$_2$β plays a role in both normal and diseased conditions

The confluence of iPLA$_2$β and bone

The process of bone resorption balances the process of bone formation to maintain bone mass and preserve bone structure and strength amid the constant remodeling of adult bone. Aging generates a loss in bone mass and strength; consequently increasing the risk of fractures (107-113). Age-related bone loss is multifactorial and the underlying cellular basis must involve decreases in bone

formation by osteoblasts and/or increases in bone resorption by osteoclasts (107,111,112,114).

Prostaglandins (PGs) and other bioactive eicosanoids (products of arachidonic acid [AA] metabolism) are potent regulators of bone formation and bone resorption (115-117). The PGs are generated via cyclooxygenase (COX)-catalyzed metabolism of arachidonic acid following its hydrolysis from membrane phospholipids by phospholipases A_2 (PLA_2s) (58,118-123). The PLA_2s catalyze hydrolysis of the *sn*-2 substituent from glycerophospholipid substrates to yield a free fatty acid and a 2-lysophospholipid (124). The recognized PLA_2s are classified based on their Ca^{2+} requirement for activation and sequence homology (5) and include low molecular weight secretory ($sPLA_2$), high molecular weight cytosolic Ca^{2+}-dependent ($cPLA_2$), and high molecular weight cytosolic and membranous Ca^{2+}-independent ($iPLA_2$) enzymes.

To date, studies have focused on the roles of $cPLA_2$ and $sPLA_2$ in regulating bone metabolism and have demonstrated that bone resorption induced by PTH (125), cadmium (126), or lipopolysaccharide (127) is associated with activation of $cPLA_2$; and by IL-1 (128) or ovariectomy (129) with activation of $sPLA_2$. Further, bone loss due to inflammation is prevented in $cPLA_2$-null mice and due to decreases in estrogen by sPLA2 inhibitors. These observations suggest that $cPLA_2$ and $sPLA_2$ participate in mediation of bone resorption induced by certain stimuli. However, no temporal relationship between PLA_2 activation and PGE_2 synthesis over the course of reduced osteoid mineralization was evident (130). In addition, inhibition of COX-2 did not prevent IL-1-induced decreases in mineralization (128). These finding

suggest that other products of arachidonic acid metabolism (i.e., leukotrienes, HETEs, PAF) (131,132) participate in bone resorption. Several reports, however, suggest that arachidonic acid has positive effects on bone formation. For piglets, AA supplementation of diet resulted in increases in whole body and bone mineral density (133) and bone mass (134,135), and decreases in bone resorption (136). Findings from various studies implicate PGE_2 as the mediator of arachidonic acid-induced bone formation (137-143).

Among the bone-related cells, PGE_2 is produced mainly by osteoblasts (144,145) and has been reported to enhance bone formation at low doses but increase bone resorption at high doses (127,137,146-148). The differential effects of PGE_2 are thought to be linked to the presence of multiple cell surface PG receptors (139,149-153). Systemic or local injections of PGE_2 have also been shown to stimulate bone formation in rats (139,146) and in piglets (136), and reduce bone loss in rats due to disuse or orchidectomy (147,148,154). Lamellar bone formation in response to mechanical strain is mediated by cyclooxygenase II (COX-2) (107,155) and COX-2 -/- mice have decreased bone density (152). PGE_2 increases bone nodule formation in low density cultures of rat bone marrow cells by recruiting osteoblast precursors present in the bone marrow (156-161). These findings suggest that PGE_2 promotes bone formation. The demonstration that PGE_2 can increase differentiation of osteoblastic cells (156,160,161) and enhance bone morphogenetic protein-induced osteoblastogenesis and expression of Runx2/cbfa1 and osterix (149), which are required for osteoblast differentiation, suggests a potential mechanism by which PGE2 may contribute to bone formation.

Activation of PLA_2 also leads to the hydrolysis of arachidonic acid and generation of PGE_2 in a variety of cells (63,81,85,162-171). Among the PLA_2s is one that does not require Ca^{2+} for activity and is classified as a Group VIA $iPLA_2$ and designated as the β-isoform of $iPLA_2$ ($iPLA_2\beta$) (86,101,172). It is activated by ATP and inhibited by a bromoenol lactone suicide substrate (BEL) (63). $iPLA_2\beta$ is an 84-88 kDa cytosolic PLA_2 and its amino acid sequence includes eight N-terminal ankyrin repeats, an ATP binding domain, a serine lipase consensus sequence (GXSXG), and C-terminal calmodulin binding domain(s) (173). $iPLA_2\beta$ has been proposed to be involved in phospholipid remodeling (174), cell proliferation and apoptosis (173), and signal transduction in a variety of cells (63,81,91,93-95,101,175,176). $iPLA_2\beta$ mRNA is expressed in a variety of wild-type tissues (177) including bone marrow and spinal cord (89), however, no studies to date have explored the role of $iPLA_2\beta$ in bone. $iPLA_2\beta$-null mice have been generated by homologous recombination, and these mice exhibit defective spermatozoa motility (177), pancreatic islet insulin secretion (178), and signaling abnormalities in macrophages (93,179). Unexpectedly, aging $iPLA_2\beta$-null female mice have lower bone mineral density than age-matched wild-type female mice. Further, there is a decreased rate of bone formation and diminished bone strength that is accompanied increased bone marrow fat content in the $iPLA_2\beta$-null mice, as compared with age-matched wild-type mice (75).

The effects of PGE_2 are propagated through interaction with four recognized PGE_2 receptor subtypes (EP1-4) differ in the triggered signal transduction pathways (180-183). *In vitro* (184-186) and *in vivo* (187,188) studies suggest that the bone

anabolic effects of PGE_2 are mediated through the prostaglandin E receptor 4 (EP_4 receptor). EP_4 receptor is also expressed by pre-adipocyte cells (189) and activation of this receptor by PGE_2 suppresses induction of adipogenesis marker gene peroxisome proliferator-activated receptor gamma (PPARγ) and inhibits adipocyte differentiation (189,190). Collectively, these observations reveal a dual role for PGE_2; in increasing bone formation and decreasing adipogenesis, giving rise to the strong possibility that decreases in PGE_2 (i.e. as in the absence of $iPLA_2\beta$) contribute to decreased osteogenesis and increased bone marrow adipogenesis.

Prostaglandins are also important mediators of skeletal mechano-responsiveness (191). Mechanical stress controls bone formation by stimulating PGE_2 production and EP_4 expression in osteoblasts (192) and mechanical stimulation of osteoblasts by fluid flow stimulates release of PGs including PGE_2 (193). Other *in vivo* studies have shown that pharmacological inhibition of COX-2 attenuates loading induced bone formation (117,194,195). These findings lend support to the hypothesis that mice lacking $iPLA_2\beta$ have an attenuated osteogenic response to mechanical loading compared to WT mice.

The fact that osteoblasts and adipocytes are differentiated cells derived from common mesenchymal progenitor cells is an important facet of our studies (110,196-200). Bone formation is regulated by transcription factor Runx2/Cbaf1 (12-14,201) and fat formation by PPARγ (202-204). Beresford et al. (199) were among the first to suggest an inverse relationship between adipocytic and osteoblastic differentiation. Since then, a number of studies demonstrated that bone formation is inversely related to adipocyte formation in the bone marrow

(197,199,205-208). PGE_2 has been shown to increase the number of bone marrow stromal cells (BMSCs) (209), accelerate osteoblast differentiation (210), and stimulate osteogenesis at the expense of adipogenesis in the bone marrow of aged OVX rats by binding to EP_4 (211). Further, strontium ranelate used in the treatment of postmenopausal osteoporosis (212,213) has been shown to promote osteoblast differentiation and mineralization of BMSCs via PGE_2 production (214).

In clinical settings, various conditions associated with decreases in bone mass (i.e., diabetes and osteoporosis) are accompanied by increases in bone marrow fat content (215-217). Gains in bone marrow fat, with accompanying losses in bone mass, are also characteristic of aging (110,218). In fact, mesenchymal progenitors in mice (219,220) and humans (208) appear to shift in lineage determination with age, such that the cells preferentially differentiate into adipocytes rather than osteoblasts (110). Among the factors that are thought to contribute to reduced bone formation in aging humans and mice include decreases in osteoblast progenitor number, proliferation, differentiation, half-life, or due to osteocyte and osteoblast apoptosis (6,143,144).

Earlier dual-energy x-ray absorptiometry (DEXA) scanning from our group revealed lower bone mineral density (BMD) in aging $iPLA_2\beta$-null mice. BMD of WT and $iPLA_2\beta$-null mice are similar at 3 months of age, but lower in the $iPLA_2\beta$-null mice at 1 year and 2 years of age, relative to age matched WT mice (75). To determine if the decreased bone volume in $iPLA_2\beta$-null mice with aging is related to changes in osteoblast density, sections were stained to identify and quantitate osteoblasts lining the endocortical bone. At 6 months of age, the osteoblast

number/mm bone surface decreased in both groups, relative to 3 months of age. However, the decrease was significantly greater ($p < 0.05$) in the 6 month old iPLA$_2$β-null mice, relative to age matched WT mice (75). These findings suggest that the lower bone volumes in iPLA$_2$β-null mice might be due, in part, to decreases in osteoblast number.

Furthermore, the aging iPLA$_2$β-null mice were found to exhibit decreased cortical and trabecular bone. WT mice show a progressive increase in bone area and moments of inertia with aging, but these parameters fail to increase in iPLA$_2$β-null mice (75). Osteoclast density is not increased in iPLA$_2$β-null mice. These findings indicate that bone resorption due to increases in osteoclast number does not contribute to lower bone volumes in iPLA$_2$β-null mice (75).

Abnormal circulating levels of sex hormones, or renal and pancreatic islet secretory functions have profound effects on bone modeling (1). Our lab therefore measured plasma levels of estradiol, FSH, creatinine, blood urea nitrogen, amylin, insulin, and glucose and found no significant differences in any between iPLA$_2$β -null and age-matched WT mice (3 months to 2 years of age). These analyses reveal that typical circulating factors that can affect bone formation and resorption are not dysregulated in the iPLA$_2$β-null mice.

Calcein labeling was administered to mice, and the distance between the two labeled surfaces is a reflection of the mineral apposition rate (MAR). These analyses reveal significant double labeling in the epiphyseal region of the tibia in both WT and iPLA$_2$β-null mice, reflecting active trabecular bone formation. At 6 months of age it is significantly reduced in the iPLA$_2$β-null mice, relative to age-matched WT

mice (75). As MAR is a reflection of osteoblast function; these findings with those presented in previous data, suggest that decreases in osteoblast number and function contribute to the low bone phenotype in iPLA$_2$β-null mice.

Our lab has shown that when BMSCs harvested from WT and iPLA$_2$β-null mice are cultured in an osteogenic-media, more bone nodules arise from WT cells, but when the BMSCs are cultured in adipogenic medium greater fat deposition occurs in iPLA$_2$β-null cells. These findings suggest that the differentiation process may be dysregulated in the absence of iPLA$_2$β (75). It might be speculated that iPLA$_2$β and/or products of its activation participate in these pathways and their absence either shifts the balance from osteoblast formation towards adipocyte formation or eliminates stimuli necessary for BMSCs differentiation to osteoblasts.

Based on these earlier findings, the corner stone of this thesis was to access the underlying molecular mechanisms by which iPLA$_2$β participates in bone formation. Such elucidation of mechanism could shed light on the importance of phospholipid metabolism in the development of bone, the onset of osteoporotic bone loss, and bone disease.

CONTRIBUTION OF Ca^{2+}-INDEPENDENT PHOSPHOLIPASE $A_2\beta$ TO OSTEOGENESIS VIA PGE_2 GENERATION AND TRANSCIPTIONAL INDUCTION OF *RUNX2* AND *PLA2G6*

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ABSTRACT

Bone modeling can be modulated by lipid signals and arachidonic acid (AA); its cyclo-oxygenase 2 (COX2) metabolite, prostaglandin E_2 (PGE_2), is an important mediator of optimal bone formation. Hydrolysis of AA from membrane glycerophospholipids is catalyzed by phospholipases A_2 (PLA_2s). We reported that mice deficient in the Ca^{2+}-independent PLA_2beta ($iPLA_2\beta$), encoded by *PLA2G6*, have decreased bone formation, relative to wild type (WT) mouse bones. Here, we examined at the mechanistic and molecular levels the role of $iPLA_2\beta$ in bone formation using bone marrow stromal cells and calvarial osteoblasts from WT and $iPLA_2\beta$-deficient mice and MC3T3-E1 osteoblast precursor cell line. Our data reveal that osteogenesis and osteogenic factors (BMP2, alkaline phosphatase, and Runx2) are decreased with $iPLA_2\beta$-deficiency. These results are corroborated and recapitulated in WT cells treated with a selective inhibitor of $iPLA_2\beta$, and rescued in $iPLA_2\beta$-deficient preparations by additions of AA and more prominently by PGE_2. Further, under osteogenic conditions we find an association of Runx2 with its promoter region, enhanced *PLA2G6* transcriptional activity, and a surprisingly strong association of $iPLA_2\beta$ with both *Runx2* and *PLA2G6* promoter regions when transcriptional activity is high. These findings reveal a strong link between osteogenesis and $iPLA_2\beta$-derived lipids and raise the intriguing possibility that $iPLA_2\beta$ itself participates in transcriptional regulation of *Runx2* and *PLA2G6*. We postulate that targeting the link

between iPLA$_2$β and Runx2 may be a means to decrease bone loss associated with disease states.

INTRODUCTION

With aging, patients experience a loss in bone mass and strength, consequently increasing the risk of fractures (1-7). Age-related bone loss is multifactorial and the underlying cellular basis involves decreases in bone formation by osteoblasts and/or increases in bone resorption by osteoclasts (2,3,7,8).

Prostaglandins (PGs) and other bioactive eicosanoids (products of arachidonic acid (AA) metabolism) are potent regulators of bone formation and bone resorption (9-11). Within the bone milieu, PGE_2 is produced mainly by osteoblasts (12,13) and has been reported to enhance bone formation at low doses but increase bone resorption at high doses (14-16). The differential effects of PGE_2 are thought to be linked to the presence of multiple cell surface PG receptors (17,18). Suggesting PGE_2 promotes bone formation are demonstrations of increasing differentiation of osteoblastic cells (19,20), enhancing bone morphogenetic protein-induced osteoblastogenesis, expression of Runx2/cbfa1 and osterix (21), and requirements for osteoblast differentiation by PGE_2.

However, no temporal relationship between PLA_2 activation and PGE_2 generation over the course of reduced osteoid mineralization is evident (22). For piglets, AA supplementation in the diet resulted in increases in whole body and bone mineral

density (23), bone mass (24,25), and decreases in bone resorption (26). Findings from various studies implicate PGE_2 as a mediator of AA-induced bone formation (13,27,28).

PGE_2 propagates signals via interaction with four recognized PGE_2 receptor subtypes (EP1-4), triggering different signal transduction pathways (24,25). *In vivo* studies (26,27,29) reveal PGE_2 anabolism is mediated by EP_4 receptor. PGE_2 is generated via cyclooxygenase (COX)-catalyzed metabolism of arachidonic acid following its hydrolysis from membrane glycero-phospholipids by phospholipases A_2 (PLA_2s) (30,31). The PLA_2s catalyze hydrolysis of the *sn*-2 substituent from glycerophospholipid substrates to yield a free fatty acid and a 2-lysophospholipid (32). Activation of PLA_2 leads to hydrolysis of AA and generation of PGE_2 (33-36). Thus, mitigation of PGE_2 signaling, via reduced PLA_2-catalyzed generation of AA, would be expected to decrease bone formation.

We previously reported that among the family of PLA_2s, the group VIA Ca^{2+}-independent PLA_2beta ($iPLA_2\beta$) plays a critical role in bone formation (37). We found that mice with $iPLA_2\beta$-deficiency experience lower bone mineral density than age-matched WT mice. This was associated with a decreased rate of bone formation and diminished bone strength in the $iPLA_2\beta$-deficient mice, as compared with WT mice (37). Our findings that osteoclast density or plasma levels of CTX in $iPLA_2\beta$-deficient mice were not different from age-matched WT mice suggest that the lower bone volume in $iPLA_2\beta$-deficient mice was most likely not due to increased bone resorption. However, decreased mean apposition rate, in addition to reduced osteoblast number and function in the $iPLA_2\beta$-deficient mice raised the possibility that $iPLA_2\beta$-derived

lipids (iDLs) had a significant impact on bone formation.

To delineate the role of iDLs on bone formation, we utilized different models (bone marrow stromal cells, calvaria, and MC3TC3 osteoblast cells) and assessed osteogenic transcripts, proteins, mineralization, and molecular links. Our findings reveal that PGE_2 generated via $iPLA_2\beta$ activation is critical for Runx2 expression and osteogenesis and that $iPLA_2\beta$ may participate in transcriptional regulation of both *Runx2* and *PLA2G6*.

RESULTS

Recapitulation of $iPLA_2\beta$-deficient osteogenesis phenotype in wild type-We previously reported that $iPLA_2\beta$-deficient mice exhibited lower bone volume. Here we assessed whether this phenotype was due to the absence of $iPLA_2\beta$. After confirming the genotype of WT and $iPLA_2\beta$-deficient (KO) mice, as described (37), BMSCs were prepared from WT and KO mice and PCR analyses were used to assess $iPLA_2\beta$ expression. Such analyses yielded an expected product size of 972 bp in WT but not KO (Fig. 1*A*), confirming $iPLA_2\beta$-deficiency in the KO group. Over a 28-day period of culturing, WT BMSCs exhibited significant mineralization upon exposure to osteogenic media, as viewed under bright field microscopy (Fig. 1*B*). In contrast, there was no visual evidence of mineralization in the KO group. To determine whether this defect in osteogenesis was associated with an $iPLA_2\beta$-deficiency, BMSCs from WT were cultured in the absence and presence of *S*-BEL, a selective inhibitor of $iPLA_2\beta$

(38) and bone nodule formation assessed by von Kassa staining. In the absence of *S*-BEL, there was dramatic nodule formation in the WT group (Fig. 1*C*, top panels). However, with the addition of *S*-BEL, nodule formation was noticeably diminished in the WT group and resembled the low mineralization phenotype of the KO group (± *S*-BEL, Fig. 1*C*, bottom panels). These findings suggest a role for iPLA$_2$β in bone formation.

Rescue of iPLA$_2$β-deficient bone mineralization phenotype by PGE$_2$-In view of the evidence supporting a role for PGE$_2$ on bone formation and our reports that iPLA$_2$β activation leads to hydrolysis of arachidonic acid and generation of PGE$_2$ (39-42), we examined the link between PGE$_2$ and bone mineralization. BMSCs isolated from WT and KO were cultured, as above, in osteogenic media in the absence or presence of *S*-BEL. Following 4 days of culturing, media [PGE$_2$] was determined by ELISA. In comparison with non-osteogenic conditions, PGE$_2$ levels in the WT group rose nearly 3-fold under osteogenic conditions. Increases in PGE$_2$ accumulations, however, were not evident in the KO and were not significantly affected by *S*-BEL treatment (Fig. 2*A*). In contrast, *S*-BEL prevented PGE$_2$ increase in the WT group. In view of these findings, we assessed the impact of PGE$_2$ on mineralization in the iPLA$_2$β-deficient group. Supplementation of the osteogenic culturing media with PGE$_2$ (Fig. 2*B*) promoted dramatic mineralization, as viewed under bright field microscopy, in comparison with the lack of mineralization in the absence of PGE$_2$ addition (vehicle group). Consistent with this, alizarin red staining, a reflection of calcium deposition, in the KO is reduced, relative to WT, and is increased upon supplementation of the media with PGE$_2$ (Fig. 2*C*). Quantitation of alizarin red staining revealed a

progressive increase over a 28-day period with PGE_2 supplementation that was mimicked by addition of EP_4 agonist. Further, the staining was significantly reduced by co-incubation of the EP_4 receptor agonist with EP_4 receptor antagonist (Fig. 2*D*). These findings suggest that $iPLA_2\beta$ activation provides important lipid mediators that facilitate osteogenesis and that enhancing PGE_2 signaling through EP_4 receptor can rescue the lower bone phenotype that arises with $iPLA_2\beta$ deficiency.

$iPLA_2\beta$-derived lipids (iDLs) promote expression of osteogenic factors and osteogenesis-We next utilized primary calvarial osteoblasts to gain insight into the osteogenic pathway impacted by iDLs by assessing select osteogenic factors: Bone morphogenetic protein 2 (BMP2) is a TGFβ superfamily member that is important in skeletogenesis and essential for fracture repair. It is initiated in differentiating osteoblasts and is a hallmark of osteogenesis (43); alkaline phosphatase hydrolyzes pyrophosphate and provides inorganic phosphate to promote mineralization (43). Inorganic pyrophosphate, pyridoxal phosphate, and phosphoethanolamine are thought to be the physiologic substrates of alkaline phosphatase; and Runt-related transcription factor 2 (Runx2) is a master osteogenic transcriptional factor (44). For these analyses, calvaria-derived osteoblasts from WT and KO mice were prepared and cultured in the absence or presence of *S*-BEL, AA, or PGE_2.

As in BMSCs, calvarial $iPLA_2\beta$ mRNA was evident in WT, but undetectable in KO, as determined by RT-qPCR analyses, (Fig. 3*A*). All three osteogenic factors were reduced in the KO group (Figs. 3*B-D*, left panels), relative to the WT group, and such

decreases were recapitulated in the WT upon treatment with *S*-BEL. Moreover, supplementation of media provided to KO with AA or PGE_2 promoted significant recoveries in all three osteogenic factors (Figs. 3*B-D*, right panels), relative to un-supplemented media. In the case of *BMP2* and alkaline phosphatase, the effects of PGE_2 tended to be greater than with AA and were similar (*BMP2*) or higher (*alkaline phosphatase* and *Runx2*), relative to WT. These findings are consistent with a role for iDLs in modulating bone formation.

Time course of iPLA$_2\beta$ and Runx2 induction in calvaria-To further address the impact of iDLs on promoting bone mineralization, we focused on Runx2, the master regulator of osteogenesis. Initial examination of temporal induction of *iPLA$_2\beta$* and *Runx2* revealed a significant rise in both by 24 h followed by a decline by 36 h (Figs. 4*A-B*), which persisted up to 72 h (data not shown). In the KO group, there was no induction of $iPLA_2\beta$ and Runx2 remained unchanged and was similar to basal WT expression over 36 h.

Consistent with these findings, both alkaline phosphatase, reflecting induction of osteogenesis (Fig. 5*A*, left panel), and alizarin red staining, reflecting successful osteogenesis (Fig. 5*B*, left panel), were reduced in the $iPLA_2\beta$-deficient group. Supplementation of the media with either AA or PGE_2 promoted both alkaline phosphatase and alizarin red staining in the WT group. Because of recognized limitations in correlating alkaline phosphatase staining with mineralization alizarin red staining was quantified. Such analyses revealed significant increases in the $iPLA_2\beta$-deficient group, relative to un-supplemented WT group (Fig. 5*C*), with PGE_2

promoting a near complete recovery. Collectively, these findings strengthen the possibility that PGE_2 derived through $iPLA_2\beta$-mediated hydrolysis of AA is a key contributor to bone formation.

Selective inhibition of Runx2 expression-In view of its prominent role in osteogenesis and function as a transcription factor, we further addressed a potential link between Runx2 and $iPLA_2\beta$. As these analyses required larger abundances of cells, the osteoblast precursor cell line, MC3T3-E1, was utilized for subsequent analyses. The cells were cultured with control or osteogenic media for two days, in view of findings described in Fig. 4, prior to immunoblotting analyses. Relative to growth media, osteogenic media promoted an increase in $iPLA_2\beta$ (Fig. 6*A*). As expected, Runx2 was also induced with osteogenic media (Fig. 6*B*). In contrast, addition of *S*-BEL prevented increase in Runx2 expression above basal levels. However, supplementation of the media with PGE_2 rescued *S*-BEL-mediated decrease in Runx2 expression. These findings suggest that iDLs modulate Runx2 expression.

Transcriptional regulation by Runx2-To assess transcriptional regulation of Runx2 and $iPLA_2\beta$ during osteoblast differentiation, we performed chromatin immunoprecipitation (ChIP) assays with primers designed to examine both the *PLA2G6* and *Runx2* promoters. *In silico* analyses identified the *PLA2G6* promoter and determined that it has a putative binding site for Runx2 located within the first 2 kbs upstream of the $iPLA_2\beta$ transcriptional start site. When Runx2 was immunoprecipitiated, we detected binding at the *PLA2G6* promoter, which was not

influenced by osteogenic differentiation (Fig. 7*A*, left panel). As a positive control, we analyzed the *Runx2* promoter, which is known to be regulated by Runx2 (Fig. 7*B*, left panel). These data suggest Runx2 is present at the *PLA2G6* promoter, but may be insufficient to drive *PLA2G6* expression in the absence of other factors. To assess transcriptional activity, we used antibodies specific for acetylated histone 3 (AcH-H3). These data confirm that both the *PLA2G6* (Fig. 7*A*, right panel) and *Runx2* (Fig. 7*B*, right side) promoter have been modified to favor transcription; these data correlate with increased *PLA2G6* and *Runx2* mRNA and protein levels. As a negative control, we used antibodies specific for IgG; this antibody did not immunoprecipitate the *PLA2G6* or *Runx2* promoter (data not shown). These findings reveal that *PLA2G6* is induced at the transcriptional level along with *Runx2* under osteogenic conditions.

Transcriptional regulation by iPLA$_2\beta$- iPLA$_2\beta$ has never been reported to function as a transcription factor. However, it is detected in the nucleus and either it or its enzymatic products may influence transcriptional events. As such, we performed ChIP with antibodies specific for iPLA$_2\beta$. Surprisingly, we found that iPLA$_2\beta$ strongly associated with both *Runx2* (Fig. 8*A*, left panel) and *PLA2G6* (Fig. 8*B*, right panel) promoter regions during oseteogenic induction. These observations correlated with increased detection of AcH-H3 (Figs. 8*A-B*, right panels), suggesting active transcription of *Runx2 and PLA2G6*. IgG failed to significantly immunoprecipitate *PLA2G6* promoter (data not shown). These data are the first to demonstrate that nuclear iPLA$_2\beta$ regulates transcription during osteogenic differentiation.

DISCUSSION

Adult bone is remodeled continuously and the process of bone resorption must be balanced by the process of bone formation to maintain bone mass and preserve bone structure and strength. With aging, there is a loss in bone mass and strength and a consequent increase in risk of fractures (1,3,4,7,45-47). The cellular basis for age-related bone loss is multifactorial and may involve decreases in bone formation by osteoblasts or increases in bone resorption by osteoclasts (1,8,45,46). Prostaglandins (PGs) and other bioactive eicosanoids (products of arachidonic acid metabolism) are potent regulators of bone formation and bone resorption (48-50). The PGs are generated via cyclooxygenase (COX)-catalyzed metabolism of arachidonic acid following its hydrolysis from membrane phospholipids by phospholipases A_2 (PLA_2s) (51-57). The PLA_2s catalyze hydrolysis of the *sn*-2 substituent from glycerophospholipid substrates to yield a free fatty acid and a 2-lysophospholipid (58). The recognized PLA_2s are classified based on their Ca^{2+} requirement for activation and sequence homology (59) and include low molecular weight secretory ($sPLA_2$), high molecular weight cytosolic Ca^{2+}-dependent ($cPLA_2$), and high molecular weight cytosolic ($iPLA_2\beta$) and membranous Ca^{2+}-independent ($iPLA_2\gamma$) enzymes.

Studies to date have focused on the roles of $cPLA_2$ and $sPLA_2$ in regulating bone metabolism and they demonstrated that bone resorption induced by PTH (60), cadmium (61), or lipopolysaccharide (62) is associated with activation of $cPLA_2$; and by IL-1 (63)

or ovariectomy (64) with activation of $sPLA_2$. Further, bone loss due to inflammation is prevented in $cPLA_2$-deficient mice and due to decreases in estrogen by $sPLA_2$ inhibitors. These observations suggest that $cPLA_2$ and $sPLA_2$ participate in mediation of bone resorption induced by certain stimuli. However, no temporal relationship between PLA_2 activation and PGE_2 synthesis over the course of reduced osteoid mineralization was evident (65). In addition, inhibition of COX-2 did not prevent IL-1-induced decreases in mineralization (63). These finding suggest that other products of arachidonic acid metabolism (i.e., leukotrienes, HETEs, PAF) (66,67) participate in bone resorption.

Several reports, however, suggest that arachidonic acid has positive effects on bone formation. Supplementation of diets fed to piglets with arachidonic acid resulted in increases in whole body and bone mineral density (68) and bone mass (69,70), and decreases in bone resorption (12). Findings from other studies implicate PGE_2 as the mediator of arachidonic acid-induced bone formation (18,71-76). Among the bone-related cells, PGE_2 is produced mainly by osteoblasts (77,78) and has been reported to enhance bone formation at low doses but increase bone resorption at high doses (62,76,79-81). The differential effects of PGE_2 are thought to be related to the presence of multiple cell surface PG receptors (18,82-86).

Systemic or local injections of PGE_2 have also been shown to stimulate bone formation in rats (18,81) and in piglets (12), and reduce bone loss in rats due to disuse or orchidectomy (79,87,88). Lamellar bone formation in response to mechanical strain is mediated by COX-2 (48,89) and COX-$2^{-/-}$ mice have decreased bone density (90). PGE_2 induces bone nodules to form in BMSC cultures (81,91) and in cultured calvaria osteoblast (20,92-94), and osteoblastogenesis from bone marrow precursors (95). BMSCs isolated

from rats injected with PGE_2 for two weeks produce four times more mineralized bone nodules than control BMSCs (95). PGE_2 increases bone nodule formation in low density cultures of rat bone marrow cells by recruiting osteoblast precursors present in the bone marrow (96). Collectively, these findings suggest that PGE_2 promotes bone formation. The demonstration that PGE_2 can increase differentiation of osteoblastic cells (20,97,98) and enhance bone morphogenetic protein-induced osteoblasto-genesis and expression of *Runx2/cbfa1* and *osterix* (86), which are required for osteoblast differentiation, suggests a potential mechanism by which PGE_2 may contribute to bone formation.

PGE_2 produces its effects through interaction with four recognized PGE_2 receptor subtypes (EP1-4), that differ in the signal transduction pathway they trigger (99-102). *In vitro* (91,103,104) and *in vivo* (105,106) studies suggest that the bone anabolic effects of PGE_2 are mediated through the EP_4 receptor. Further, an EP_4 receptor-selective PGE_2 agonist was reported to stimulate cortical bone formation and restore bone mass and strength in aged OVX rats (107). Mesenchymal stem cells express COX-2 and EP_4 receptors and constitutively synthesize PGE_2 (82). The secreted PGE_2 has been shown to activate the EP_4 receptor leading to induction *of Runx2/cbaf1* and *osterix* (106) and BMP2 (82), which are all essential for bone formation.

In view of our earlier findings that $iPLA_2\beta$ deficiency promotes a low bone phenotype, we sought to determine the lipid signaling and potential underlying molecular mechanisms. Utilizing multiple models (BMSCs and calvarial osteoblasts from WT and $iPLA_2\beta$-deficient mice and MC3T3-E1 osteoblast cell line) and analyses (mineralization, mRNA, and protein), we find that (a) $iPLA_2\beta$ deficiency leads to significant reductions in osteogenesis and that this phenotype is recapitulated in WT preparations treated with a

selective inhibitor of iPLA$_2$β; (b) the low bone phenotype is associated with reduced production of PGE$_2$ and that the osteogenic capacity of iPLA$_2$β-deficient preparations is significantly restored by supplementation of the media with PGE$_2$; (c) expression of BMP2, alkaline phosphatase, and Runx2 is reduced with iPLA$_2$β deficiency, recapitulated in WT preparations by the iPLA$_2$β inhibitor, and rescued in iPLA$_2$β-deficient preparations with addition of PGE$_2$ or an EP$_4$ agonist and inhibited by an EP$_4$ antagonist; (d) induction of osteogenesis promotes parallel increases in transcription of *Runx2* and *PLA2G6* mRNA and Runx2 and iPLA$_2$β protein; (e) Runx2 expression is inhibited by iPLA$_2$β-selective inhibitor and rescued with addition of PGE$_2$; and intriguingly; (e) there is a strong association of iPLA$_2$β protein under osteogenic conditions with both *Runx2* and *PLA2G6* promoter regions.

Collectively, our findings reveal the importance of iPLA$_2$β-derived lipids in bone formation, where activation of iPLA$_2$β in osteoblasts leads to hydrolysis of arachidonic acid, which is then metabolized to PGE$_2$ by COX-2. The PGE$_2$ acting via EP$_4$ triggers signaling pathways that lead to induction of osteogenesis factors. The findings that the decreases in mineralization and associated osteogenic factor expression are evident in an iPLA$_2$β-deficient model, and that these outcomes are rescued by addition of arachidonic acid or PGE$_2$, and that expression of the master regulator, Runx2, is mitigated by an inhibitor of iPLA$_2$β, suggest that the predominant pool of PGE$_2$ that contributes to bone formation is derived through iPLA$_2$β-catalyzed hydrolysis of arachidonic acid from membrane glycero-phospholipids. The observation that the impact of PGE$_2$ supplementation was in general more profound than with arachidonic acid is consistent with the fact that arachidonic acid is a substrate for multiple metabolic pathways (108),

thus, only a fraction of it is likely converted to PGE_2. While the mechanism by which PGE_2 induces Runx2 was not examined here, a recent report offers a plausible explanation (109). In that study, PGE_2 signaling via EP_4 receptor promoted gene transcription. Additionally, localization of EP_4 in the nuclear envelope (110) and presence of COX and PGE_2 in the nuclear membrane (111) have been reported. It is therefore plausible to speculate that $iPLA_2\beta$-derived PGE_2, through EP_4 signaling, leads to *Runx2* induction.

As noted, the finding of $iPLA_2\beta$ presence at promoter regions of *PLA2G6* and *Runx2* was unexpected. These data offer the intriguing possibility that $iPLA_2\beta$ under certain stimulatory conditions can induce its own gene transcription. As $iPLA_2\beta$ is not recognized to be a transcription factor, it is most likely an indirect effect where $iPLA_2\beta$ recruits factors that facilitate transcription. Further detailed molecular studies are needed to discern the role of $iPLA_2\beta$ at the transcription level.

In summary (Fig. 9), or findings provide a scheme for how $iPLA_2\beta$ participates in bone formation. Under osteogenesis stimuli, $iPLA_2\beta$ is induced leading to accumulation of arachidonic acid and generation of PGE_2. Subsequent PGE_2-mediated signaling leads to induction of Runx2 (and $iPLA_2\beta$). It might be proposed that modulating the $iPLA_2\beta$-Runx2 link may be a means to improve bone health in diseased states associated with compromised bone formation.

EXPERIMENTAL PROCEDURES

Animals-Breeders (C57BL/6J background) obtained from Dr. John Turk (Washington University School of Medicine, St. Louis, MO) were used to generate wild-type (WT) and iPLA$_2$β-deficient mice at the University of Alabama at Birmingham (UAB), as described (112-115). PCR validation of genotype performed, using mRNA forward/reverse primers: ccaaacgactttggggagact /ctggatgccgaccatctcg (expected product size, 972 bp). Animal experiments were conducted according to approved Institution Animal Care and Use Committee (IACUC) guidelines at UAB.

Materials-Coomassie reagents, SDS-PAGE reagents, kaleidoscope pre-stained molecular mass standards, and Triton X-100 (161-0324, 161-0407; BioRad, Hercules, CA) ; *S*-BEL (iPLA$_2$□ inhibitor), PGE$_2$, and PGE$_2$ EIA Kit (10006801, 14010, and 514010; Cayman Chemicals, Ann Arbor, MI); paraformaldehyde (Electron Microscopy Sciences, Ft. Washington, PA); acetyl histone H3 antibody, normal mouse IgG, protein A beads (06-911, 12-371, 16-125; EMD Millipore, Billerica, MA); Collagenase 2, fetal bovine serum (FBS) and common cell culture reagents (17101-015; Gibco, Carlsbad, CA); Immobilin-P PVDF membrane (IPVH00010; Millipore Corp., Bedford, MA); Runx2 antibody (D130-3; MBL International, Wobrurn, MA); GAPDH, iPLA$_2$β (T-14), and tubulin antibodies, and

other 2° antibodies (FL-335, sc-14463, sc-2004, sc-2418, sc-2302 Santa Cruz Biotechnology Inc., Santa Cruz, CA); dispase, protease inhibitor cocktail, alpha-MEM media, common reagents (D4693, P8340; Sigma Chemical Co., St. Louis, MO); enhanced chemiluminescence reagent, (34095; Thermo-Fisher, Waltham, MA). EP_4 agonist ONO-328 and EP_4 antagonist ONO-208 were a kind gift from ONO Pharmaceuticals, Osaka, Japan.

Mineralization Analyses-(a) bright field microscopy was visualized on an Olympus IX81, (b) Von Kassa staining was performed on fixed cells with a 5% silver nitrate solution, rinsed well, and exposed to UV light, (c) alizarin red staining was performed on fixed cells with a 2% alizarin red solution, rinsed well, and scanned for imaging, and quantified using Image J software (National Institutes of Health).

Primary BMSC Isolation-6-week old female WT and iPLA$_2$β-deficient mice (KO) were euthanized and femurs surgically isolated. The femurs were transferred to a sterile bio-cabinet before being flushed with culture media (alpha-MEM with 10% FBS, 1% pen/strep). The media was then centrifuged and resuspended in fresh culture media. Plated cells were grown to confluence and differentiation (100 μg/ml ascorbic acid and 10 mM β-glycerol phosphate) or culture media applied. In some experiments, cells were treated with iPLA$_2$β-selective inhibitor *S*-BEL (10 μM), 30 min treatment at each media change), EP_4 receptor agonist (10 μM ONO-AE1-329), or EP_4 antagonist (10 μM ONO-AE3-208), cultured for up to 28 days and

osteogenesis assessed under bright field microscopy, Von Kassa staining, or alizarin red staining.

Primary Calvaria Isolation-4-day old female WT and iPLA$_2$β-deficient mice were euthanized and calvaria surgically isolated. The calvaria were transferred to a sterile bio-cabinet before being minced and digested. Digestion media consisted of dispase and collagenase, each at 6 mg/ml. Cells were plated and grown to confluence prior to exposure to differentiation or control media for 48 h and osteogenesis assessed under bright field microscopy and with alizarin red staining.

Cell Culture and Treatments-MC3T3-E1 cells, a gift from Dr. Greg Clines (University of Michigan, Detroit, MI), were cultured and maintained, as described (116). At confluence, cells were treated with vehicle (DMSO), or AA or PGE$_2$ (each at 10 μM, applied at time of differentiation) and osteogenesis assessed under bright field microscopy, alkaline phosphatase, or alizarin red staining.

Protein Analyses-Cells were harvested, aliquoted, and analysed by SDS-PAGE. Resolved proteins were transferred from a 10% gel onto Immobilin-P PVDF membranes for immunoblotting analyses. Immunoreactive bands were visualized by ECL, as described (117).

RNA Isolation and RT-qPCR-Cells were treated with vehicle (DMSO), osteogenic media for 48 h. Total RNA was isolated, as described (37), and 1 μg of

total RNA was reverse-transcribed and analyzed by RT-qPCR using primers sets, based on known mouse sequences (**Table 1**). Reactions for each sample were performed in triplicate using a PCR protocol (95 °C activation for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min) in an ABI StepOnePlus Detection System (Applied Biosystems, Foster City, CA). Delta Ct values for genes examined were determined using Ct values generated by StepOnePlus software (Applied Biosystems). HPRT was used as the housekeeping control.

Chromatin Immunoprecipitation (ChIP) Analyses-ChIP assays were performed with 5 μg of antibodies directed against iPLA$_2$β, RunX2, AcH-H3, or IgG, as described (118). Immune complexes were absorbed with protein A beads blocked with salmon sperm DNA. After pre-clearing and before immunoprecipitation, equal amounts of sonicated DNA (10% volume of each sample) were reserved for qPCR (input) analysis. *PLA2G6* and *Runx2* promoters were probed with specific primers against the immunoprecipitated DNA by qPCR using primers sets based on known mouse sequences (**Table 2**), based on known mouse sequences. Reactions for each sample were performed as before, comparing input and immunoprecipitation samples.

Statistical Analyses-Data are presented as means ± standard error of the means (SEs). Statistical significances between groups were determined using Student's *t*-test. Values of $p < 0.05$ were considered significant.

Author contributions-WDH designed and performed experiments, analyzed data, prepared figures, and wrote the paper. YGT bred and genotyped mice. SEN provided expertise with the ChIP analyses. SR conceived and coordinated the study, analyzed data, contributed to preparation of the figures, and edited the paper.

Acknowledgements-We thank the support of the Department of CDIB, UAB Center for Metabolic Bone Disorders, UAB Comprehensive Diabetes Center and Diabetes Research Center, and Dr. Amjad Javed for offering advice on the RT-qPCR analyses.

Conflict of interest statement-The authors declare that they have no conflicts of interest with the contents of this article.

Footnotes:

[1]Abbreviations: 18S, 18S ribosomal RNA; 12-lipoxygenase (12-LO); Ac-H3, acetylated histone H3; AA, arachidonic acid; BMCSs, bone marrow stromal cells; BMP2, bone morphogenetic protein 2; ChIP, chromatin immunoprecipitation; COX, cyclooxygenase; cPLA$_2$, cytosolic phospholipase A$_2$; HA3, Anti-acetyl-Histone H3; HPRT, hypoxanthine phosphoribosyltransferase; IgG, immunoglobulin G; iPLA$_2$beta, group VIA phospholipase A$_2$β; iPLA$_2$γ, group VIA phospholipase A$_2$gamma; KO, knockout; PCR, polymerase chain reaction; PGE$_2$, prostaglandin E$_2$; PLA$_2$, phospholipase A$_2$; RT-qPCR, real time quantitative PCR; Runx2, Runt-related transcription factor 2; *S*-BEL, *S*-enantiomer of bromoenol lactone; WT, wild type

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FIGURE LEGENDS

FIGURE 1. **Bone Phenotype of WT and iPLA$_2$β-deficient BMSCs.** Bone marrow stromal cells (BMSCs) from WT and iPLA$_2$β-deficient (KO) mice were harvested and subsequently exposed to osteogenic induction (Ost) media. *A*, Validation of iPLA$_2$β-deficient genotype via PCR. *B*, BMSCs were exposed to culture or Ost media for 28 days and mineralization was assessed under bright field microscopy. *C*, Bone nodule formation. WT and iPLA$_2$β-deficient BMSCs were cultured with Ost media for 28 days without and with *S*-BEL (10 μM) and bone nodule formation was assessed by Von Kassa staining.

FIGURE 2. **Rescue of iPLA$_2$β-deficient bone mineralization phenotype.** *A*, PGE$_2$ production. Bone marrow stromal cells (BMSCs) were harvested from WT and iPLA$_2$β-deficient (KO) mice and cultured in control media or osteogenic induction (Ost) media ± *S*-BEL (10 μM). At 4 days, media accumulations in PGE$_2$ were determined by ELISA. (*, WT group significantly different from all other groups, $p < 0.05$). Data are the means ± SE (4-5 independent measurements). *B*, Osteogenesis ± PGE$_2$ (10 μM). BMSCs harvested from iPLA$_2$β-deficient mice were cultured in osteogenic media without and with PGE$_2$ supplementation and visualized under bright field microscopy. *C*, Mineralization ± PGE$_2$, representative images are shown. *D*, Mineralization quantitation. BMSCs harvested from KO mice were cultured in media supplemented

with PGE_2 (10 μM), EP_4 agonist (10 μM), or EP_4 antagonist (10 μM). Alizarin red staining was quantified by ImageJ analysis. Data are the means ± SE (3 independent measurements. (*, Antagonist group significantly different from agonist group, $p < 0.05$.)

FIGURE 3. **Impact of $iPLA_2\beta$-derived lipids on osteogenic factors in calvaria.** Calvarial osteoblasts were prepared from 4-day old female WT and $iPLA_2\beta$-deficient (KO) mice. *A*, Genotype verification. The cells were processed for $iPLA_2\beta$ RT-qPCR. *B-D*, Osteogenic factors. WT cells were cultured with osteogenic media in the absence or presence of *S*-BEL (10 μM) and KO cells in the absence or presence of arachidonic acid (AA, 10 μM) or PGE_2 (10 μM) at each media change. The cells were then processed for RT-qPCR analyses for *BMP2* (*B*), *alkaline phosphatase* (*C*), and *Runx2* (*D*). *HPRT* was used as housekeeping control. Data are the means ± SE (3 independent measurements). (#, KO group significantly different from age-matched WT group, $p < 0.05$; *, WT treatment-group significantly different from age-matched WT vehicle-treated group, $p < 0.05$; †, KO supplemented groups significantly different from vehicle-treated KO group, $p < 0.05$.)

FIGURE 4. Time course of $iPLA_2\beta$ and Runx2 induction in calvarial osteoblasts. Calvarial osteoblasts prepared from WT and $iPLA_2\beta$-deficient (KO) mice were cultured in osteogenic induction media for up to 36 h. At 12, 24, and 36 h the calvarial cells were processed for RT-qPCR analyses of *$iPLA_2\beta$* (*A*) and *Runx2* (*B*). *HPRT* was used as

housekeeping control. Data are the means ± SE (3 independent measurements). (*WT group significantly different from corresponding 0 h groups, $p < 0.05$.)

FIGURE 5. **Effects of AA and PGE_2 on mineralization in calvarial osteoblasts.** Calvaria of WT and $iPLA_2\beta$-deficient mice (KO) were harvested and cultured with osteogenic induction (Ost) media with vehicle (DMSO), AA (10 μM), or PGE_2 (10 μM) for 7 days and stained for alkaline phosphatase (*A*) or alzarin red (*B*). *C*, Quantitation of alizarin red staining. Data are the means ± SE (3 independent measurements). (#, KO group significantly different from age-matched WT group, $p < 0.005$; *, KO treatment-groups significantly different from age-matched KO vehicle-treated group $p < 0.005$. n = 3.)

FIGURE 6. **Expression of $iPLA_2\beta$ and Runx2 protein during osteogenesis.** MC3T3-E1 cells exposed to osteogenic induction (Ost) media for 2 days with vehicle, *S*-BEL, or PGE_2. *A and B (left panels),* Representative immunoblots of $iPLA_2\beta$, Runx2, and GAPDH. *A and B (right panels)* quantification of $iPLA_2\beta$ and Runx2, relative to corresponding GAPDH, expression. Data are the means ± SE (3 independent measurements). (*****, Ost group significantly different from Veh group, $p < 0.05$.)

FIGURE 7. **Association of Runx2 with the *Runx2* and *PLA2G6* promoter during osteogenic induction.** MC3T3-E1 cells were treated with vehicle (Veh) or cultured with osteogenic induction (Ost) media for 2 days. *A*, ChIP analyses of the *PLA2G6* promoter

region when immunoprecipitated for Runx2 (left panel) or AcH-H3 (right panel). *B*, ChIP analyses of the *Runx2* promoter region when immunoprecipitated for Runx2 (left panel) or AcH-H3 (right panel). Data are representative analyses from 3-4 independent measurements.

FIGURE 8. **Association of iPLA$_2$β with the *Runx2* and *PLA2G6* promoter regions during osteogenic induction.** MC3T3-E1 cells were treated with vehicle (Veh) or cultured with osteogenic induction (Ost) media for 2 days. *A*, ChIP analyses of the *Runx2* promoter region when immunoprecipitated for iPLA$_2$β (left panel) or AcH-H3 (right panel). *B*, ChIP analyses of the *PLA2G6* promoter region when immunoprecipitated for iPLA$_2$β (left panel) or AcH-H3 (right panel). Data are representative analyses from 3-4 independent measurements.

FIGURE 9. **Proposed model of iPLA$_2$β involvement in Runx2-mediated induction of osteogenesis.** Potential mechanism of action of iPLA$_2$β in modulating *Runx2* through iDL production and mediation of nuclear events in differentiating osteoblasts.

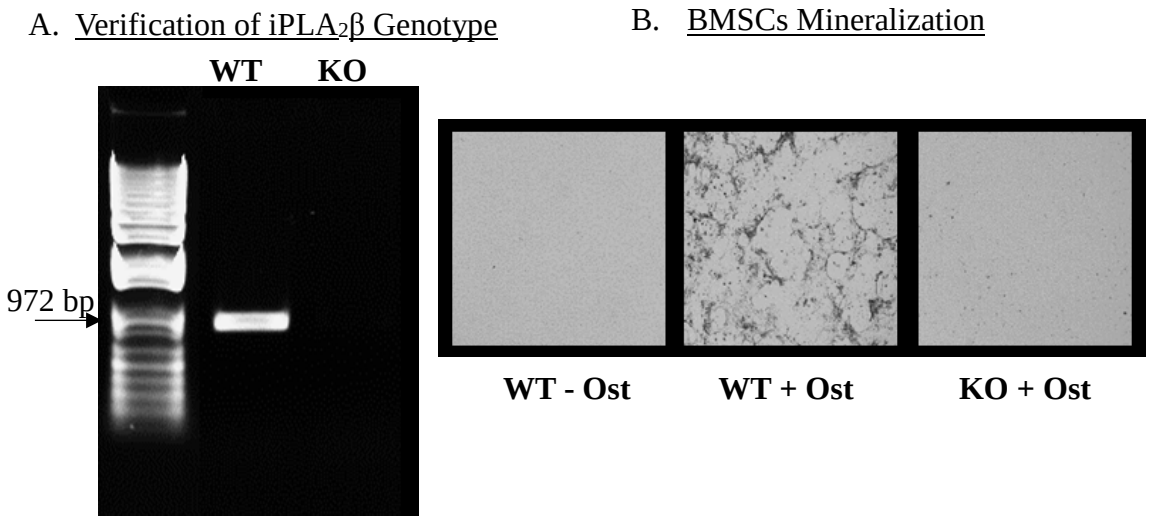
Table 1. Primers for Targets Analysed by RT-qPCR

Target	Sequence (5' to 3')	Product Size (bp)
HPRT	**gcagcgtttctgagccattg**	**165**
	taatcacgacgctgggactg	
iPLA$_2$β	**tgtctctggggacaggaaa**	**264**
	cagcactgcatcactgacct	
Runx2	**atcagttcccaatggtacccg**	**215**
	atcagttcccaatggtacccg	
Alkaline Phosphatase	**ttgtgccagagaaagagagaga**	**075**
	gtttcagggcatttttcaaggt	
BMP2	**gggtggcgagagcttttcta**	**101**
	ttcagagtggttgtcaatccg	

Table 2. ChIP Primers for Targets Analysed by RT-qPCR

Target	Sequence (5' to 3')	Product Size (bp)
PLA2G6 **Promoter**	**tacagggccacactggtcac**	**489**
	atgggcagttcacatgatcg	
Runx2 **Promoter**	**tgacgccatagtccctcctt**	**284**
	ccaaccgagtcagtgagtgc	

Figure 1. Bone Phenotype of WT and iPLA$_2$β-Deficient BMSCs



C. Inhibition of iPLA$_2$β Mimics iPLA$_2$β-Deficient Phenotype in WT

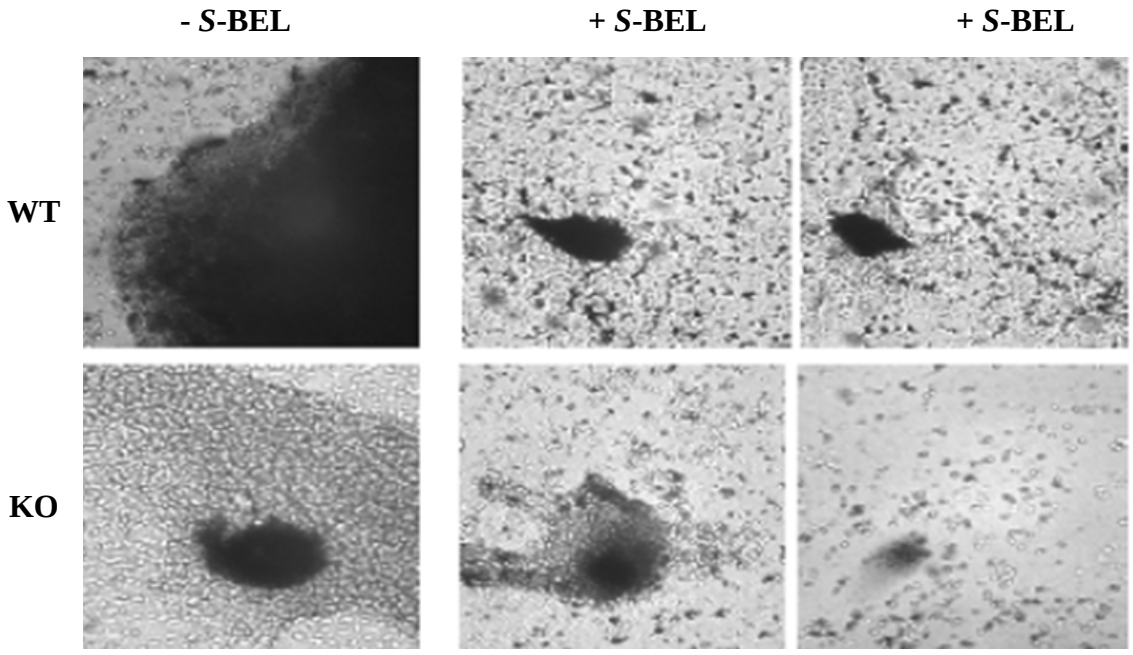


Figure 2. Rescue of iPLA$_2$β-Deficient Bone Phenotype with PGE$_2$ Supplementation

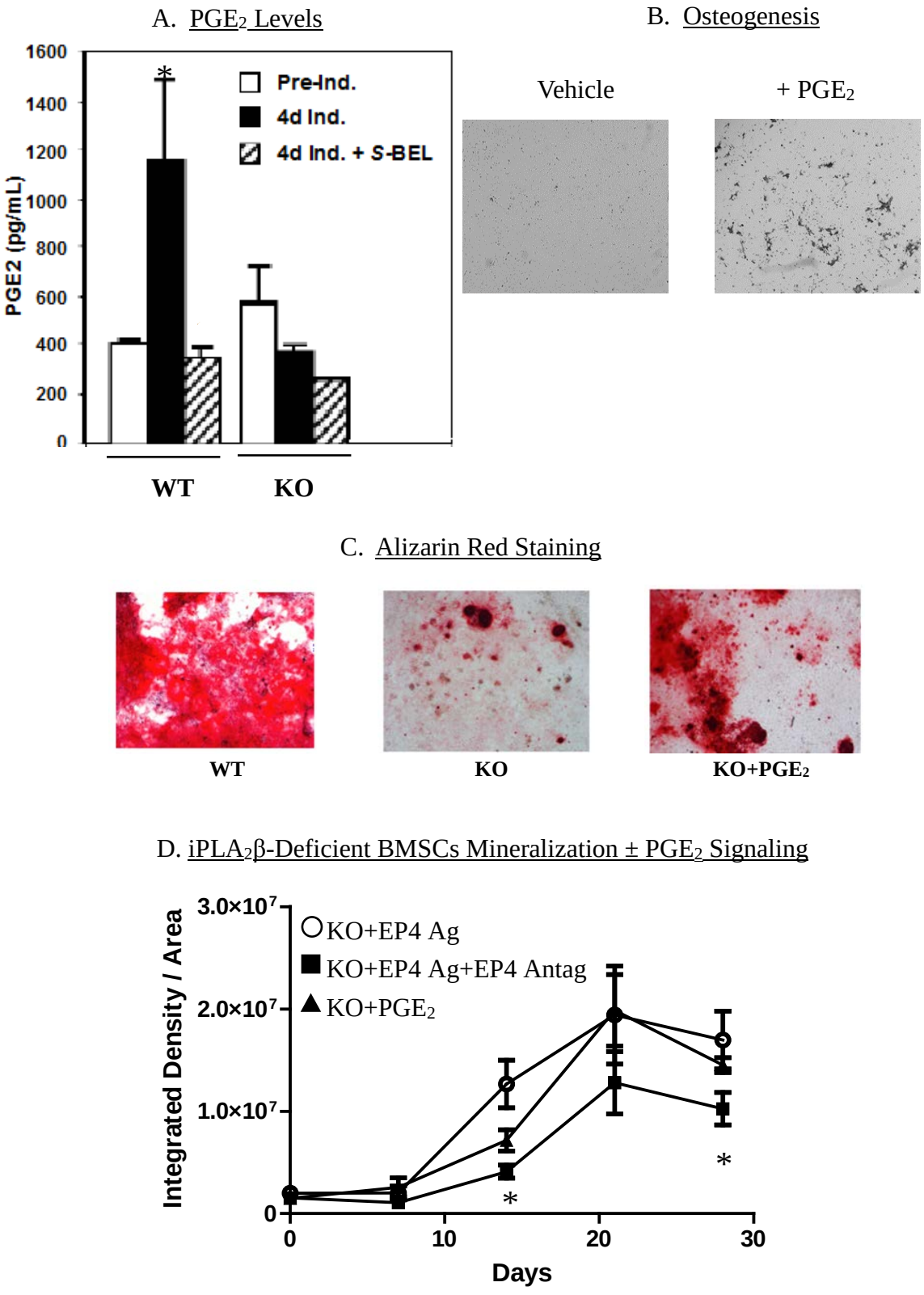
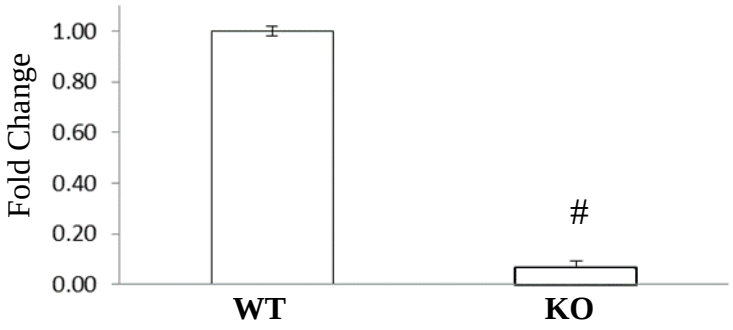
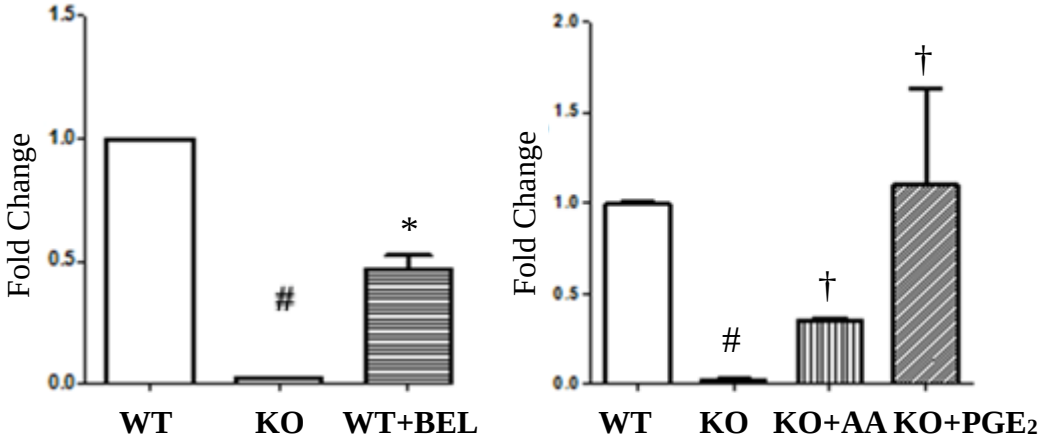


Figure 3. Impact of iPLA$_2$β-Derived Lipids on Osteogenic Factors in Calvaria

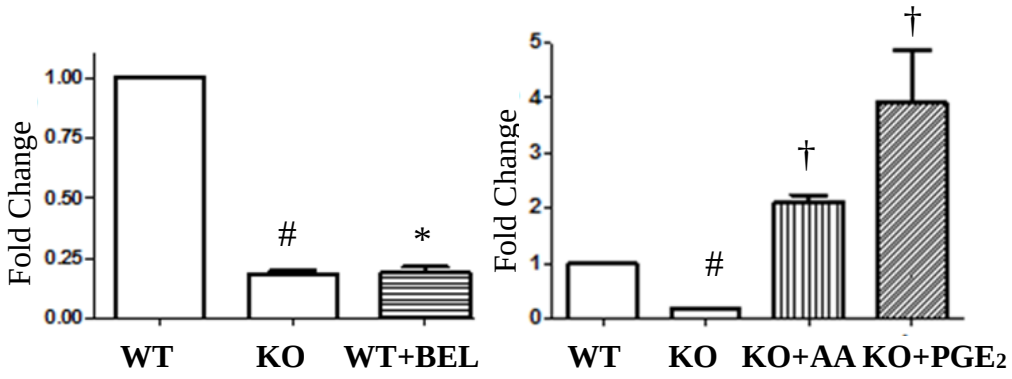
A. *iPLA$_2$β*



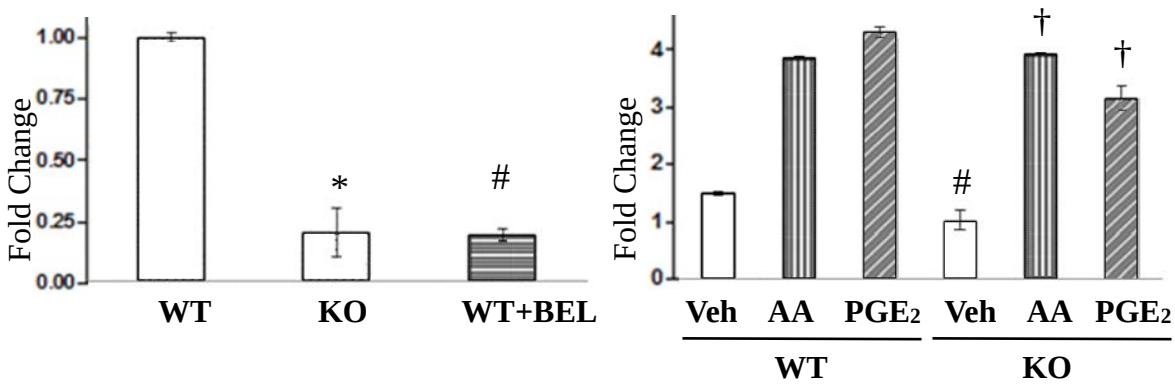
B. *BMP2*



C. *Alkaline Phosphatase*



D. *Runx2*


Fold Change
1.00
0.75
0.50
0.25
0.00
*
#
WT
KO
WT+BEL
Fold Change
4
3
2
1
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Veh
AA
PGE2
Veh
AA
PGE2
WT
KO

Figure 4. Time Course of iPLA$_2$β and Runx2 Induction in Calvarial Osteoblasts by Osteogenic Media

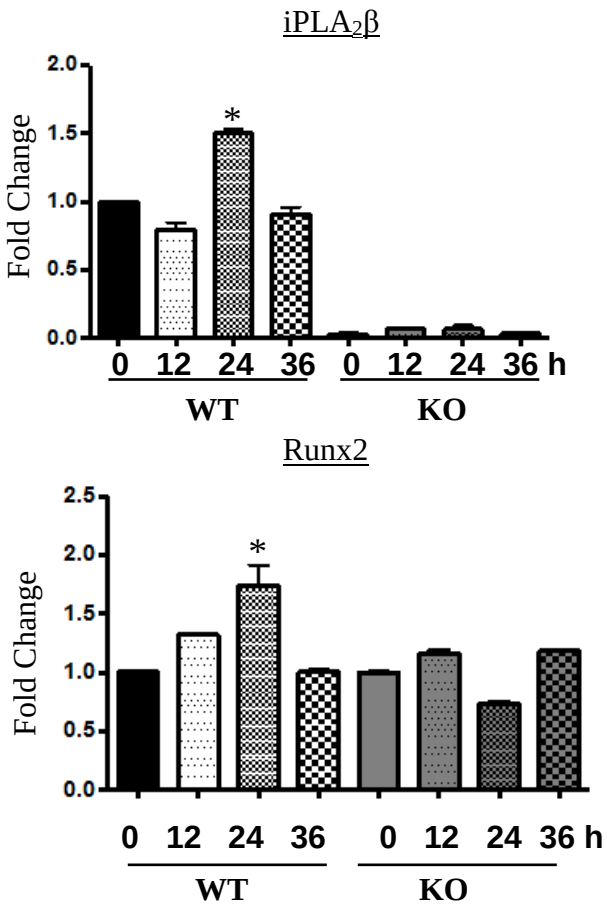
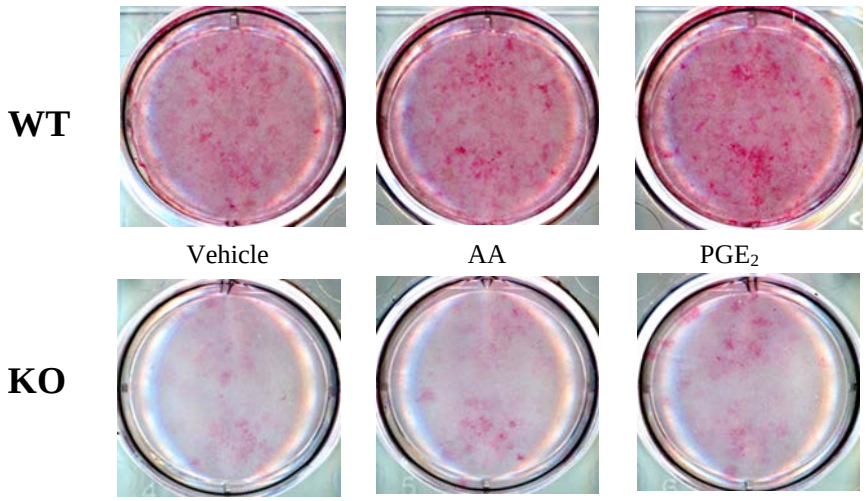
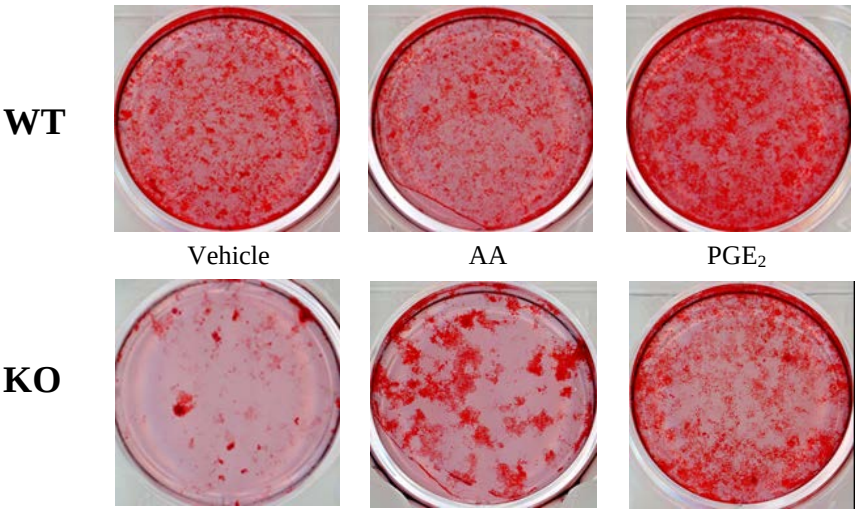


Figure 5. Effects of AA and PGE_2 on Mineralization in Calvarial Osteoblasts

A. Alkaline Phosphatase Staining



B. Alizarin Red Staining



C. Alizarin Red Staining Quantification

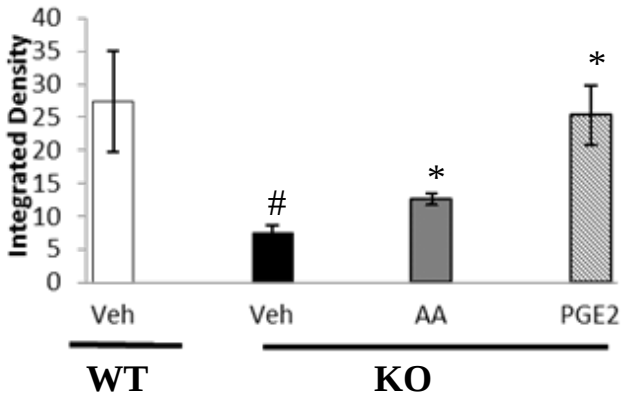
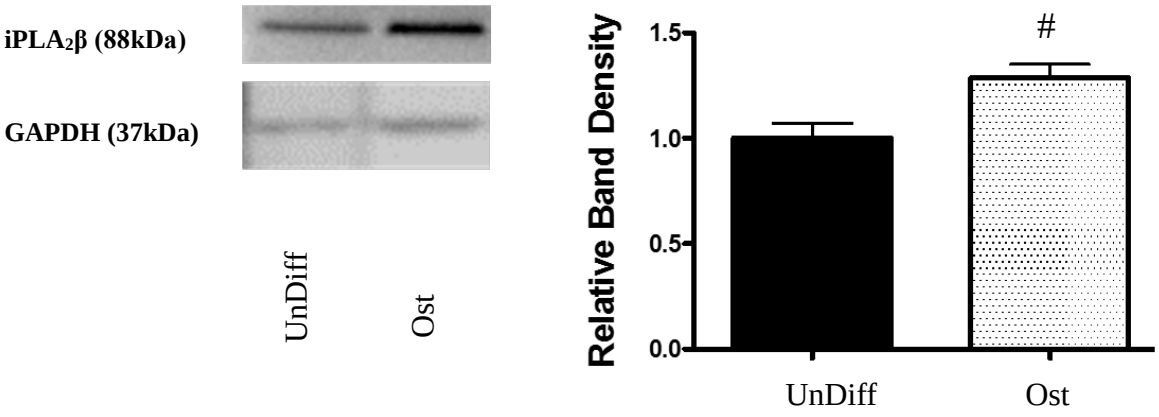


Figure 6. Expression of Protein by MC3T3 cells During Osteogenesis

A. Representative Western Blotting of iPLA$_2$β



B. Representative Western Blot of Runx2

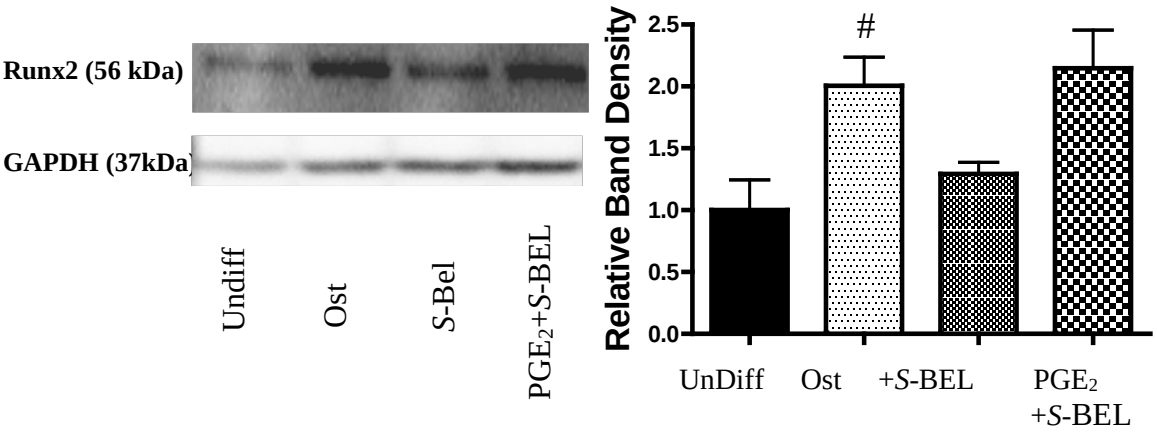
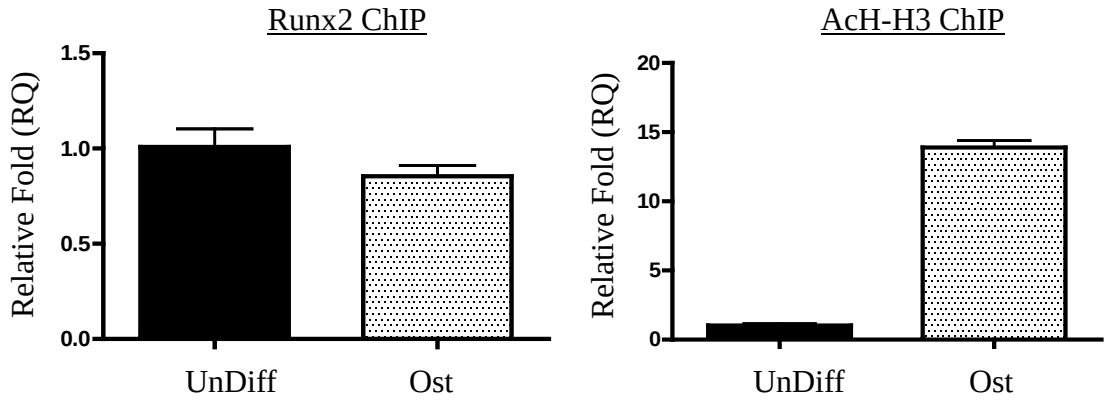


Figure 7. Association of Runx2 with the *Runx2* and *PLA2G6* Promoter During Osteogenic Induction

A. *PLA2G6* Promoter



B. *Runx2* Promoter

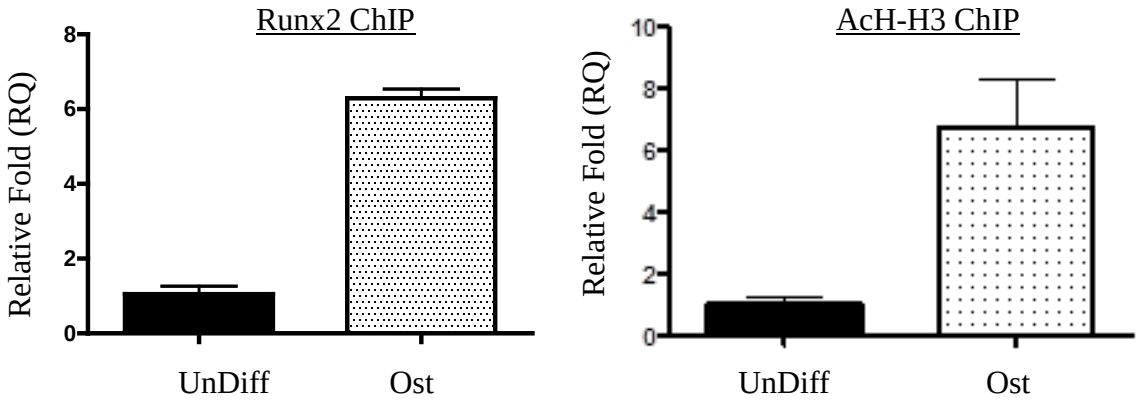
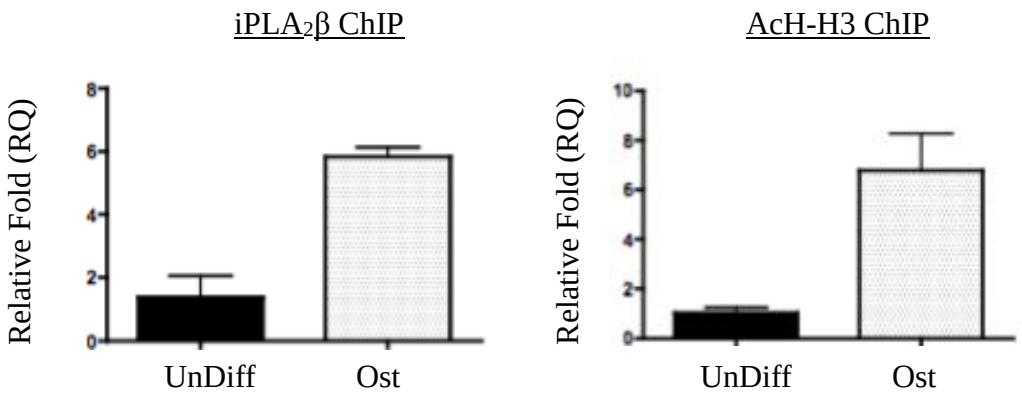


Figure 8. Association of iPLA$_2$β with the *Runx2* and *PLA2G6* Promoter Regions During Osteogenic Induction

A. *Runx2* Promoter



B. *PLA2G6* Promoter

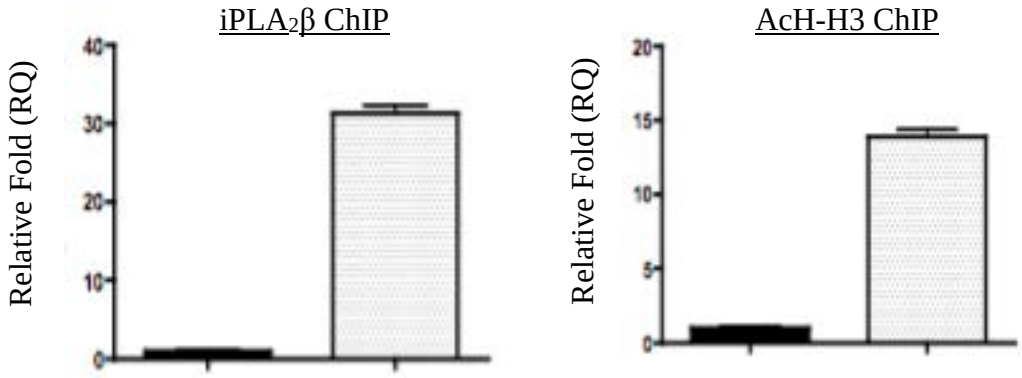
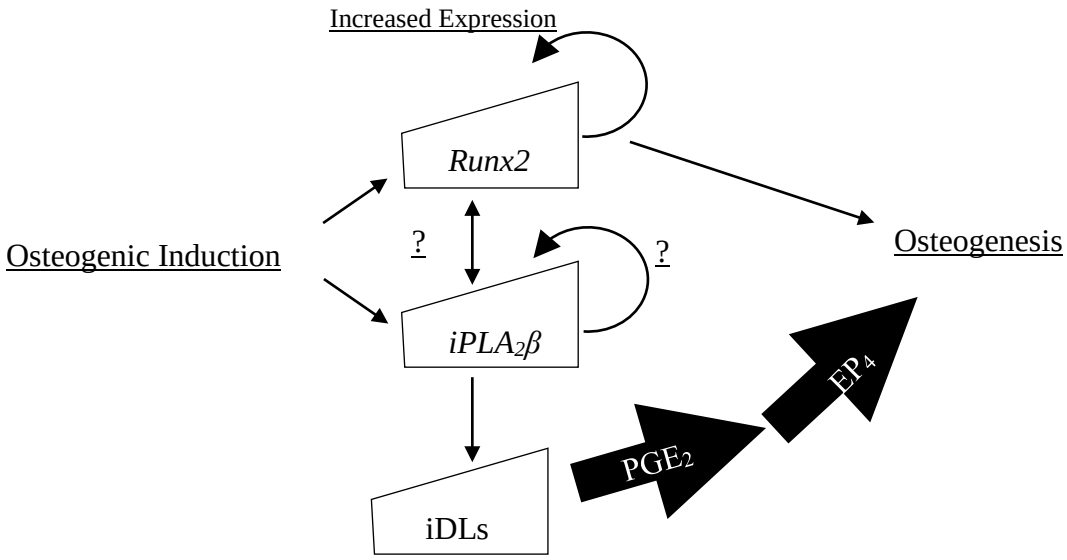


Figure 9. Proposed Model of iPLA$_2$β Involvement in Runx2-Mediated Induction of Osteogenesis



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Ca^{2+}-INDEPENDENT PHOSPHOLIPASE A_2 BETA AT THE DNA

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ABSTRACT

The group VIA calcium-independent phospholipase A_2 beta ($iPLA_2\beta$) has been shown to translocate to the nucleus. Nuclear localization for phospholipases have been explored since the 1970s, with implications in cell cycle control, proliferation, differentiation, and apoptosis. Current studies demonstrate that nuclear localization of $iPLA_2\beta$ may be interacting with complexes at the chromatin.

INTRODUCTION

A cursory survey of the profoundly intricate actions of cells will make clear the need for a tight regulation of gene transcription; often relying on bewildering complex mechanisms of control. Types of cells, even subtypes of the same differentiated cells, will rely heavily on a gene of interest within one context; finding expression of the same gene antagonistic or dire-consequence inducing in other

contexts. Initially, studies of transcriptional regulation displayed a comparatively simple method of maintaining order. Sequences of DNA in 6 to 20 base pairings, located within several kilobases of transcription initiation sites, exhibit binding to transcription factors activating gene expression(2). Potentially, any gene's expression could be predicted based solely on transcription factor binding site and the relevant transcription factor expression; yielding an understanding of the developmental regulation of expression in all cells.

Of course, the true nature of gene regulation exhibits bewildering complexity in place of this simple picture. Controlling sequences interact with an entire milieu of proteins and protein complexes, transcription factors gain functionality as multimers of unique polypeptide chains; different combinations of subunits are able to yield different functions. Combinations of control sequences produce varying expression patterns differing markedly from the individual effects of the separate control sequences(3). The diversity of combinations in transcription factors and control sequences marks the starting point for this exploration of the nuclear activity of iPLA$_2$β.

While primarily cytosolic, stimulation can induce iPLA$_2$β to localize to a variety of sub-cellular compartments. From the ER and the nucleus of β cells to leading-edge of the plasma membrane in migrating macrophages, iPLA$_2$β exhibits localization to drive multiple cellular functions (4). In previous work, immunoaffinity, activity, and immunofluorescence evidence for expression and

localization of iPLA$_2$β to the nucleus has been shown in transfected INS-1 cells(1). **Figure 1A** shows immunoaffinity within lysates from INS1 cells overexpressing iPLA$_2$β in cell homogenate (CH), nuclear extracts (NE); not shown are control preparations showing no iPLA$_2$β in particulate fractions nor antibody control peptide preparations (1).

Figure 1B illustrates the activity of iPLA$_2$β within these fractions through a radiometric assay utilizing radiolabeled phospholipids. Again these experiments illustrate the localization to the nucleus of iPLA$_2$β with maintained phospholipase activity (1).

iPLA$_2$β visibly localizes to the nucleus when observed with immunoflouresence; under stimulation of glucose, iPLA$_2$β over-expressing INS1 cells display both cytosolic and nuclear localizations. The potential function within the nucleus could easily be remodeling of the inner leaflet of the nuclear membrane. With no nucleotide binding domain visible *in silico*, involvement beyond membrane remodeling and generation of lipid signaling molecules in the nucleus is unclear.

EXPERIMENTAL PROCEDURES

Materials-Coomassie reagents, and Triton X-100 (161-0324; BioRad, Hercules, CA) ; paraformaldehyde (Electron Microscopy Sciences, Ft. Washington, PA); acetyl histone H3 antibody, normal mouse IgG, protein A beads (06-911, 12-371; EMD Millipore, Billerica, MA); fetal bovine serum (FBS), common cell culture reagents (17101-015; Gibco, Carlsbad, CA); GAPDH, IgG, iPLA$_2$β (T-14) antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA); protease inhibitor cocktail, alpha-MEM media, common reagents (D4693, P8340; Sigma Chemical Co., St. Louis, MO).

Cell Culture and Treatments-MC3T3 cells, a gift from the laboratory of Dr. Greg Clines (University of Michigan, Detroit, MI), were cultured and maintained as described (5).

Chromatin Immunoprecipitation (ChIP) Analyses-ChIP assays were performed with 5 ug of antibodies directed against iPLA$_2$β, Ac-H3, or IgG, as described (6). Immune complexes were absorbed with protein A beads blocked with salmon sperm DNA. After pre-clearing and before immunoprecipitation, equal amounts of sonicated DNA (10% volume of each sample) were reserved for qPCR (input)

analysis. *PLA2G6* promoters were probed with specific primers against the immunoprecipitated DNA by qPCR using primers sets based on known mouse sequences (**Table 1**), based on known mouse sequences. Reactions for each sample were performed as before, comparing input and immunoprecipitation samples.

RESULTS

iPLA$_2$β in Chromatin Immunoprecipitation

Implicating iPLA$_2$β as present in the nucleus, but without knowing the function therein, the role of iPLA$_2$β influence on osteoblast differentiation and function at the DNA was examined. Analysis of promoter regions of iPLA$_2$β, due to a lack of known DNA-binding sequences or DNA binding partners for a complex involving iPLA$_2$β. Chromatin immunoprecipitation assays were performed to expose potential interaction at the chromatin of lysates from undifferentiated and osteogenically induced MC3T3-E1 cells. These immunoprecipitations were then probed for promoter regions of interest, as determined by *in silico* analysis of the mouse sequences.

A representative iPLA$_2$β chromatin immunoprecipitation did bind the iPLA$_2$β promoter region **(Fig. 3)**; at the 48 hour timepoint previously described. Anti-acetyl-Histone H3 (HA3) antibody identifies acetylated histone H3, a positive control for open histones; this is an indicator of open transcription. This also illustrates active transcription of iPLA$_2$β. The negative control for antibodies used, IgG, showed no effect.

DISCUSSION

Previous reports indicate the presence of iPLA$_2$β in the nucleus. Several potential roles within the nucleus are possible, complexation with DNA is a novel and exciting new role that iPLA$_2$β is playing in regulating important cell functions.

Unique distinction between iPLA$_2$β and other PLA$_2$s is the presence of a variable number of Ank repeats in iPLA$_2$β, as mapped in Figure 4(7). Ank regions may confer iPLA$_2$β protein-protein activity. The active form of iPLA$_2$β appears to be an oligomer of interacting protein subunits, as supported by radiation inactivation and gel filtration chromatography analyses that reveal association of the 85 kDa iPLA$_2$β activity with an apparent molecular mass of 250–350 kDa (8-10). The active form of iPLA$_2$β may be an oligomer. The 85 kDa subunits could associate with each other via their Ank repeat regions (9); similar to the involvement of Ank repeats in other protein-protein

interactions (11). In a long isoform of human iPLA$_2$β, a proline-rich insert interrupts the last iPLA$_2$β Ank repeat with some similarities to the Smad4 domain that mediates interactions with signaling partners (12).

This raises the possibility that the proline-rich insert in human iPLA$_2$β allows it to interact with other proteins. While direct interaction with the DNA is difficult to include as a possible function, requiring a novel DNA binding motif, the formation of a complex that incorporated other binding partners remains a strong possibility.

Figure 1. iPLA2β Presence in Sub-Cellular Fractions

A. Western Blotting of Sub-Cellular Fractions

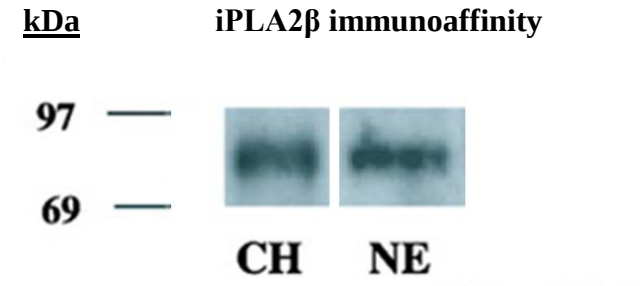

Figure 1. INS1 cells over expressing $iPLA_2\beta$ were fractionated and probed for subcellular localization of $iPLA_2\beta$ via (A) SDS-PAGE/Western Blotting (B) radiometric measures of $iPLA_2\beta$ activity (1).

(CH, cellular homogenate; NE, nuclear extract)

B. Enzymatic Activity of Sub-Cellular Fractions

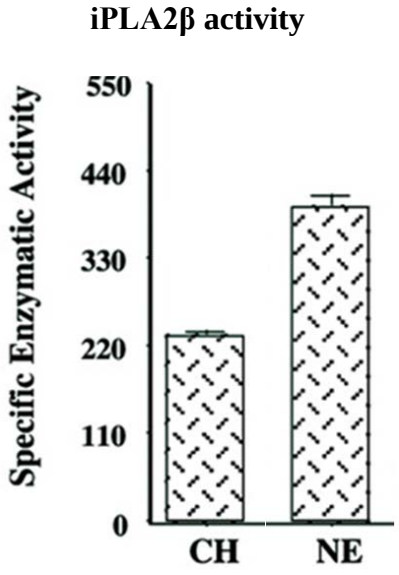

Figure 2: Visualization of iPLA2β in Cells by Confocal Microscopy

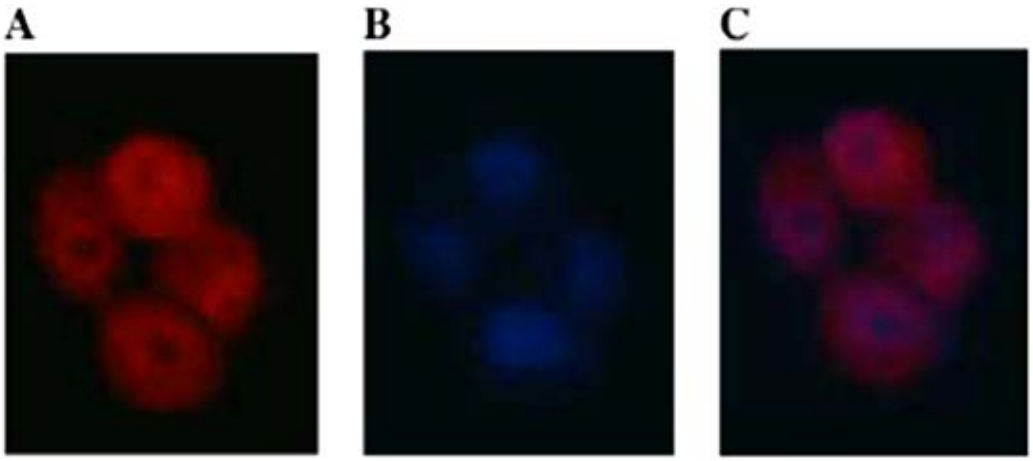

Figure 2. INS1 cells over expressing $iPLA_2\beta$, stimulated with 2mM glucose, were imaged with confocal microscopy and probed for $iPLA_2\beta$ fluorescence (red) was visualized with a rhodamine filter, and the nuclear fluorescence (blue) was visualized with a DAPI filter with a Nikon Inverted Scope. (1)

Figure 3: Effects on iPLA2β promoter During Osteogenic Induction of MC3T3 Cells

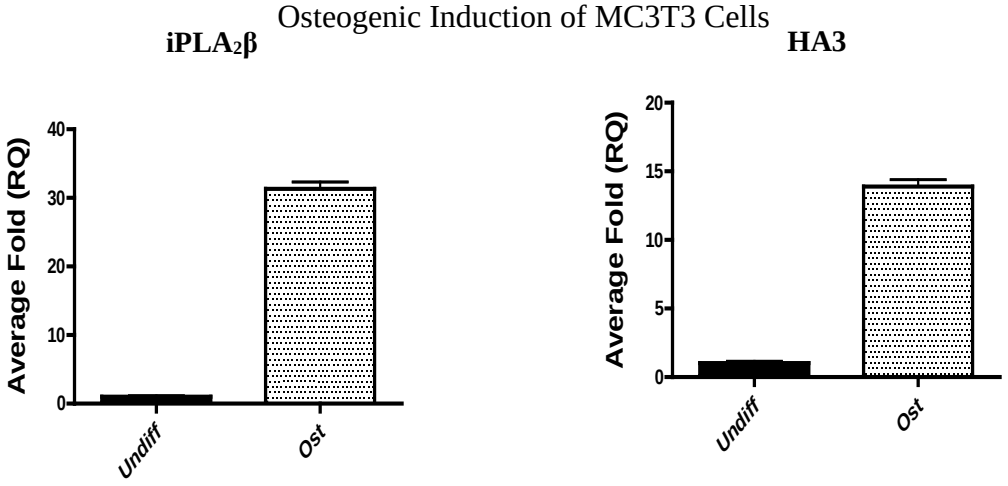

Figure 3. MC3T3 cells in growth media (Undiff) and osteogenic media (ost) were lysed and nuclei prepared. These nuclei were probed for (a) $iPLA_2\beta$, (b) acetylated histone 3, or (c) normal IgG binding at the chromatin.

Figure 4: Schematic of iPLA2β

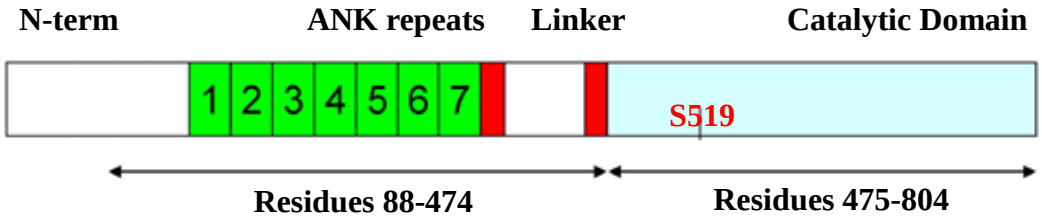

Figure 4. Schematic representation of iPLA$_2$β, illustrating the seven ankyrin repeats in green; the eighth ankyrin repeat (depicted in red) is interrupted by a 54-residue insertion (white). The catalytic serine (519) within the catalytic domain is marked.

Table 1. ChIp Primers for Targets Analysed by RT-qPCR

Target	**Sequence (5' to 3')**	**Product Size (bp)**
PLA2G6 Promoter	tacagggccacactggtcac	489
	atgggcagttcacatgatcg	

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DISCUSSION

iPLA$_2$β and Osteogenesis

Remodeled continuously, adult bone undergoes resorption balanced by osteogenesis. This process ensures bone mass, structure, and strength are maintained throughout life. Under aging, a negative balance forms as a loss in bone mass and strength; this effect is concomitant with increases in risk of fractures and subsequent fatalities (221-223). The principle cells of bone turnover are mineralizing osteoblasts and resorptive osteoclasts; the cellular mechanisms driving age-related bone loss is multifactorial and may involve changes in bone formation rate of osteoblasts or increases in bone resorption by osteoclasts (224,225). While osteoblasts and osteoclasts crosstalk, the parity of bone turnover becomes unhinged with age.

Bioactive eicosanoids are potent regulators of bone formation and bone resorption (226,227). Prostaglandins are among the principal eicosanoids of interest in the bone milieu. Prostaglandins are generated via cyclooxygenase-catalyzed metabolism of arachidonic acid; this follows arachidonic acid hydrolysis from membrane phospholipids by phospholipases A2 (PLA2s) (228-231). Catalyzing the liberation of the sn-2 substituent from glycerophospholipid substrates, PLA2s derives a 2-lysophospholipid and

a free fatty acid (232). PLA2s are codified by their Ca2+ necessities for activation and sequence (233). These include low molecular weight secretory (sPLA2), high molecular weight cytosolic Ca2+-dependent (cPLA2), and high molecular weight cytosolic and membranous Ca2+-independent (iPLA2β) enzymes.

Previous works have centered on the roles of cPLA2 and sPLA2 as mitigators of bone turnover; important studies have demonstrated that bone resorption can be perturbed by activation of both cPLA2 and sPLA2. cPLA2-deficient mice experience a rescue of bone loss due to inflammation. Bone loss due to decreases in estrogen are ameliorated by sPLA2 inhibitors. These results offer that cPLA2 and sPLA2 are involved in mediation of bone resorption induced by other stimuli. These studies exhibited no temporal relationship between PLA2 activation and PGE2 synthesis amid the course of failing osteoid mineralization was evident (234). Additionally, suppression of COX-2 activity did not prevent IL-1-induced decreases in mineralization (235). These results allude to other products of arachidonic acid metabolism (236,237) play a role in bone resorption processes.

Reports have suggested that arachidonic acid can have positive effects on bone formation. Piglets with a diet supplemented with arachidonic acid yielded higher bone mineral density (238) and bone mass (239,240); they also exhibited decreases in bone resorption (241). Other studies provide support for PGE_2 serving as the principal metabolite of arachidonic acid-induced bone effects (242-244). Within the bone microenvironment, PGE_2 is primarily produced by osteoblasts (245,246). PGE_2 has been

reported, with low doses, to enhance bone formation; however, it increases bone resorption at high doses (247,248). This interesting dichotomy is proposed to be a product of activity at multiple cell surface prostaglandin receptors (243,249,250).

PGE_2, both systemically and locally injected, has been shown to stimulate bone formation in rats (243,248) and in piglets (241). PGE_2 also lessened bone loss in rats due to unloading or orchidectomy (251,252). The quality of lamellar bone formation, as a result of mechanical strain, shows mediation by COX-2 (253). COX-2 deficient mice exhibit reduced bone density (254). PGE_2 enhances bone nodule formation in bone marrow stromal cell cultures (255) and in primary culture calvarial osteoblast (256). Osteoblastogenesis from mesenchymal precursors is also enhanced in PGE_2 treatment (257). Bone marrow stromal cells from rats, having undergone a two week PGE_2 injection scheme, were able to produce four times more mineralization than control isolates (257). This literature supports the potential of PGE_2 in promoting bone formation and as a target for expanding understanding of osteoblasts.

The impact of PGE_2 on differentiation potential of pre-osteoblasts (258-260), combined with the impact on expression of Runx2/cbfa1 (261) implies a possible mechanistic connection. Runx2 is widely considered the “master regulator” of osteoblast differentiation. PGE_2 interacts with four recognized receptor subtypes (EP_{1-4}); these receptors initiate separate signal transduction pathways (262-265). *In vitro* (255,266,267) and *in vivo* (268,269) reports offer that the bone anabolic effects of PGE_2 are directed through activity at the EP4 receptor. An EP4 receptor-selective PGE_2 agonist enhanced

cortical bone formation, also restoring bone mass and strength OVX rats at age (270). Mesenchymal stem cells express COX-2 and EP4 receptors; also constitutively synthesizing PGE_2 (249). Secreted PGE_2 has exhibited activation of the EP_4 receptor leading to induction of Runx2/cbaf1 (269) and BMP2 (249), both are essential for healthy bone formation.

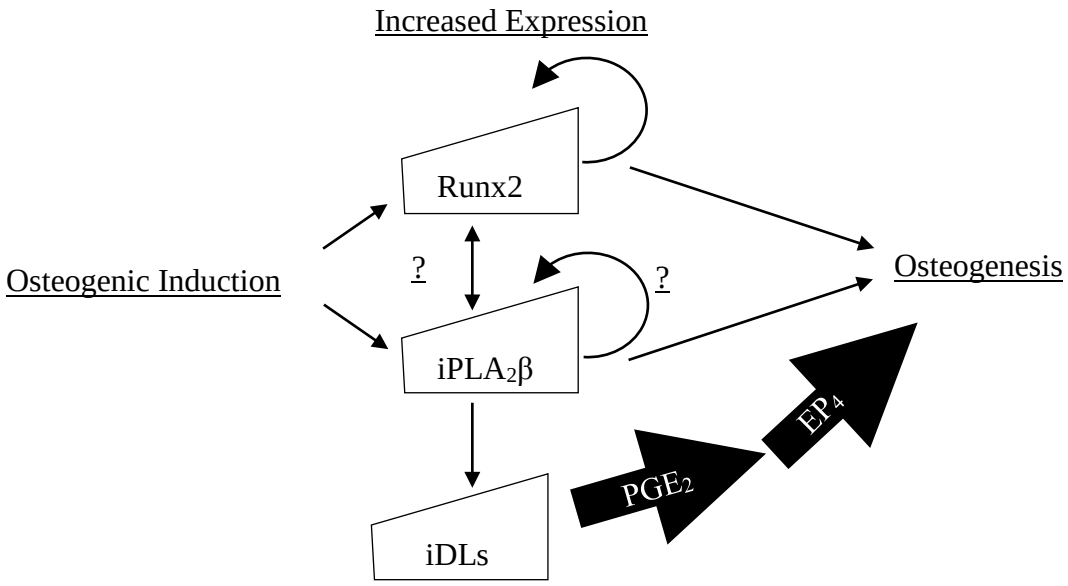
In view of the previous reports of iPLA2β-deficiency producing a low bone phenotype, this thesis aimed to further elucidate the impact lipid signaling could have on osteogenesis; also hoping to interrogate the potential underlying molecular mechanisms. Exploring different primary models for osteogenesis, bone marrow stromal cells and calvarial osteoblasts from WT and iPLA2β-deficient mice provided a unique tool to explore the relationship of phospholipase derived lipid signals. The MC3T3-E1 pre-osteoblast cell line provided a robust means to accentuate the primary cultures. Analyses like mineralization staining, RNA message, and protein quantification, elucidated that (a) $iPLA_2\beta$ deficiency leads to significant reductions in osteogenesis and that this phenotype is recapitulated in WT preparations treated with a selective inhibitor of $iPLA_2\beta$; (b) the low bone phenotype is associated with reduced production of PGE_2 and that the osteogenic capacity of $iPLA_2\beta$-deficient preparations is significantly restored by supplementation of the media with PGE_2; (c) expression of BMP2, alkaline phosphatase, and Runx2 is reduced with iPLA2β deficiency, recapitulated in WT preparations by the $iPLA_2\beta$ inhibitor, and rescued in $iPLA_2\beta$-deficient preparations with addition of PGE_2 or an EP4 agonist and inhibited by an EP4 antagonist; (d) induction of osteogenesis promotes parallel increases in transcription of Runx2 and PLA2G6 and Runx2 and

iPLA$_2$β protein; (e) Runx2 expression is inhibited by iPLA$_2$β-selective inhibitor and rescued with addition of PGE$_2$; and intriguingly; (e) there is a strong association of iPLA2β protein under osteogenic conditions with both Runx2 and PLA2G6 promoter regions.

Collectively, these findings demonstrate the role of iPLA$_2$β-derived lipids in osteogenesis; activation of iPLA$_2$β in osteoblasts initiates hydrolysis of arachidonic acid, which is then metabolized to PGE$_2$ by COX-2. PGE$_2$ acting via EP$_4$ receptor triggers signaling pathways that lead to induction of osteogenesis factors. The observations of decreases in mineralization and associated osteogenic factor expression in the iPLA$_2$β-deficient model represent a clear phenotype. The subsequent rescue of these outcomes by addition of iPLA$_2$β-derived lipids arachidonic acid or PGE$_2$ furthers the elucidation that iPLA$_2$β is at work in osteogenesis. The expression of master regulator Runx2 is mitigated by an inhibitor of iPLA$_2$β extends these observations to suggest that an impactful sum of iPLA$_2$β-derived PGE$_2$ contributes to bone formation. The derivation of this PGE$_2$ is through iPLA$_2$β-catalyzed hydrolysis of arachidonic acid from membrane glycerophospholipids in osteoblasts. The observation that PGE$_2$ supplementation provided a greater effect than arachidonic acid is consistent with the variety of potential fates for arachidonic acid, as a substrate for multiple metabolic pathways (271) would provide an effectively reduced concentration of PGE$_2$. The mechanism by which PGE$_2$ induces Runx2 was not examined herein, recent literature offers a plausible explanation (272). Within that paper, PGE2 signaled via EP$_4$ receptor, promoting gene transcription; also, localization of EP$_4$ to the nuclear envelope (273) and presence of COX and PGE$_2$

inside the nuclear membrane have been reported (274). These data converge on the speculation that the iPLA$_2$β-derived PGE$_2$, acting through the EP4 receptor signals induction of Runx2 activation.

Figure 1. iPLA$_2$β Influence and Osteogenesis



Nuclear Involvement of iPLA$_2$β

iPLA$_2$β has been reported to localize to the nucleus under stimulation (275). Potential roles within the nucleus include complexation with DNA-binding proteins and deriving lipid-signaling molecules from the inner leaflet of the nuclear envelope. Lacking a known DNA-binding homology domain, the expectation is that iPLA$_2$β would require binding to a partner protein to have a direct effect on DNA transcription. iPLA$_2$β has a

unique distinction among other PLA2s, it contains a variable number of ankyrin repeats; these commonly accompany sites of protein-protein interaction. These ankyrin regions may confer iPLA2β protein-protein activity required to associate with DNA to affect transcription. Active iPLA2β appears as an oligomer of interacting protein subunits, this is supported by radiation inactivation and gel filtration chromatography analyses; this data reveals an association of the 85 kDa iPLA2β activity with an apparent molecular mass of 250–350 kDa (271,276-278). Active forms of iPLA2β may contain an oligomer of 85 kDa subunits; with the subunits associating with each other via their ankyrin repeat regions (277), similar to the involvement of ankyrin repeats in other protein-protein interactions (279). A long isoform of human iPLA2β contains a proline-rich insert, interrupting the last iPLA2β ankyrin repeat domain. This bears some similar qualities with the Smad4 domain that mediates interactions with signaling partners (280).

Tantalizingly, this offers the possibility that the ankyrin motif and the proline-rich insert in iPLA2β allows it to interact with other proteins in a mechanism involving modulation and multiple binding partners. The finding of $iPLA_2\beta$ presence at promoter regions of PLA2G6 and Runx2 was an unexpected. This offers the possibility that $iPLA_2\beta$, under certain stimulatory conditions, can induce its own gene transcription. Most likely an indirect effect, $iPLA_2\beta$ may recruit factors that facilitate transcription; further detailed molecular studies are needed to discern the role of iPLA2β at the transcription level.

Extensions

Bone

While our findings provide for iPLA$_2$β involvement in osteogenesis; the disease relevance for this study requires greater investigation. Finding iPLA$_2$β derived lipids, via PGE$_2$-mediated signaling, lead to induction of Runx2 (and iPLA$_2$β) provides an exploitable link from lipid signaling to osteogenesis for use in bone disease. Osteoporosis and osteopenia are the most common causes of disease-related fractures. Disease-related fractures result in over 1.5 million incidents each year. 10 million patients in the United States have osteoporosis; 33.6 million have osteopenia. By the year 2020, it is projected that 1 in 2 Americans over age 50 will be at risk for osteoporosis of the hip; the hip being a critical site for fracture presents more than the vertebra and forearm fractures common in osteoporosis. It is possible to propose that modulating the transcriptional iPLA$_2$β-Runx2 link may be a means to improve bone health in diseased states associated with compromised bone formation.

The Nucleus

An exciting way to link lipid metabolism with epigenetic changes may be iPLA$_2$β generating free fatty-acids within the nucleus for synthesis of the acetyl groups needed to modulate histones. The relaxation of chromatin is accomplished by addition of acetyl groups to available lysine residues in the N-terminus of histone subunits in the nucleosome. These acetyl groups are donated by acetyl coenzyme A inside the nucleus; the addition of acetyl groups to histone tails provides a changes the overall charge from positive to neutral. Neighboring histone subunits have a prevalent negative charge that must be mediated for tight packaging of the nucleosome; this is achieved by predominately positive charge for the histone tail. Shifting this positive charge to neutral frees the histone tail from the neighboring subunit to allow relaxation of the nucleosomal fiber. This relaxation allows greater access to the chromatin; enabling promoter recognition and initiation of transcription. The provision of acetyl groups for this purpose is critical to proper regulation of gene transcription. Histone acetyltransferases are the enzymes responsible for linking the acetyl groups to histone tails. These enzymes are directed by large multi-protein assemblies towards substrate specificities and histone targets. These large complexes could be an area of iPLA$_2$β association.

Pyruvate dehydrogenase complex, ATP citrate lyase, and acyl-CoA synthetase short-chain family member 2 are three known metabolic pathways to shown to work in generation of Acetyl coenzyme A in the nucleus. While unorthodox, the generation of acetyl groups from free fatty acids outside the mitochondria may take place; this could be derivatively localized to histone acetyltransferase activity within large complexes known

to drive specificity in histone acetylation. This would provide an interesting new avenue for exploring epigenetic regulation mediated by iPLA$_2$β.

Another extension of the translocation of iPLA$_2$β to the nucleus, under stimulation, could be to drive changes in gene expression indirectly via eicosanoid generation. As eicosanoids can be made within the nucleus; they are also known to signal there. Examples include, eicosanoids like the 5-hydroxyeicosatetraenoic acid family; these act as ligands for nuclear receptors, like the peroxisome proliferator-activated receptors. Also evidence shows the eicosanoid leukotriene C_4 can signal a receptor inside eosinophils; moreover, a leukotriene C_4 receptor has been identified within the nucleus of adenocarcinomas cells. Additionally, the direct product of 5-lipoxygenase metabolism, Leukotriene A_4, has been found to covalently bind to DNA. These actions support the hypothesis that specific eicosanoids interact with targets within the nucleus; and these interactions are biologically meaningful. Derivative to this hypothesis is the corollary that generation of these eicosanoids, within the nucleus, could be a potent effect of iPLA$_2$β.

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APPENDIX

Institutional Animal Care and Use Committee Approval

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM
Institutional Animal Care and Use Committee (IACUC)

MEMORANDUM

DATE: 04-Oct-2016

TO: Ramanadham, Sasanka

FROM: 

Robert A. Kesterson, Ph.D., Chair

Institutional Animal Care and Use Committee (IACUC)

SUBJECT: **NOTICE OF APPROVAL**

The following application was approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on 04-Oct-2016.

Protocol PI: Ramanadham, Sasanka

Title: iPLA2b-Derived Lipid Signals and Bone Integrity

Sponsor: UAB DEPARTMENT

Animal Project Number (APN): IACUC-09775

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), is registered as a Research Facility with the USDA, and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

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