LYSOPHOSPHATIDIC ACID, VITAMIN D, AND P53: A NOVEL SIGNALING AXIS IN CELL DEATH AND DIFFERENTIATION

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LYSOPHOSPHATIDIC ACID, VITAMIN D, AND P53: A NOVEL SIGNALING AXIS IN CELL DEATH AND DIFFERENTIATION

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To my husband, Sean

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LIST OF ABBREVIATIONS

$1,25(OH)_2D_3$	1,25-dihydroxyvitamin D3
$24R,25(OH)_2D_3$	24R,25-dihydroxyvitamin D3
AA	Arachidonic Acid
AGK	Acyl Glycerol Kinase
ATX/lysoPLD	Autotaxin/lysophospholipase D
CHEL	Chelerythrine
COMP	Cartilage Oligomeric Matrix Protein
ColII	Collagen Type 2
ColX	Collagen Type 10
DAG	Diacyl Glycerol
Edg	Endothelial Differentiation Gene
GC	Growth/Proliferative Zone Chondrocytes
GPCR	G-Protein Coupled Receptor
LPA	Lysophosphatidic Acid
LPA1-6	LPA Receptors 1-6
LPC	Lysophosphatidylcholine
LPE	Lysophosphatidylethanolamine
LPP	Lipid Phosphate Phosphatase
LPS	Lysophosphatidylserine
MAG	Monoacyl Glycerol
Mdm2	Murine Double Minute-2
OMPT	(2S)-1-Oleoyl-2-O-Methyl-Glycero-3-Phosphothionate
PA	Phosphatidic Acid

PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PGE2	Prostaglandin E2
Pi	Inorganic Phosphate
PI_3K	Phosphoinositol 3-Kinase
PKC	Protein Kinase C
$PLA_{1/2}$	Phospholipase A1/2
PLC	Phospholipase C
PLD	Phospholipase D
PPAR-γ	Peroxisome proliferator-activated receptor-gamma
PS	Phosphatidylserine
RC	Resting/Reserve Zone Chondrocytes
VPC32183(S)	((S)-Phosphoric acid mono-{2-octadec-9-enoylamino-3-[4-(pyridine-2-ylmethoxy)-phenyl]-propyl} ester)

SUMMARY

Lysophosphatidic acid (LPA) is a bioactive acid lysophospholipid that regulates a wide array of cellular processes such as migration, proliferation, inhibition of apoptosis, wound healing, and morphological changes through activation of G-protein coupled receptors. The overall goal of this thesis was to understand the mechanisms by which LPA enhances cell survival by inhibiting apoptosis. The project was divided into three studies: 1) to determine the mechanism of LPA-mediated inhibition of p53 in A549 lung carcinoma cells, 2) to investigate the regulation of growth plate chondrocytes by LPA, and 3) to determine the mechanisms of LPA-mediated effects in the growth plate.

In the first study, evidence is provided that LPA reduces the cellular abundance of the tumor suppressor p53 in A549 lung carcinoma cells, which express endogenous LPA receptors. The LPA effect depends upon increased proteosomal degradation of p53 and it results in a corresponding decrease in p53-mediated transcription. Inhibition of phosphatidylinositide 3-kinase (PI_3K) protected cells from the LPA-induced reduction of p53, which implicates this signaling pathway in the mechanism of LPA-induced loss of p53. LPA partially protected A549 cells from actinomycin D induction of both apoptosis and increased p53 abundance. These results identify p53 as a target of LPA action and provide a new dimension for understanding how LPA stimulates cancer cell division, protects against apoptosis, and thereby promotes tumor progression.

In the second study, the role of LPA in resting zone chondrocytes (RC cells) was investigated. RC cells are regulated by 24,25-dihydroxyvitamin D3 [$24,25(OH)_2D_3$] via a phospholipase D-dependent pathway, suggesting downstream phospholipid metabolites

are involved. In this study, we showed that 24R,25$(OH)_2D_3$ stimulates rat costochondral RC cells to release LPA. RC cells also expressed LPA receptors 1-5 (LPA1-5) and the LPA-responsive intracellular fatty acid receptor peroxisome proliferator-activated receptor-gamma (PPAR-γ). LPA and the LPA1/3-selective agonist OMPT increased proliferation and maturation markers. The anti-apoptotic effects of LPA and 24R,25$(OH)_2D_3$ were inhibited by the LPA1/3-selective antagonist VPC32183(S). Furthermore, apoptosis induced by either inorganic phosphate (Pi) or chelerythrine was attenuated by LPA. LPA prevented apoptotic signaling by decreasing the abundance, nuclear localization, and transcriptional activity of p53. LPA treatment also regulated the expression of the p53-target genes Bcl-2 and Bax to enhance cell survival. Collectively, these data suggest that LPA promotes differentiation and survival in RC chondrocytes, demonstrating a novel physiological function of LPA signaling.

In the final study, the mechanism of LPA and 24R,25$(OH)_2D_3$-mediated inhibition of chondrocyte apoptosis was further investigated. We found that both 24R,25$(OH)_2D_3$ and LPA rescue of Pi-induced caspase-3 activity. The actions of 24R,25$(OH)_2D_3$ were dependent upon $G_{\alpha i}$, LPA1/3, phospholipase D (PLD), phospholipase C (PLC), and calcium (Ca^{++}); whereas, those of LPA were dependent on $G_{\alpha i}$ and PI_3K signaling and nuclear export. 24R,25$(OH)_2D_3$ decreased both p53 abundance and p53-medaited transcription and inhibited Pi-induced cytochrome c translocation. Moreover, LPA induced an increase mdm2 phosphorylation, a negative regulator of p53. Taken together, these data show that 24R,25$(OH)_2D_3$ inhibits Pi-induced apoptosis through Ca^{++}, PLD, and PLC signaling and through

LPA/LPA1/$G_{\alpha i}$/PI_3K/mdm2-mediated degradation of p53, resulting in decreased caspase-3 activity.

CHAPTER 1

BACKGROUND AND SIGNIFICANCE

1.1 Lysophosphatidic Acid (LPA) and LPA Receptors

Lysophosphatidic acid (LPA) (1-acyl-2-*sn*-glycerol-3-phosphate) is a small, serum-borne lysophospholipid. It was first isolated in *E. coli* and identified as an intermediary in glycerol lipid metabolism (Pieringer and Hokin 1962). Early work demonstrated a role for LPA in platelet aggregation, establishing LPA as a biologically active lipid (Gerrard, Kindom et al. 1979; Tokumura, Fukuzawa et al. 1981). Now, LPA is recognized as a versatile, bioactive lysophospholipid that elicits broad phenotypic effects such wound healing, morphological changes, migration, proliferation, and cell survival (Chun 1999; Goetzl, Kong et al. 1999; Fang, Yu et al. 2000; Fukushima, Weiner et al. 2000; Inoue, Nagano et al. 2001; Deng, Wang et al. 2003; Zhang, Baker et al. 2004) (Table 1.1). LPA is produced by activated platelets and a number of cancer cells (Borges, Davanzo et al. 1998; Eder, Sasagawa et al. 2000) and exerts its effects on cells by activating the cell surface G-protein coupled receptors (GPCRs) LPA1/Edg2, LPA2/Edg4, LPA3/Edg7, LPA4/GPR23/P2Y9, LPA5/GPR92, and LPA6/P2Y5 (Goetzl and An 1999; McIntyre, Pontsler et al. 2003; Noguchi, Ishii et al. 2003; Lee, Rivera et al. 2006; Yanagida, Masago et al. 2009). These receptors collectively stimulate the $G_{\alpha i}$ (LPA1,2,3), $G_{\alpha q}$ (LPA1,2,3,5), $G_{\alpha s}$ (LPA5), and $G_{\alpha 12/13}$ (LPA1,2,5) signaling pathways (Fukushima, Kimura et al. 1998; Chun 1999; Anliker and Chun 2004; Lee, Rivera et al. 2006; Lee, Rivera et al. 2007) (Fig. 1.1).

LPA GPCRs can be divided into two groups: Endothelial Differentiation Gene (Edg) family receptors and the P2Y family receptors. LPA1 (Edg2), LPA2 (Edg4), and

LPA3 (Edg7) are members of the Edg family. Other members of the Edg GPCR family (Edg1, 3, 5, 6, and 8) respond to sphingosine 1-phosphate (S1P) (Hla 2001; Takuwa, Takuwa et al. 2002). Edg family LPA receptors share sequence homology with one another and contain a conserved intron that codes for transmembrane domain 6 (Takuwa, Takuwa et al. 2002; Anliker and Chun 2004). Additionally, LPA1 and LPA2 contain C-terminal protein-protein binding motifs that facilitate LPA-mediated actions (Lin and Lai 2008; Murph, Nguyen et al. 2008). Of the Edgs, LPA1 is the most abundantly expressed LPA receptor and it is found in almost every tissue (An, Bleu et al. 1998; Anliker and Chun 2004). LPA2 and LPA3 are less widely distributed. LPA2 is expressed primarily in the lungs, kidney, and nervous system, whereas LPA3 is found in the heart, brain, lungs, and kidneys (Takuwa, Takuwa et al. 2002). Collectively, the Edg receptors have captured the interest of cancer researchers as potential therapeutic targets. LPA1-3 receptor antagonists are under investigation for use as chemotherapeutic agents (Chang, Kim et al. 2007; Murph and Mills 2007; Ma, Matsumoto et al. 2009).

As previously stated, LPA also stimulates some P2Y GPCR family members. One such P2Y GPCR is LPA4, also know as P2Y9 (Noguchi, Ishii et al. 2003). LPA4 is only ~25% homologous with the Edg LPA receptors, suggesting a different evolutionary ancestor. In comparison to LPA1-3, little is known about the function of LPA4. However, recent studies have implicated LPA4 in embryogenesis and morphological changes (Yanagida, Ishii et al. 2007; Ohuchi, Hamada et al. 2008). LPA also stimulates LPA5/GPR92 (Lee, Rivera et al. 2006), a GPCR with a sequence more similar to that of LPA4 than those of LPA1-3. As with LPA4, the physiological role of LPA5 is not well

TABLE 1.1. Activation of LPA Receptors Elicit Broad Phenotypic Effects.

Phenotypic Effect	LPA Receptor(s)	Target Tissue/Cell Type	Reference
Skeletal maintenance/development	LPA1	Chondrocytes, osteoblasts	(Kim, Lee et al. 2005; Aki, Kondo et al. 2008)
Promotion of spermatogenesis	LPA1, LPA2, LPA3	Male germ-line cells	(Ye, Skinner et al. 2008)
Oocyte maturation	---	Female germ-line cells	(Yue, Yokoyama et al. 2004)
Blastocyst implantation	LPA3	---	(Ye, Hama et al. 2005)
Stimulation of hair growth	LPA6	Hair follicles	(Pasternack, von Kugelgen et al. 2008)
Neurite retraction	Multiple	Neurons	(Nurnberg, Brauer et al. 2008)
Interleukin secretion; Inflammation	LPA1	Osteoblasts, epithelial cells, synovial fluid	(Aki, Kondo et al. 2008; Nochi, Tomura et al. 2008; Kalari, Zhao et al. 2009)
Morphological changes	LPA4	Neurons	(Yanagida, Ishii et al. 2007)
Inhibition of apoptosis	Multiple	T-cells, cancer cells, chondrocytes	(Goetzl, Kong et al. 1999; Murph, Hurst-Kennedy et al. 2007; Hurst-Kennedy, Boyan et al. 2009)
Angiogenesis, Vascularization	---	Cancer cells	(Hu, Tee et al. 2001; Lee, Park et al. 2006)
Stimulation of proliferation	LPA1, LPA3	Cancer cells, astrocytes, chondrocytes	(Kim, Lee et al. 2005; Billon-Denis, Tanfin et al. 2008; Shano, Moriyama et al. 2008)
Tumor formation	LPA2	---	(Lin, Wang et al. 2009)
Cerebral cortical development	LPA1	Cortical neural precursors	(Estivill-Torrus, Llebrez-Zayas et al. 2008)
Smooth muscle contraction	LPA3	Stomach, heart	(Ainslie, Shi et al. 2004; Sriwai, Zhou et al. 2008)
Platelet Activation	LPA5	Platelets	(Williams, Khandoga et al. 2009)
Migration	LPA1, LPA2, LPA3	Cancer cells, osteoblasts	(Karagiosis, Chrisler et al. 2009; Komachi, Tomura et al. 2009)
Wound healing	LPA1	Lungs	(Tager, LaCamera et al. 2008)

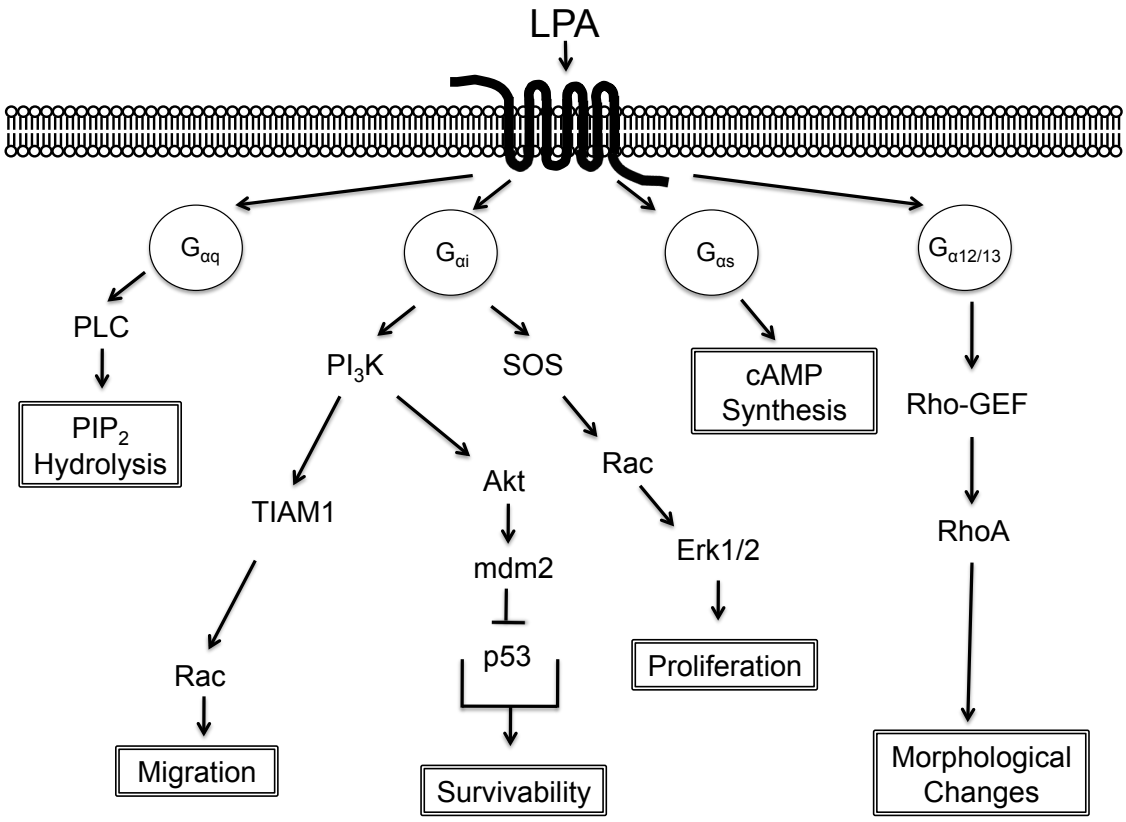

FIGURE 1.1. LPA GPCRs couple to many G alpha subunits. The LPA GPCRs, LPA_1/Edg2, LPA_2/Edg4, LPA_3/Edg7, LPA4/GPR23, LPA5/ GPR92, and LPA6/P2Y5, collectively stimulate the $G_{\alpha i}$, $G_{\alpha q}$, $G_{\alpha s,}$ and $G_{\alpha 12/13}$ signaling pathways. As a result LPA modulates a number of cellular processes such as proliferation, cell survival, migration, and morphological changes.

understood. LPA5 has, however, been show to activate platelets (Williams, Khandoga et al. 2009). Recently, a new GPCR has been identified as a potential LPA receptor: P2Y5/LPA6 (Pasternack, von Kugelgen et al. 2008; Yanagida, Masago et al. 2009). This receptor shares the most sequence homology with LPA4, the other P2Y family member, and induces Rho-mediated motility.

In addition to G-protein coupled receptors, LPA is a ligand for one intracellular receptor: peroxisome proliferator-activated receptor gamma (PPAR-γ), a nuclear, fatty acid receptor that binds a number of other lipid mediators (McIntyre, Pontsler et al. 2003; Tsukahara, Tsukahara et al. 2006). LPA-mediated stimulation of PPAR-γ has been linked to prevention of endotoxemia-induced organ injury (Murch, Collin et al. 2007) and inhibition of adipocyte differentiation (Simon, Daviaud et al. 2005).

1.2 LPA Metabolism

LPA is the simplest of the phospholipids consisting of a single fatty acid chain, a glycerol backbone, and a phosphate head group (Tokumura 1995). LPA can be derived from a number of precursor sources including lysophosphatidylcholine (LPC), phosphatidic acid (PA), and monoacylglycerol (MAG) (Stracke, Krutzsch et al. 1992; Aoki, Taira et al. 2002; Umezu-Goto, Kishi et al. 2002; Bektas, Payne et al. 2005) (Fig. 1.2). Additionally, LPA is synthesized through at least two distinct mechanisms: extracellular metabolism of lysophospholipids and intracellular cleavage of PA (Aoki 2004).

In biological fluids such as serum and salvia, LPA is primarily generated via an extracellular mechanism. Moreover, overabundant extracellular LPA synthesis in ascites

from ovarian cancer patients has been strongly linked to tumorigenesis (Xu, Fang et al. 1995; Lee, Swaby et al. 2006; Yu, Murph et al. 2008). Extracellular LPA production begins with the generation of lysophospholipids such as LPC, LPE, and LPS. These lysophospholipids are derived from membrane phospholipids (PC, PE, PS) through the actions of membrane and/or secretory phospholipase $A_{1/2}$ ($PLA_{1/2}$) (Aoki 2004). Additionally, LPC can be produced as a byproduct of lecithin cholesterol acyltransferase (LCAT)-mediated cholesterol ester synthesis (Aoki, Taira et al. 2002). Next, lysophospholipid head groups are cleaved by autotaxin (ATX), a multi-subunit ecto-enzyme also known as lysophospholipase D (lysoPLD) (Stracke, Krutzsch et al. 1992; Yuelling and Fuss 2008), to yield LPA. LPA can be degraded extracellularly as well. Lipid phosphate phosphatases (LPPs, LPP1-3) remove the phosphate head group from LPA, resulting in the production of monoacylglycerol (MAG) (Brindley and Pilquil 2009; Tomsig, Snyder et al. 2009). This is a reversible process. MAG can be converted back into LPA when phosphorylated by acylglycerol kinase (AGK) (Bektas, Payne et al. 2005; Kalari, Zhao et al. 2009).

The second mechanism by which LPA is produced occurs intracellularly. Membrane phospholipids such as PC and PE are substrates for phospholipase D (PLD), which catalyzes the conversion of PE/PC to PA (Jenkins and Frohman 2005). LPA can be generated from PA through the actions of PLA1 or PLA2 (Eder, Sasagawa et al. 2000; Sonoda, Aoki et al. 2002). Conversely, LPA can be acylated by lysophospholipid acyl tranferases (LPATs) to regenerate PA (Yamashita, Nakanishi et al. 2007). Intracellular

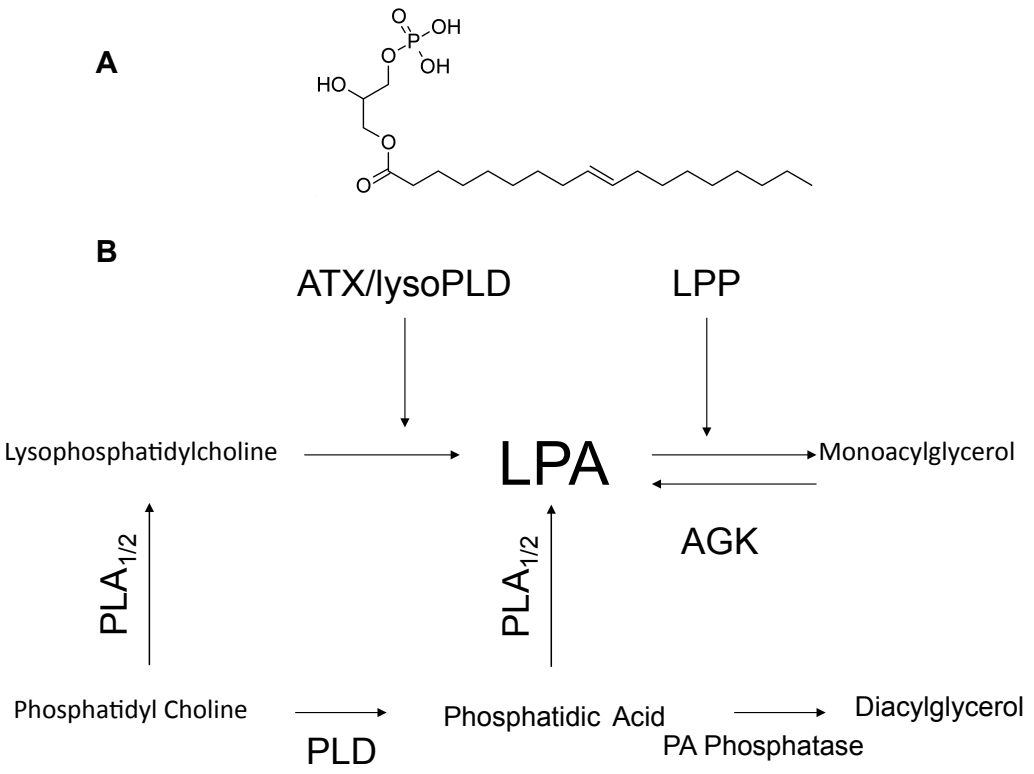

FIGURE 1.2 LPA structure and metabolism. (A) LPA consists of a single fatty acyl chain, a glycerol backbone, and a phosphate head group. (B) LPA is derived from at least three precursor lipids: lysophosphatidyl choline (LPC), monoacylglycerol (MAG), and phosphatidic acid (PA). Autotaxin/lysophospholipase D mediates the cleavage of choline from LPC yielding LPA. LPA is reversibly converted to MAG through the actions of lysophospholipid phosphatases (LPPs) and acyl glycerol kinase (AGK). Moreover, phospholipase A1 (PLA_1) or phospholipase A2 (PLA_2) mediates the metabolism of phosphatidic acid (PA) to LPA. LPA acylation resulting in the regeneration of PA is controlled by lysophosphatidic acid acyl transferases (LPATs).

synthesis of LPA has been identified in osteoblasts (Panupinthu, Zhao et al. 2007; Panupinthu, Rogers et al. 2008) and cancer cells (Eder, Sasagawa et al. 2000; Billon-Denis, Tanfin et al. 2008).

1.3 The Role of LPA in Physiology and Disease

1.3.1 Bone and Cartilage

Research carried out during the past decade has shown that LPA is a regulator of cartilage. The first of these studies, conducted by *Koople et al*, demonstrated that LPA induces an increase in intracellular calcium in human articular chondrocytes (Koolpe, Rodrigo et al. 1998). This was followed by the observations that LPA stimulates $G\alpha_{i/o}$ and ERK-mediated proliferation in primary rat articular chondrocytes (Kim, Lee et al. 2005), while it decreases proliferation in T/C-28a2 cells, an immortalized, juvenile human articular chondrocyte cell line (Kokenyesi, Tan et al. 2000; Facchini, Borzi et al. 2005). Collectively, these studies demonstrate that chondrocytes are LPA-responsive cells. Moreover, the data suggest chondrocyte responses to LPA may vary in different species and in different subtypes of chondrocytes. Recently, a research group has identified a single nucleotide polymorphism (SNP) in the LPA1 receptor that is associated with the progression of osteoarthritis (Mototani, Iida et al. 2008), a cartilage degenerative disease, suggesting that LPA and LPA1 are involved in cartilage-related pathogenesis.

Actions of LPA in bone are better characterized that those in cartilage. The pre-osteoblastic cell line MC3T3-E1 (Karagiosis, Chrisler et al. 2009), the immature osteoblast cell line MG63 (Gidley, Openshaw et al. 2006), and primary osteoblasts (Panupinthu, Rogers et al. 2008) all respond to LPA, resulting in increased maturation.

Panupinthu et al has found that LPA-mediated osteoblast maturation occurs through stimulation of the P2X7 receptor. Activation of P2X7 in osteoblasts leads to PLD-mediated LPA production, which subsequently enhances osteogenesis (Panupinthu, Zhao et al. 2007; Panupinthu, Rogers et al. 2008). Additionally, studies conducted in osteoblast cell lines have shown that LPA and the vitamin D metabolite $1,25(OH)_2D_3$ act synergistically to promote maturation by increasing alkaline phosphatase activity and cytoskeletal rearrangement (Gidley, Openshaw et al. 2006; Mansell, Farrar et al. 2009). These studies suggest the existence of a $1,25(OH)_2D_3$, LPA, PLD signaling axis in bone formation. LPA also regulates cell migration and morphological changes in bone cells. After injury, LPA stimulates dendrite formation in osteocytes to reestablish osteocyte connectivity (Karagiosis and Karin 2007), which mediates the transmission of physical signals in bone. These data indicate a role for LPA in mechanosensation. Moreover, LPA is an osteoblast chemoattractant (Masiello, Fotos et al. 2006). Together, these data support a role for LPA in bone fracture repair. LPA has been identified as an osteoblast mitogen as well, as evidenced by the ability to stimulate DNA synthesis in osteoblasts via a $G_{\alpha i}$-dependent mechanism (Caverzasio, Palmer et al. 2000; Grey, Banovic et al. 2001). Lastly, emerging research indicates that LPA1 may be significant in bone maintenance. In osteoblasts, LPA1 is necessary for LPA-mediated induction of IL-6 and IL-8 expression (Aki, Kondo et al. 2008) and for the induction of chemotaxis by LPA (Masiello, Fotos et al. 2006). Furthermore, the LPA1-null mouse has a smaller skeleton relative to its wild-type counterpart (Contos, Ishii et al. 2002). Finally, LPA1 has been shown the mediate metastasis of breast cancer cells to bone (Boucharaba, Serre et al. 2004). As a whole, these studies establish LPA as a regulator of chondrocytes,

osteoblasts, and osteocytes, implicating LPA signaling in the regulation of skeletal development and in cartilage and bone-related pathogenesis.

1.3.2 LPA, Cancer, and p53

As previously mentioned, LPA is regulator of cell proliferation, migration, and cell survival. These functions implicate the lipid mediator in cancer progression. This is most notable in ovarian cancer (Ren, Xiao et al. 2006). LPA is found in high levels in ascites fluid as a result of increased ATX (Tokumura, Kume et al. 2007) and $PLA_{1/2}$ (Ren, Xiao et al. 2006) activity. Ovarian cancer cells also express abnormally high levels of LPA receptors (Yu, Murph et al. 2008). The result of the overabundance of LPA and LPA receptors is increased vascularization (Sako, Kitayama et al. 2006), motility (Kim, Yun et al. 2008), and invasion (Sengupta, Kim et al. 2007) of ovarian tumors. Recent work has shown that many of these LPA-mediated effects in ovarian cancer are dependent upon LPA2 signaling (Jeong, Park et al. 2008; Wang, Wen et al. 2008; Hope, Wang et al. 2009). LPA signaling contributes to breast cancer progression as well. Overexpression of ATX or any of the Edg family LPA receptors in transgenic mice increases the abundance of estrogen positive, aggressive breast cancer tumors (Liu, Umezu-Goto et al. 2009). Furthermore, exogenous LPA increases motility and invasion in breast cancer cell lines though stimulation of LPA1 and LPA2 (Stadler, Knyazev et al. 2006; Chen, Towers et al. 2007). Lastly, emerging research indicates that LPA is also a regulator of prostate cancer. A recent clinical study revealed that LPA3 expression levels are higher in prostate tumors relative to benign prostate tissue (Zeng, Kakehi et al. 2009), implicating this receptor in tumorigenesis. LPA3, along with LPA1 and LPA2, have also been shown to be involved in LPA-mediated motility in the prostate cancer cell line PC3

(Hasegawa, Murph et al. 2008). Taken together, these studies strongly suggest that LPA is a key stimulatory factory in cancer progression.

Despite the established role of LPA in cancer progression, little was known about the role of LPA signaling in cell cycle regulation in cancer cells or in normal cells prior to the publication of *Lysophosphatidic Acid Decreases the Nuclear Localization and Cellular Abundance of the p53 Tumor Suppressor in A549 Lung Carcinoma Cells* (Murph, Hurst-Kennedy et al. 2007). Our data indicate a role for LPA in the regulation of the protein p53, a key regulator of the cell cycle that is mutated in over 50% of all cancers (Bartek, Iggo et al. 1990). p53 is a transcription factor with over 50 transcriptional targets that promote cell cycle arrest, DNA damage repair and apoptosis (Pietenpol, Tokino et al. 1994; Miyashita and Reed 1995; Slee, O'Connor et al. 2004). In response to genotoxic stress, p53 is phosphorylated at residue Serine-15 by ataxia telangiectasia mutated (ATM) (Zhang, Li et al. 2005), thus blocking a nuclear export site resulting in the accumulation of p53 in the nucleus and increased p53-mediated transcription (Zhang and Xiong 2001; Inoue, Wu et al. 2005) (Fig. 1.3). p53 exerts non-transcriptional effects in the cell as well by directly interacting with the Bcl-2 family proteins in the mitochondrial membrane to promote the release of cyotochrome c into the cytoplasm resulting in increased caspase activity (Sayan, Sayan et al. 2006; Speidel, Helmbold et al. 2006). Recent studies have demonstrated that p53 regulates the transcriptional activity of Runx2, an osteoblast-specific transcription factor (Lengner, Steinman et al. 2006). This study demonstrated that p53 signaling participates in cell differentiation in addition to its function as a cell cycle regulator. In the absence of

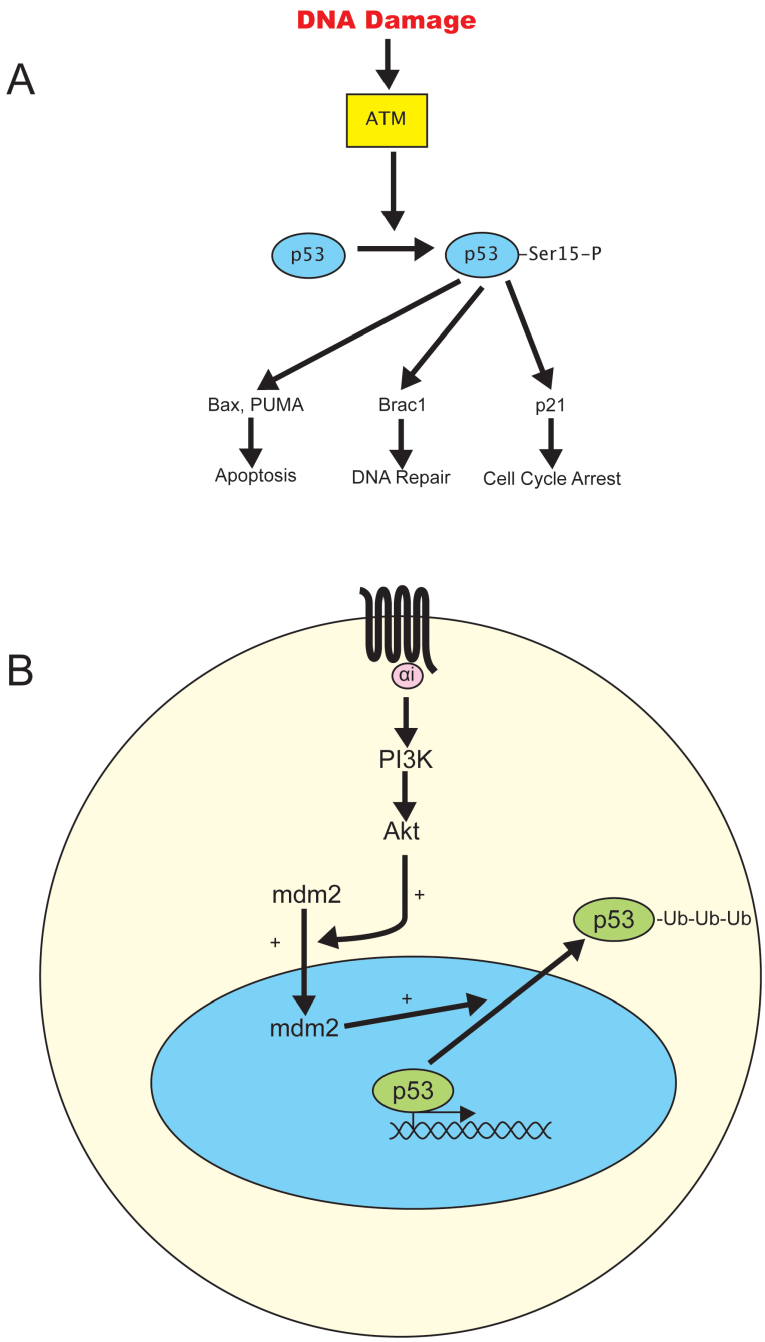

FIGURE 1.3. P53 Signaling. (A) The DNA damage-sensing molecule ATM phosphorylates p53 at residue Serine-15 in response to double-stranded DNA breaks. This phosphorylation results in the masking of a nuclear export site and stabilizes p53, allowing p53 to modulate the transcription of genes that control DNA damage repair, cell cycle arrest, and apoptosis. (B) $G_{\alpha i}$ stimulates the PI_3K-Akt signaling cascade. The E3-ubiquitin ligase mdm2 is an Akt phosphorylation target. Phosphorylated mdm2 translocates to the nucleus where it can mono- or poly-ubiquitinate p53, resulting in nuclear export and degradation of p53.

genotoxic stress, the E3-ubiquitin ligase, mdm2, itself a p53 target gene, promotes the degradation and nuclear export of p53 (Haupt, Maya et al. 1997). When cellular mdm2 activity is low, p53 is mono-ubiquitinated promoting nuclear export. Conversely, when mdm2 activity is high, p53 is poly-ubiquitinated by mdm2 resulting in increased proteosomal degradation of the p53 protein (Li, Brooks et al. 2003). Mdm2 is primarily localized in the cytoplasm until phosphorylated by Akt. This phosphorylation event induces the translocation of mdm2 to the nucleus where it can ubiquitinate p53 (Zhou, Liao et al. 2001).

1.3.3 Other LPA-Regulated Processes

1.3.3.1 The Cardiovascular System

The first identified biological function of LPA in vertebrates was its ability to induce platelet aggregation (Gerrard, Kindom et al. 1979; Tokumura, Fukuzawa et al. 1981). Since that time, LPA has been shown to regulate many other processes in the cardiovascular system such as angiogenesis/vascularization (Hu, Tee et al. 2001; Lee, Park et al. 2006), vascular smooth muscle contraction (Cremers, Flesch et al. 2003), and cardiomyocyte hypertrophy after vascular injury (Hilal-Dandan, Means et al. 2004). Moreover, excessive LPA signaling contributes to the progression of diseases such as atherosclerosis (Siess, Zangl et al. 1999) and hypertension (Tokumura, Fujimoto et al. 1999). Collectively, these studies establish LPA as a key regulator of the cardiovascular system. Many of these LPA-mediated effects can be attributed to activation of the Edg family LPA receptors (Chen, Chen et al. 2008; Panchatcharam, Miriyala et al. 2008), suggesting that receptor-specific modulators could be used as therapeutic agents.

Furthermore, others have suggested ATX activity regulators could be used to control LPA signaling in the treatment of cardiovascular disease progression (Federico, Pamuklar et al. 2008).

1.3.3.2 Obesity

Obesity and obesity-related diseases correlate with the elevated storage of triglycerides in adipose tissue (Calabro and Yeh 2007). Studies suggest that LPA signaling may contribute to this phenomenon. *In vitro* work has shown that pre-adipocytes express ATX mRNA, resulting in ATX-mediated LPA production (Gesta, Simon et al. 2002; Ferry, Tellier et al. 2003). This causes autocrine/paracrine LPA signaling that promotes adipocyte differentiation. Moreover, genetically obese mice express more adipose ATX mRNA than their wild-type counterparts (Ferry, Tellier et al. 2003). Additionally, LPA increases glucose uptake in the 3T3-L1 adipocyte cell line through LPA1/3 and PI_3K-dependent signaling (Yea, Kim et al. 2008). LPA also lowered serum glucose levels in wild type and diabetic mice without altering insulin levels. These data collectively suggest that LPA is a pro-adipogenic factor. However, in contrast to the aforementioned findings, one group has observed that LPA inhibits differentiation of adipocytes through LPA1-mediated inhibition of PPARγ (Simon, Daviaud et al. 2005). This suggests that LPA signaling in adipose tissue is complex and may elicit different effects depending on adipocyte maturation state.

1.3.3.3 Fertility and Pregnancy

LPA is involved in both male and female gamete maturation (Yue, Yokoyama et al. 2004; Ye, Skinner et al. 2008), indicating that the lipid mediator is an important factor in reproduction. Recent studies in bovine models have shown that LPA is significant in

the maintenance of early pregnancy (Woclawek-Potocka, Brzezicka et al. 2009; Woclawek-Potocka, Komiyama et al. 2009). LPA is produced by bovine endometrial cells and acts as an autocrine/paracrine factor to increase prostaglandin production, resulting in enhanced corpus luteum viability. Similar observations have been made in mice. LPA3-null mice have smaller litters due to delayed blastocyst implantation (Ye, Hama et al. 2005). This phenotype can be rescued with the administration of prostaglandins. Additionally, *Tokumura et al* observed altered ATX activity in pregnant patients with preeclampsia or pre-term labor (Tokumura, Kume et al. 2009), suggesting that LPA acts to sustain pregnancy in the later stages as well. Collectively, these studies suggest that modulation of LPA signaling may be important in the treatment of infertility and the prevention of miscarriage and abnormal pregnancy.

1.4 The Growth Plate and Vitamin D Metabolite Signaling

1.4.1 Endochondral Ossification and the Growth Plate

Endochondral ossification is the mechanism by which embryonic skeletal formation and longitudinal bone growth in children and adolescents occurs. This process is mediated by growth plates, specialized regions of developing cartilage located between the epiphysis and the metaphysis (Ballock and O'Keefe 2003) (Fig 1.4). The growth plate is divided into four zones of maturation: the resting zone, the proliferative zone, the pre-hypertrophic/hypertrophic zone, and the calcifying cell zone. The resting zone, also know as the reserve zone, serves as a pool of chondrocytes for the remainder of the growth plate. Resting zone chondrocytes express early chondrogenic markers such as collagen II and aggrecan (Reichenberger, Aigner et al. 1991; Yamane, Cheng et al. 2007). In response to growth stimuli, these chondrocytes begin to deposit extracellular matrix,

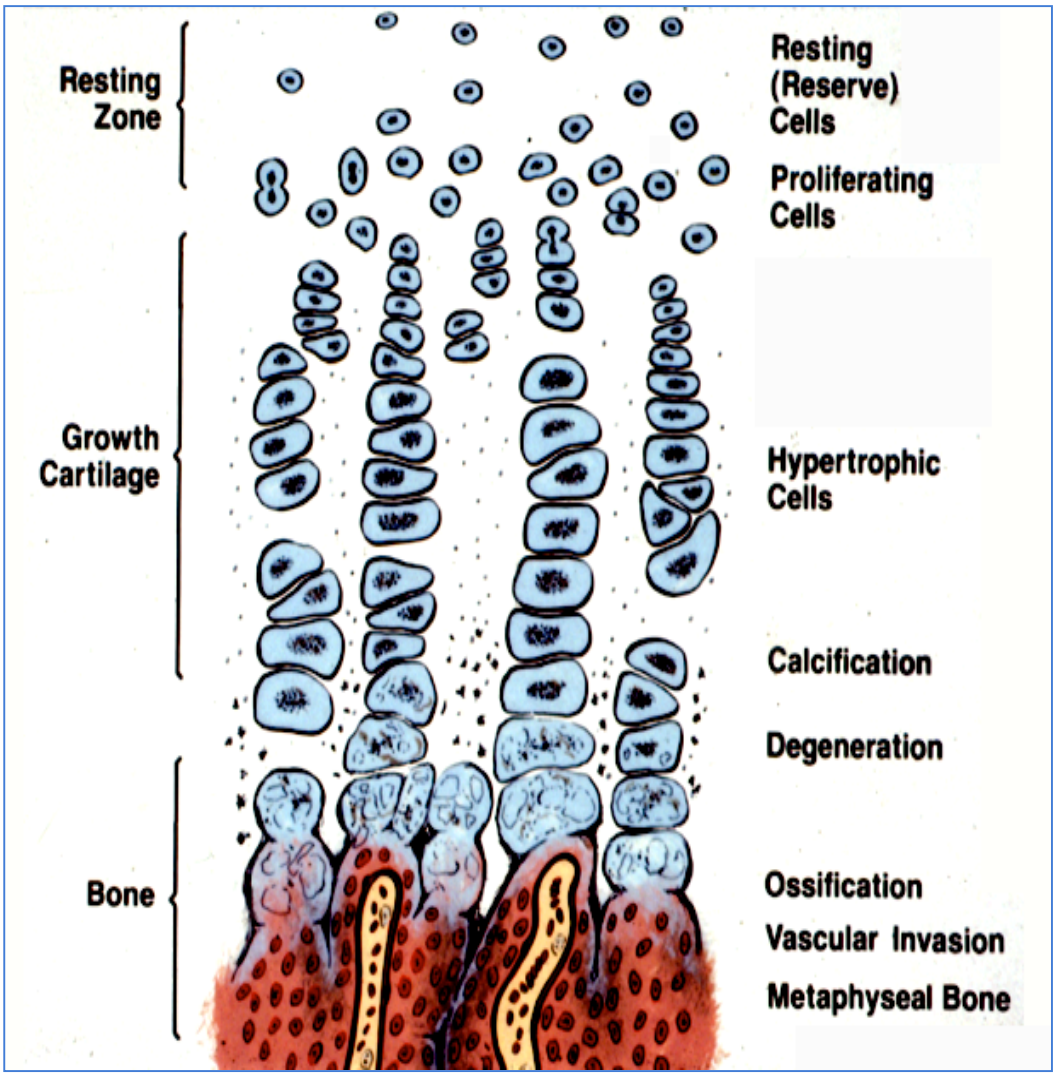

FIGURE1.4. **The growth plate.** The growth plate is divided into three zones of maturation: the resting zone, the growth zone, and the hypertrophic zone. The resting zone serves as a pool of chondrocytes for the remainder of the growth plate. These cells proliferate, calcify, and undergo terminal differentiation and apoptosis as the growth plate matures. Resting zone chondrocytes respond preferentially to the vitamin D3 metabolite 24R,25-dihydroxyvitamin D3, resulting in PLD-mediated maturation and matrix production. Image taken from Boyan and Schwartz et al with permission (Boyan, Schwartz et al. 1988).

proliferate, and organize themselves into columns (growth zone). In the pre-hypertrophic/hypertrophic zone, chondrocytes increase in size and remodel their extracellular matrix (hypertrophy). They express late chondrocyte markers such as cartilage oligomeric matrix protein (COMP), an extracellular calcium binding protein, and collagen X (Reichenberger, Aigner et al. 1991; Ekman, Reinholt et al. 1997). In the calcifying cell zone, the hypertrophic chondrocytes deposit calcium phosphate crystals. This allows for the resorption of the calcified matrix by chondroclasts, permitting the invasion of blood vessels and the formation of new bone (Teixeira, Mansfield et al. 2001; Mansfield, Pucci et al. 2003; Zhong, Carney et al. 2008).

Growth plate maturation is regulated by a number of hormones, cytokines, and growth factors. Among these effectors, vitamin D metabolites are crucial in maintaining the tissue (Boyan, Schwartz et al. 1992). In the absence of vitamin D signaling, the extracellular matrix of cartilage is not calcified, resulting in rickets in children (Ballock and O'Keefe 2003), a disorder characterized by a softening of the bones, bowed limbs, and increased fracture risks. Recent studies have shown that the prevalence of rickets is increasing in the US (Kreiter, Schwartz et al. 2000; DeLucia, Mitnick et al. 2003), particularly in exclusively breastfed African American children. In comparison to normal growth plates, rachitic growth plates exhibit a decreased resting zone and increased growth and hypertrophic zones (Itakura, Yamasaki et al. 1978). Moreover, the columns in the growth zone are less organized and expression of collagen types II and X is decreased (Reginato, Shapiro et al. 1988). This same growth plate phenotype is observed in vitamin D nuclear receptor-null mice (Yagishita, Yamamoto et al. 2001).

In addition to mediating long bone growth and skeletal development, endochondral ossification also plays a role in bone fracture healing. Fracture healing is divided into three stages (Gerstenfeld, Cullinane et al. 2003). The first of these is the reactive stage, characterized by inflammation, hematoma formation, and the recruitment of stem cells to the damage site. This is followed by the reparative stage, which involves the formation of the fracture callus, a cartilaginous region that is structurally similar to the growth plate (Sandberg, Aro et al. 1989; Gerstenfeld, Cullinane et al. 2003). Finally, in the remodeling stage, the cartilage is degraded and replaced by bone. Studies have shown that vitamin D metabolite signaling stimulates bone fracture healing (Kato, Seo et al. 1998; Fu, Tang et al. 2009). Due to the similarities between the growth plate and the fracture callus, this can most likely be attributed to vitamin D metabolite-mediated regulation of the fracture callus.

1.4.2 Vitamin D Metabolites

Vitamin D is a steroid hormone that regulates a number of physiological processes such as calcium and phosphate transport, bone remodeling, and immune cell activity. As with other steroid hormones such as estrogen and testosterone, vitamin D is derived from cholesterol (Glover, Glover et al. 1952). However, unlike other hormones, vitamin D contains four fused rings instead of the typical five (Procsal, Okamura et al. 1976). For this reason, vitamin D is referred to as a "seco-steroid" hormone. Two major isoforms of vitamin D exist: vitamin D2 (ergocalciferol) and vitamin D3 (cholecalciferol) (Procsal, Okamura et al. 1976). The two isoforms are distinguished structurally by the presence of a double bond in D2 that is absent in D3. Furthermore, D2 is primarily synthesized in plants whereas animals produce D3.

Circulating vitamin D3 in the body is derived from both dietary sources and through *de novo* synthesis. *De novo* synthesis occurs when cholesterol-derived 7-dehydroxycholesterol (pro-vitamin D3) in cell membranes undergoes photolysis in response to UVB radiation from the sun. This results in the conversion of pro-vitamin D3 to vitamin D3, also known as cholecalciferol (Glover, Glover et al. 1952). Dietary sources of vitamin D3 include oily fishes, cod liver oil, and fortified dairy items and fruit juices (Lamberg-Allardt 2006). D3 from these foods is absorbed into the circulation via the intestines and is transported in chylomicrons. In the liver, vitamin D3 is hydroxylated to form the metabolite 25-hydroxyvitamin D3 (25-OHD_3), which is then further hydroxylated in the kidneys to form 1,25-dihydroxyvitamin D3 [1,25$(OH)_2D_3$] and 24R,25-dihydroxyvitamin D3 [24,25$(OH)_2D_3$] (Holick, Schnoes et al. 1972). 1,25$(OH)_2D_3$ exerts its effects through the classical nuclear vitamin D receptor (nVDR) (Tsai and Norman 1973) and at least one membrane receptor, protein disulfide isomerase family A, member 3 (PDIA3, also known as Erp60/57 and 1,25-MARRS) (Boyan, Wong et al. 2007). Actions of 1,25$(OH)_2D_3$ are broad and include calcium and phosphate intestinal absorption and maintenance of bone, cartilage, and immune cells (Deeb, Trump et al. 2007; St-Arnaud 2008).

The biological actions of 24,25$(OH)_2D_3$ are less understood than those of 1,25$(OH)_2D_3$. Unlike 1,25$(OH)_2D_3$, 24,25$(OH)_2D_3$ has little to no binding affinity for nVDR (Tanaka, Wichmann et al. 1983). Moreover, early studies indicated that 24,25$(OH)_2D_3$ functioned solely as a negative regulator of 1,25$(OH)_2D_3$, acting to reduce 1,25$(OH)_2D_3$ serum levels when they became too high (Tanaka and DeLuca 1984; Brommage and DeLuca 1985). These contributed to the notion that 24,25$(OH)_2D_3$ was a

"waste" vitamin D metabolite with no biological function of its own. This idea was challenged primarily through the work of *Henry, et al* and *Norman, et al*. Together, they first identified 24,25$(OH)_2D_3$ as an inhibitor of parathyroid hormone (PTH) secretion in chickens and dogs (Henry, Taylor et al. 1977; Canterbury, Lerman et al. 1978). This was followed by the observations that 24,25$(OH)_2D_3$ is necessary for normal chicken egg hatching (Henry and Norman 1978; Norman, Leathers et al. 1983) and that it regulated calcium and phosphate homeostasis (Boyle, Omdahl et al. 1973; Norman, Henry et al. 1980). Collectively, these studies identified 24,25$(OH)_2D_3$ as a physiologically relevant vitamin D metabolite capable of eliciting cellular responses.

1.4.3 Rapid Actions of 24R,25-dihydroxyvitamin D3 in the Growth Plate

Chondrocytes isolated from the resting zone of rat growth plates respond preferentially to 24,25$(OH)_2D_3$ with increased alkaline phosphatase activity and [S^{35}]-incorporation, decreased DNA synthesis, and increased matrix metalloproteinase (MMP) activity (Maeda, Dean et al. 2001; Schwartz, Ehland et al. 2002). Similar observations have been made in avian growth plate chondrocytes (Wu, Genge et al. 2006). 24,25$(OH)_2D_3$-mediated effects are maintained in mice lacking nVDR (Boyan, Sylvia et al. 2003) and are not inhibited by Ab99, a blocking antibody targeted against PDIA3 (Boyan, Bonewald et al. 2002). Additionally, the effects of 24,25$(OH)_2D_3$ are rapid, inducing protein kinase C (PKC) activation in as little as 9 minutes (Helm, Sylvia et al. 1996). Taken together, these data indicate that 24,25$(OH)_2D_3$ acts through a membrane associated receptor ($mVDR_{24,25}$) that is distinct from the 1,25$(OH)_2D_3$-responsive membrane receptor(s). This conclusion is supported by the findings of *Kato et al* and *Larsson et al* demonstrating the presence of a membrane-associated 24,25$(OH)_2D_3$

receptor in chicken fracture calluses and cod enterocytes, respectively (Kato, Seo et al. 1998; Larsson, Nemere et al. 2001).

The actions of $24,25(OH)_2D_3$ in the resting zone chondrocytes are mediated through rapid activation of PKC. $24,25(OH)_2D_3$ increases the abundance of diacylglycerol (DAG) (Helm, Sylvia et al. 1996), an activator of many PKC isoforms. PKC activation by $24,25(OH)_2D_3$ is maintained in the presence of chemical inhibitors targeted against either phosphatidyl choline (PC) or phosphoinositol (PI)-specific phospholipase C (PLC) (Schwartz, Sylvia et al. 2001), indicating that the source of DAG stimulating PKC activation is not phosphoinositide derived. Inhibition of tyrosine kinase signaling also does not attenuate rapid actions of $24,25(OH)_2D_3$, eliminating tyrosine kinases as a cause of PKC activation (Helm, Sylvia et al. 1996). Instead, activation of PKC by $24,25(OH)_2D_3$ is dependent upon DAG derived from phosphatidic acid (PA) generated the actions of by phospholipase D (PLD), specifically PLD2 (Schwartz, Sylvia et al. 2001; Sylvia, Schwartz et al. 2001). Another consequence of PLD activation is the production of LPA (Luquain, Singh et al. 2003; Aoki 2004). These findings implicated LPA as a second messenger in $24,25(OH)_2D_3$-directed signaling. We found that $24,25(OH)_2D_3$ increased the abundance of extracellular LPA and LPA receptor 1 (LPA1) mRNA. Additionally, inhibition of LPA1 and LPA3 attenuated $24,25(OH)_2D_3$-induced chondrocyte maturation and cell survival (Hurst-Kennedy, Boyan et al. 2009). Resting zone chondrocytes responded to LPA with increased DNA synthesis, alkaline phosphatase activity, and [^{35}S]-incorporation. Furthermore, LPA protected chondrocytes against inorganic phosphate (Pi)-induced apoptosis by activating the phosphoinositol 3-kinase (PI_3K) and murine double minute 2 (mdm2) signaling, resulting in the degradation

of p53 and a decrease in p53-mediated transcription. Interestingly, this is the same mechanism by which LPA enhances cell survival in cancer cells (Murph, Hurst-Kennedy et al. 2007). We have also observed that Gβγ-mediated PLC activation also contributes to the inhibition of Pi-induced apoptosis by $24,25(OH)_2D_3$ (Hurst-Kennedy, Boyan, et al, unpublished data). The stimulation of the pro-survival actions of LPA by $24,25(OH)_2D_3$ establishes an anti-apoptotic function for the metabolite.

A second phospholipid-dependent mechanism also contributes to the rapid actions of $24,25(OH)_2D_3$. Inhibition of phospholipase A2 (PLA_2) enhances PKC activation by $24,25(OH)_2D_3$, whereas PLA2 activating protein (PLAA) decreases this (Boyan, Jennings et al. 2004). This is in contrast to $1,25(OH)_2D_3$, which elicits its rapid effects via PLA_2-activation (Schwartz, Graham et al. 2005). Inhibition of PLA_2 by $24,25(OH)_2D_3$ results in a rapid decrease in arachidonic acid (AA) abundance and cyclooxygenase-1 (COX-1) activity (Boyan, Sylvia et al. 1998; Schwartz, Sylvia et al. 2000). Following the initial decrease in PLA2, $24R,25(OH)_2D_3$ upregulates arachidonic acid turnover (Schwartz, Swain et al. 1990; Swain, Schwartz et al. 1992), altering fluidity of the plasma membrane(Swain, Schwartz et al. 1993), and increasing the production of prostaglandins E1 and E2 (PGE1, PGE2) to induce protein kinase A (PKA) activity (Schwartz, Sylvia et al. 1999). Inhibition of PKA mitigated $24,25(OH)_2D_3$-induced rapid signaling and chondrocyte maturation, demonstrating the importance of this signaling pathway (Helm, Sylvia et al. 1996; Schwartz, Sylvia et al. 1999). Together, activated PKC and PKA promote MEK and ERK1/2 signaling (Schwartz, Ehland et al. 2002) in response $24,25(OH)_2D_3$ to induce changes in gene transcription to promote chondrocyte maturation (Fig 1.5).

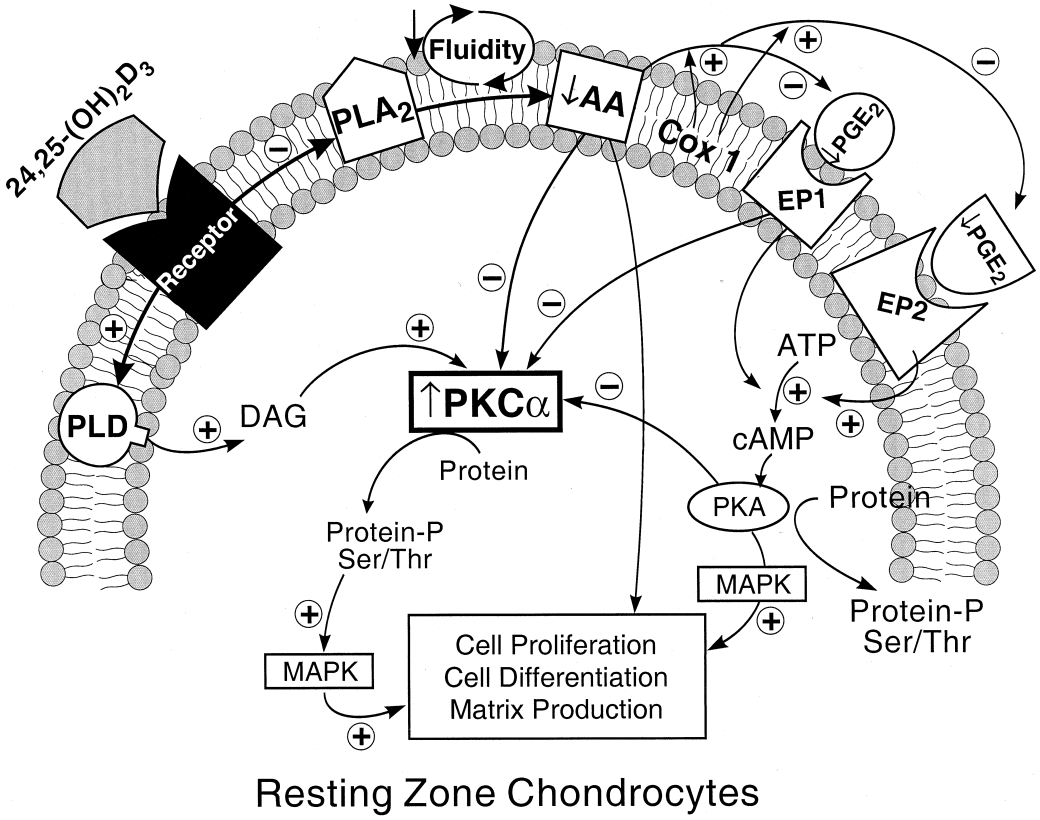

FIGURE 1.5. Actions of 24R,25-dihydroxyvitamin D3 in growth plate resting zone chondrocytes. $24,25(OH)_2D_3$ stimulates phospholipase D (PLD), resulting in diacylglycerol (DAG)-mediated protein kinase C (PKC) activation. Additionally, $24,25(OH)_2D_3$ rapidly negatively regulates PLA_2 activity leading to a reduction in arachidonic acid production, an inhibitor of PKC. Together, this results in MAPK activation to induce differentiation, proliferation, and matrix synthesis. Image taken from "24,25-(OH)2D3 regulates cartilage and bone via autocrine and endocrine mechanisms" (Boyan, Sylvia et al. 2001) with permission.

PART I. LPA-MEDIATED PROTEOSOMAL DEGRADATION OF P53 IN A459 LUNG CARCINOMA CELLS

CHAPTER 2

INTRODUCTION

Lysophosphatidic acid (LPA) is a bioactive lipid found in body fluids that elicits a wide range of effects including cell proliferation, migration, morphological changes, survival and neointimal formation (Chun 1999; Goetzl, Kong et al. 1999; Fang, Yu et al. 2000; Fukushima, Weiner et al. 2000; Inoue, Nagano et al. 2001; Deng, Wang et al. 2003; Zhang, Baker et al. 2004). LPA is produced in blood by activated platelets to facilitate wound healing (Xu, Shen et al. 1998; Lee, Goetzl et al. 2000) and it is also produced by a variety of cancer cells (Shen, Belinson et al. 1998; Eder, Sasagawa et al. 2000). The diverse actions of LPA are primarily mediated by three seven-transmembrane, G protein-coupled receptors (GPCRs): LPA_1/Edg2, LPA_2/Edg4, LPA_3/Edg7, and possibly also by the metabolic receptor, PPAR-γ (An, Dickens et al. 1997; An, Bleu et al. 1998; Bandoh, Aoki et al. 1999; McIntyre, Pontsler et al. 2003; Noguchi, Ishii et al. 2003). Recent studies also indicate that the orphan receptors GPR23 and GPR92 are high affinity LPA receptors (Noguchi, Ishii et al. 2003; Lee, Rivera et al. 2006). The LPA-binding GPCRs can collectively activate G_i, G_s, G_q, and $G_{12/13}$ (Ishii, Contos et al. 2000; Noguchi, Ishii et al. 2003; Radeff-Huang, Seasholtz et al. 2004). The resultant effects of LPA are mediated through the activation of downstream signaling pathways controlled by these G alpha proteins (Mills and Moolenaar 2003).

LPA potently stimulates the growth, survival, and motility of a variety of cancer cells, some of which can themselves, produce LPA (Mills and Moolenaar 2003). One mechanism for this is through the secretion of autotaxin, a secreted lysophospholipase D, that produces LPA from extracellular lysophosphatidylcholine (LPC) (Stracke, Krutzsch

et al. 1992; Umezu-Goto, Kishi et al. 2002). Autotaxin is well known to be involved in promoting tumor development, metastasis and angiogenesis and its effects can be explained by the extracellular production of LPA. The levels of LPA are high in ascites fluid and plasma of patients with ovarian tumors (Fang, Schummer et al. 2002). LPA protects against apoptosis caused by chemotherapeutic agents (Deng, Wang et al. 2003). It promotes ovarian tumor development, possibly involving increased cyclin D expression (Hu, Albanese et al. 2003). LPA also increases vascular endothelial growth factor production in some cancer cells, which stimulates angiogenesis (Hu, Tee et al. 2001). In a colon cancer cell line, LPA increases the synthesis of macrophage migration inhibitory factor, which promotes tumor growth (Sun, Nishihira et al. 2003). LPA levels are elevated in the blood of patients with multiple myeloma (Sasagawa, Okita et al. 1999).

LPA signaling leads to the activation of both cell-proliferative signaling pathways, such as the Ras/Raf/ERK pathway, and cell survival pathways, such as those involving phosphatidylinositol-3 kinase (PI3-K) and Akt (Weiner and Chun 1999; Brindley 2004). These effectors are primarily activated via pertussis toxin-sensitive, G_i signaling. Although much is known about the effects of LPA signaling on cancer cell growth and motility, relatively little is known about LPA's effects on cell cycle regulators, such as the p53 tumor suppressor. p53 is a transcription factor that controls the expression of genes encoding proteins that regulate apoptosis and cell cycle progression (Shen, Real et al. 1983; Raycroft, Schmidt et al. 1991; Symonds, Krall et al. 1994). p53 is inactivated in about 50% of all cancers (Hainaut, Soussi et al. 1997).

Given the strong mitogenic effects of LPA and the commonly observed loss of p53 function in cancer cells, we determined whether LPA signaling affected the function of the p53 tumor suppressor. We found that signaling initiated by the LPA receptors (LPA_1, LPA_2, and LPA_3) potently inhibited p53-dependent transcription, promoted the loss of p53 protein, and protected A549 lung tumor cells from actinomycin D-induced apoptosis. These results demonstrate that p53 is a target for the actions of LPA and they provide a new dimension for understanding how LPA promotes tumor growth.

CHAPTER 3

MATERIALS AND METHODS

3.1 A549 Cell Culture

A549 cells were purchased from the American Type Culture Collection (Manassas, VA) and grown at 37°C and 5% CO2 in F12K Kaighn's modification medium (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum, 100 I.U./ml penicillin, 100 μg/ml streptomycin (HyClone, Logan, UT) and 1.5 g/liter $NaHCO_3$ (Biosource International, Camarillo, CA).

3.2 Reagents

Lysophosphatidic acid (18:1 LPA; 1-oleoyl-2-hydroxy-sn-glycero-3-phosphate), OMPT ((2S)-1-oleoyl-2-o-methyl-glycero-3-phosphothionate), and VPC32183(S) ((S)-phosphoric acid mono-{2-octadec-9-enoylamino-3-[4-(pyridine-2-ylmethoxy)-phenyl]-propyl} ester) (Avanti Polar Lipids, Alabaster, AL) were reconstituted in 1% fatty-acid free, charcoal-stripped BSA as previously described (Lee, Thangada et al. 1998; Pustilnik, Estrella et al. 1999). Mouse anti-p53 antibody (DO-1) and rabbit anti-p53 (FL-393), which both detect total p53, were from Santa Cruz Biotechnology Inc (Santa Cruz, CA). Mouse anti-paxillin and mouse anti-Bip antibodies (BD Biosciences, San Jose, CA) where used to monitor loading accuracy. Mouse anti-p21 (556431) and rabbit Akt1/protein kinase B (88-100) antibodies were purchased from BD Biosciences and Calbiochem (San Diego, CA) respectively. All other reagents were from Sigma or VWR.

3.3 Transfections

For all transcriptional reporter gene assays, A549 cells were transiently transfected using Lipofectin (Invitrogen) according to the Manufacturer's guidelines.

A549 cells were transfected with 50 μM Akt1 siRNA (Santa Cruz) with DharmaFECT (Dharmacon, Lafayette, CO) using antibiotic-free, complete media for gene silencing experiments.

3.4 Immunoblotting

A549 cells were grown in 150 mm dishes for 24 h before washing with serum-free medium (SFM) and starving in SFM for 12-16 h prior to the treatments indicated above and in figure legends. Cells were rinsed with ice-cold PBS supplemented with phosphatase inhibitors (Active Motif, Carlsbad, CA), detached by scraping and collected by centrifugation. Pellets were then solubilized on ice for 30 min with intermittent agitation in lysis buffer (10 mM Tris pH 7.4, 100mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM Na4P2O7, 2 mM Na3VO4, 1% Triton X-100, 10% glycerol, 0.1% SDS and 0.5% deoxycholate, (Biosource International) supplemented with 1mM PMSF from a 0.3 M stock and protease inhibitor cocktail (1:10 dilution; Sigma). Protein concentration was quantified using a BCA protein assay (Pierce, Rockford, IL). The samples (12-20 μg protein per lane) were then separated by 10% SDS-PAGE and transferred to nitrocellulose. The binding of primary antibodies was detected using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, Il).

3.5 Indirect Immunofluorescence and Quantification

Cells were grown on glass cover slips, treated as described, and fixed in 2% formaldehyde in PBS. After blocking non-specific sites with 10% fetal calf serum (PBS-serum), the cells were permeabilized with 0.02% saponin and incubation with primary antibodies. Cover slips were subsequently rinsed three times with PBS-serum and incubated with fluorescently-labeled secondary antibodies. During the final wash,

Hoechst 33342 dye (Molecular Probes, Eugene, OR) was added to label the DNA. Samples mounted on glass slides were observed with an Olympus BX40 epifluorescence microscope equipped with a 60X plan-apochromat lens and digital photomicrographs were obtained with a MagnaFire SP digital camera. All photographs were obtained using the same exposure time. For quantification of fluorescence intensity, photomicrographs of 15-25 cells per time point or experimental treatment were obtained from each experiment, which itself was repeated at least three independent times. The fluorescence intensity of the nuclear-localized p53 was measured in each cell using Metamorph Imaging Software (Universal Imaging Corporation, Downington, PA). The p53 fluorescence intensity was normalized by subtracting the background and then dividing that value by the fluorescence intensity of DNA labeled with Hoechst dye for each cell. Normalized data averages of each photomicrograph from all experiments were combined to obtain a grand average throughout. The data are presented as the mean ± S.E.M. Metamorph image analysis showed that cells with bright nuclear p53 had an average fluorescence intensity of 0.83 ± 0.06 (n=15 cells); whereas $p53^{NUC}$-diminished cells had an average fluorescence Intensity of 0.38 ± 0.03 (n=15 cells). Changes in the proportion of p53NUC-bright cells were quantified by scoring cells whose nuclear p53 fluorescence intensity was judged as being approximately equal to the average fluorescence intensity of cells with bright nuclear p53 staining (e.g., ~ 0.8). One hundred cells per sample were randomly selected and scored as exhibiting either bright nuclear p53 staining or as diminished nuclear p53 staining, based on relative fluorescence intensity. The data is presented as the mean percentage of $p53^{NUC}$-bright cells ± S.E.M.

3.6 Luciferase reporter gene assay

Both firefly luciferase and *Renilla* (pRL-TK) luciferase activities were measured 36 h post-transfection using a dual luciferase assay kit (Promega, Madison, WI) as previously described (Murph, Scaccia et al. 2003; Nguyen, French et al. 2004). The pp53-TA-luc vector contains a p53-response element that is upstream of the luciferase reporter (BD Biosciences Clontech). This plasmid, therefore, provides a measure of the transcriptional activity of p53. The plasmid, pRL-TK, which constitutively expresses *Renilla* luciferase (Promega), was used to normalize for differences in transfection efficiency between samples. The normalized value is defined as the ratio of the p53-induced firefly luciferase activity to *Renilla* luciferase. The data are presented as percent of control and were compared to untreated controls. They are shown as the mean ± S.E.M. of triplicate measurements from a representative experiment that was repeated at least three times.

3.7 Cell Viability

1 x 10^4 A549 cells were grown in the wells of a 96-well plate for 12 h in complete medium and then rinsed three times in SFM before incubating in either SFM, 10 μM LPA, 2 μg/ml actinomycin D, or both 10 μM LPA and 2 μg/ml actinomycin D for 24 h. After this incubation, 20μl of WST-1 cell viability reagent (Roche) was added to each well for 1 h at 37°C. This assay quantifies cell proliferation and cell viability, based on the cleavage of the tetrazolium salt WST-1 to formazan. The absorbance of each well was measured at 450 nm. The results were normalized to cells grown in SFM alone (Control) and are the mean ± S.E.M. of six replicates per condition from a representative experiment that was repeated three times with similar results.

3.8 Statistical analysis

The data was analyzed using either a single-factor or two-factor ANOVA followed by a Tukey's statistical test.

CHAPTER 4

RESULTS

4.1 LPA decreases p53 expression in A549 cells

We first determined how the bioactive lipid, LPA, affected the cellular distribution of p53 protein in A549 human lung carcinoma cells since these cells proliferate rapidly, express wild-type p53, and contain endogenous LPA receptors (Lu, Lin et al. 2002; Hama, Aoki et al. 2004). p53 was localized by using mouse anti-p53 antibody (DO-1) and indirect immunofluorescence; the nucleus was visualized by staining with Hoechst dye. In the majority of A549 cells (~70%), p53 was localized in the nucleus and, to a lesser extent, in a diffuse cytoplasmic pattern (Fig. 4.1A). The nuclear p53 labeling in these cells was bright; thus, we will refer to these cells as $p53^{NUC}$-bright cells. In contrast, p53 staining was greatly reduced or completely absent from the nucleus in approximately 30% of control A549 cells (Fig. 4.1A arrows and Fig.4.2 B, SFM); no detectable difference was observed in either the fluorescence intensity or distribution of p53 in the cytoplasm of these 'nuclear p53 diminished' cells (we will henceforth refer to these cells as $p53^{NUC}$-diminished cells). We quantified the fluorescence intensity of p53 staining in the nucleus of these two populations of cells by using MetaMorph image analysis software and normalized it to the fluorescence intensity of DNA labeled with Hoechst dye. In cells exhibiting bright nuclear p53 staining, the nuclear fluorescence intensity of p53 was 2.2-fold greater than that observed in cells exhibiting reduced p53 staining ($p53^{NUC}$-bright cells: Fluorescence Intensity = 0.83 ± 0.06, n=15 cells; $p53^{NUC}$-diminished cells: Fluorescence Intensity = 0.38 ± 0.03, n=15 cells).

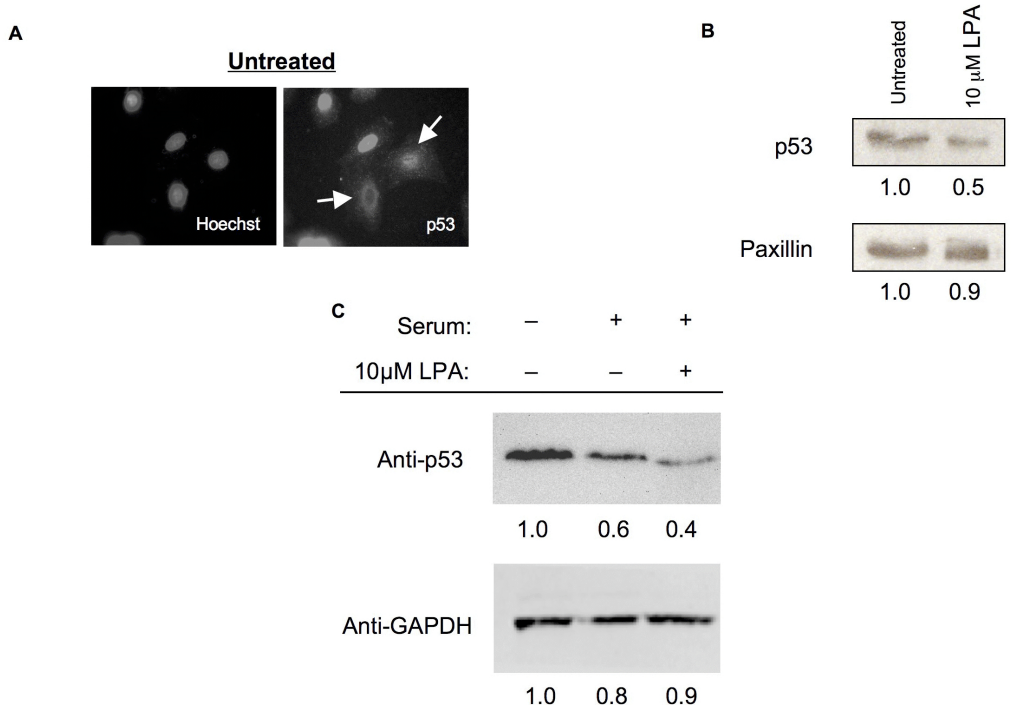

FIGURE 4.1. LPA stimulation reduces the nuclear localization and cellular abundance of p53 in A549 cells. *A). Localization of endogenous p53 in A549 lung carcinoma cells:* Endogenous p53 was localized in A549 cells using mouse anti-p53 antibody and fluorescently-labeled anti-mouse secondary antibodies as described in materials and methods; DNA was labeled with Hoescht dye. Arrows indicate cells that displayed reduced nuclear staining of p53, which are referred in the text as $p53^{NUC}$-diminished cells. (B,C) *Cellular abundance of p53:* A549 cells were treated for 6 h without LPA (Untreated) or with 10 μM LPA (B). Alternatively, cells were treated with serum-free media, complete media, or complete media supplemented with 10μM LPA for 6 h (C). Prior to immunoblotting of whole-cell extracts with mouse anti-p53 antibodies or mouse anti-paxillin antibodies. The protein band intensities were quantified by MetaMorph image analysis, following background subtraction and were normalized to the untreated samples as described in materials and methods. The relative band intensities are indicated below each band.

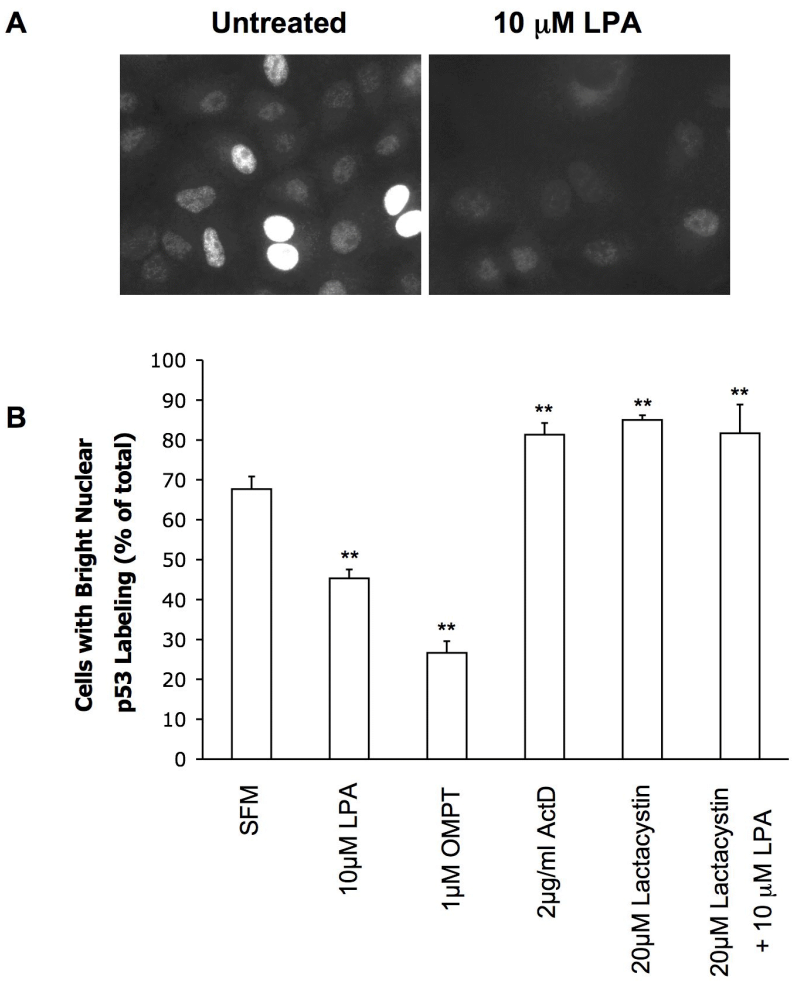

FIGURE 4.2. The LPA-induced reduction of nuclear p53 is inhibited by lactacystin. (A) *Population effect of LPA on nuclear p53 localization:* Serum-starved A549 cells were incubated in the presence or absence of 10 μM LPA for 6 h prior to indirect immunofluorescence localization of p53. Note the marked reduction in the nuclear p53 staining intensity in LPA-treated cells. (B) *Quantification of p53^{NUC}-bright cells:* Serum-starved A549 cells were stimulated for 6 h with serum-free medium (SFM), 10 μM LPA, 1 μM OMPT, 2 μg/ml actinomycin D, 20μM lactacystin, or lactacystin and LPA prior to the localization of p53 by indirect immunofluorescence of triplicate samples. One hundred cells per sample were randomly selected and scored as either having bright or diminished nuclear p53 staining. The data are presented as the mean percentage of p53^{NUC}-bright cells ± S.E.M. **P< 0.01 compared to control SFM cells.

We next investigated how LPA regulates p53 cellular abundance. Western blotting of whole-cell extracts also showed that treatment with 10 μM LPA for 6 h reduced the total cellular abundance of p53 by approximately 50% (Fig. 4.1B,C). In contrast, LPA treatment only slightly reduced the cellular abundance of the focal adhesion protein paxillin, which was used as a loading control. Taken together, these results indicated that LPA reduced the nuclear localization and total cellular abundance of the p53 tumor suppressor in A549 cells.

In addition to a reduction in the nuclear localization of p53 within a given cell, we observed that LPA also decreased the proportion of $p53^{NUC}$-bright cells (Fig. 4.2A,B). We quantified the proportion of $p53^{NUC}$-bright cells in control and LPA-treated samples and found that treatment of A549 cells with a physiological concentration of 10 μM LPA for 6 h decreased the proportion of $p53^{NUC}$-bright cells by almost 35%, relative to control cells (Fig. 4.2B, 10 μM LPA). Treatment of A549 cells with the LPA_3-selective agonist, (2S)-1-Oleoyl-2-O-Methyl-Glycero-3-Phosphothionate (OMPT) (Hasegawa, Erickson et al. 2003), decreased the proportion of $p53^{NUC}$-bright cells, even more than LPA, by approximately 60%, relative to control cells (Fig. 4.2B, 1 μM OMPT). Genotoxic drugs, such as the transcription inhibitor, actinomycin D, enhance the nuclear accumulation of p53, which ultimately induces apoptosis in cells (Kirk 1960; Cavalieri and Nemchin 1968; Kelley, Green et al. 1994). Treatment of A549 cells with 2 μg/ml actinomycin D indeed increased the proportion of $p53^{NUC}$-bright cells relative to untreated cells (Fig. 4.2B).

The rapid degradation of p53 in non-transformed cells is facilitated by ubiquitin- and proteosome-mediated degradation, which can occur both in the nucleus and in the

cytoplasm (Haupt, Maya et al. 1997; Kubbutat, Jones et al. 1997; Shirangi, Zaika et al. 2002). We next tested the effects of inhibiting proteasomal degradation on the proportion of $p53^{NUC}$-bright cells by using the proteasomal inhibitor, lactacystin (20 μM). Lactacystin has been demonstrated to inhibit nuclear proteasomal degradation (Joseph, Zaika et al. 2003; Rockel, Stuhlmann et al. 2005). Treatment of cells with lactacystin alone also increased the proportion of $p53^{NUC}$-bright cells relative to untreated cells (Fig. 4.2B). More importantly, incubation of A549 cells with both lactacystin (20 μM) and LPA (10 μM) prevented the LPA-induced decrease in $p53^{NUC}$-bright cells (Fig. 4.2B). These results indicate that the LPA-induced reduction of p53 in A549 cells is likely mediated by ubiquitin- and proteasomal-induced degradation.

4.2 LPA inhibits p53-stimulated transcription

A major function of p53 in cells is to stimulate the transcription of proteins involved in DNA repair, apoptosis, or cell-cycle arrest (Hofseth, Hussain et al. 2004). To investigate whether LPA stimulation resulted in the reduction of an endogenous p53-regulated gene product, we tested the effects of 10 μM LPA on the cellular distribution of the cyclin D inhibitor, $p21^{CIP1}$. Activated p53 stimulates the transcription of the cyclin inhibitor, $p21^{CIP1}$, which in turn promotes G_1 cell-cycle arrest (Zuo, Dean et al. 1998). In untreated cells, both p53 and $p21^{CIP1}$ were predominantly localized to the nucleus (Fig. 4.3A). Approximately 50% of all cells in the population showed $p21^{CIP1}$ labeling in the nucleus (Fig. 4.3B). Treatment with LPA (10 μM) reduced both p53 and $p21^{CIP1}$ labeling and decreased the proportion of cells showing nuclear $p21^{CIP1}$ staining to about 25% of the total population (Fig. 4.3B). In contrast, treatment of A549 cells with a genotoxic agent, doxorubicin (1 μg/ml), enhanced the nuclear staining of both p53 and $p21^{CIP1}$ (Fig.

4.3A). Doxorubicin significantly increased the proportion of cells showing $p21^{CIP1}$ staining in the nucleus to more than 85% of the population (Fig. 4.3B).

Since the expression of $p21^{CIP1}$ is transcriptionally regulated by p53, we hypothesized that the LPA-induced reduction in cellular $p21^{CIP1}$ was caused by a reduction in the transcriptional activity of p53. To directly test this hypothesis, we used a p53-stimulated luciferase reporter gene assay to determine the effects of LPA stimulation on p53-dependent transcription (M. Murph, data not shown, (Murph, Hurst-Kennedy et al. 2007)). A549 cells were transiently co-transfected with a plasmid encoding a firefly luciferase reporter gene, whose expression is driven by a basal promoter and an upstream p53 response element, and with the plasmid pRL-TK, which constitutively expresses *Renilla* luciferase. The latter plasmid serves to control for variations in transfection efficiency. Stimulation of native A549 cells with 10 μM LPA caused a significant reduction in p53-dependent transcription of luciferase to approximately 40% of that observed in untreated cells. This indicated that the observed reduction in p53 abundance correlated with a decrease in p53-dependent transcription, which likely explains the concurrent loss of $p21^{CIP1}$.

4.3 The LPA-dependent reduction of p53 abundance is dependent upon PI_3K, Akt, and nuclear export

To investigate the LPA signaling pathways that facilitate the reduction of p53, we quantified the effects of different pharmacological inhibitors of known LPA signaling pathways on the LPA-induced reduction in the proportion of $p53^{NUC}$-bright cells. Treatment of these cells with LPA alone (10 μM) decreased the proportion of $p53^{NUC}$-bright cells to approximately 35% of the total population (Fig. 4.4, Control). Pre-

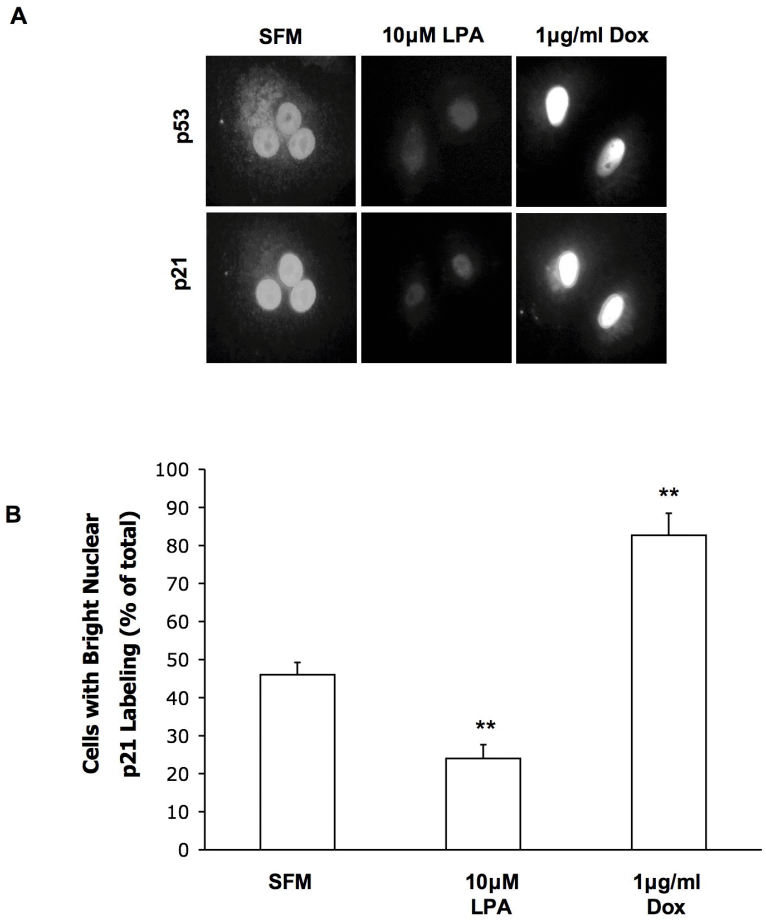

FIGURE 4.3. LPA stimulation decreases the abundance of p21^{CIP1}. (A) *Localization of p21^{CIP1}:* A549 cells were serum starved for 24 h and then stimulated for 6 h with serum-free medium, 10 μM LPA, or 1μg/ml doxorubicin. The cells were then fixed and double-labeled for endogenous p53 and p21 by indirect immunofluorescence. The majority of LPA-stimulated cells showed a reduction in the nuclear labeling of p53 and p21 relative to untreated cells. (B) The proportion of cells that showed nuclear labeling of p21 following LPA (10 μM) or doxorubicin (1 μg/ml) stimulation was quantified by evaluation of 100 cells/replicate of triplicate samples. The data are shown as the mean percentage of cells with nuclear p21 labeling ± S.E.M. **P< 0.01 compared to control SFM cells.

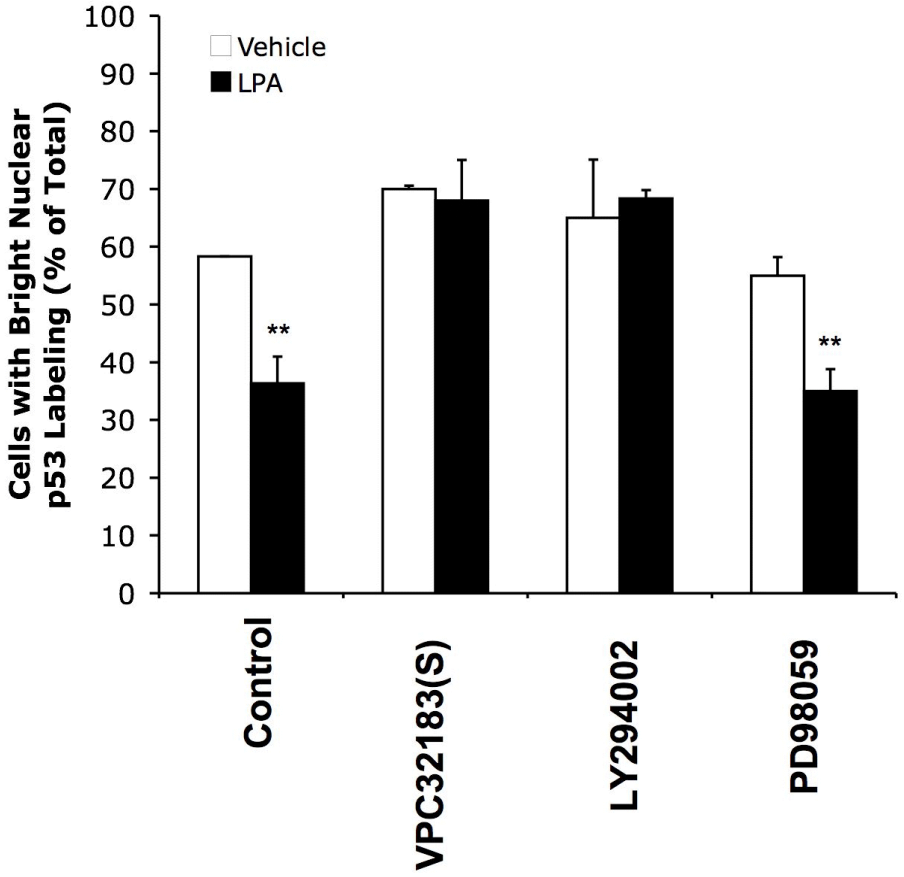

FIGURE 4.4. LPA-dependent reduction of cellular p53 is dependent on PI3Kinase and LPA1/3 signaling. A549 cells were grown on glass cover slips and were serum starved for 24 h. The cells were incubated with SFM, 10 μM LPA, 50 μM LY294002, 10 μM of VPC 32183(S), 50 μM PD980559, or a combination of LPA with the aforementioned reagents for 6 h prior to fixation and indirect immunofluorescence localization of p53. Quantification of cells exhibiting bright p53 nuclear staining was conducted as previously described in Figure 1B. **P< 0.01 compared to control, LPA-treated cells. The data shown are from a representative experiment that was repeated three times with similar results.

incubation of these cells with the LPA_1/LPA_3-selective antagonist, VPC32183(S), by itself increased the proportion of $p53^{NUC}$-bright cells. As expected, the antagonist completely prevented the LPA-induced reduction of nuclear p53 staining. Previous studies have shown that LPA receptors promote cell survival of cultured Schwann cells by activation of the PI_3K pathway (Weiner and Chun 1999). Consistent with this, incubation with the PI_3K inhibitor, LY294002, also prevented the LPA-induced reduction of nuclear p53 labeling in A549 cells. In contrast, the MEK inhibitor, PD98059, did not protect cells from the LPA-induced reduction of nuclear p53 staining. These results were consistently observed over three independent experiments.

A major function of PI_3K in cell survival signaling is to activate the serine/threonine protein kinase, Akt (Downward 2004). We next tested the role of Akt in the LPA-induced reduction of cellular p53, by using siRNA to specifically reduce the levels of Akt1 (Fig. 4.5A,B). Immunoblotting of whole cell extracts with anti-p53 antibodies showed that LPA stimulation of mock siRNA-transfected cells reduced cellular p53 levels by 30% relative to mock-transfected and untreated cells (Fig. 4.5B, Mock, + LPA). The p53 abundance in mock-transfected, untreated cells was set to 100% and all other p53 samples were normalized to this sample. LPA did not alter the cellular abundance of the cytoskeletal protein, paxillin (loading control), and only slightly reduced the cellular abundance of Akt. When A549 cells were treated with Akt1 siRNA oligonucleotides, the cellular levels of Akt were reduced by 30% (Fig. 4.5A, Akt siRNA). Although the reduction of cellular Akt1 was modest, we consistently observed a reduction in Akt1 levels that ranged from 30% to 50% over multiple independent experiments (n=3). Since siRNA treatment is performed using a standard transient

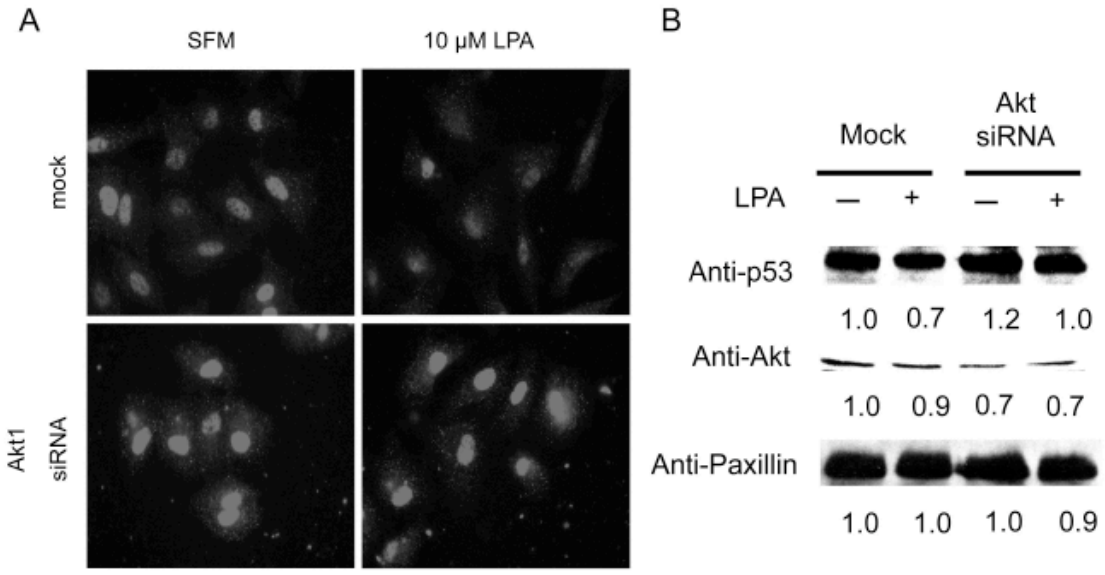

FIGURE 4.5. LPA promotes p53 reduction through activation of Akt1. A549 cells were grown in 150 mm dishes were either mock transfected or transfected with 50 nM Akt siRNA. Forty-eight h post-transfection cells were treated with either serum free media or 10 μM LPA for six h. (A) p53 was localized by indirect immunofluorescence in mock- and Akt1 siRNA-transfected cells after treatment with vehicle or 10 μM LPA. The data shown is from a representative experiment that was repeated three times with similar results. (B) Immunoblotting was conducted with mouse anti p53 (DO-1), mouse anti-paxillin, and rabbit anti-Akt1. Band intensities were determined by MetaMorph analysis. The numbers below the gels indicate the band intensities relative to untreated, mock-transfected cells. The blot is from a representative experiment that was repeated two times with similar results.

transfection protocol, transfection efficiencies are typically less than 100%. We anticipate that the extent of reduction of Akt in individual cells is likely to be greater. Nevertheless, the reduction of Akt1 in cells prevented the LPA-induced reduction of cellular p53 abundance (Fig. 6B, upper panel, Akt siRNA). Reduction of Akt1 did not alter the abundance of paxillin (Fig. 6B, lower panel, Akt siRNA).

The E3 ubiquitin ligase murine double minute 2 (mdm2) is a negative regulator of p53 and a phosphorylation target of Akt (Milne, Kampanis et al. 2004). Mono- and poly-ubiquitination of p53 results in the nuclear export and proteosomal degradation of the p53 protein (Li, Brooks et al. 2003). We determined whether LPA-mediated inhibition of p53 was dependent upon nuclear export by using the nuclear export inhibitor leptomycin B (LMB) (Kudo, Wolff et al. 1998). LPA reduced the abundance of nuclear p53 relative to untreated control (Fig. 4.6). However, in the presence of LMB, nuclear p53 levels were similar in both control and LPA-treated samples, indicated LPA-mediated inhibition of p53 is dependent upon nuclear export.

Taken together, these data indicate that stimulation of LPA1/3 initiates reduction PI_3K/Akt1-mediated nuclear export and degradation of p53.

4.4 LPA partially protects A549 cells from genotoxic stress-induced cell death

Several genotoxic drugs, such as the transcription inhibitor, actinomycin D, enhance the nuclear accumulation of p53, which ultimately induces apoptosis in cells (Kirk ; Cavalieri and Nemchin 1968; Kelley, Green et al. 1994). To investigate the physiological significance of LPA-induced reduction of p53, we examined the effects of LPA on actinomycin D-induced cell death in A549 cells. LPA protects both ovarian cancer cells and normal intestinal epithelial cells from apoptosis induced by

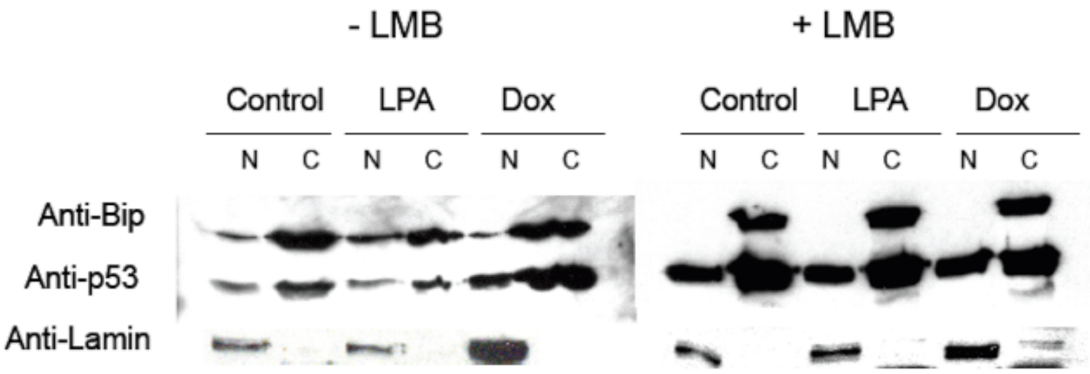

FIGURE 4.6. Degradation of p53 by LPA is dependent upon nuclear export. Confluent cultures of A549 cells were treated with serum-free media (control), 1μM LPA, 1μg/ml doxorubicin, 30ng/ml leptomycin B (LMB), or a combination of the afore mentioned treatments for 6 hours. After the treatment period, whole cell lysates were harvested and then fractionated in nuclear (N) and cytoplasmic (C) fractions using the Nuclear Fraction Kit (Active Motif). Immunoblotting was conducted using mouse anti p53 (DO1) antibody, mouse anti Bip (ER marker), and mouse anti lamin (nuclear marker). The blot is from a representative experiment that was repeated two times with similar results.

chemotherapeutic agents (Frankel and Mills 1996; Deng, Balazs et al. 2002). A549 cells were incubated with, or without 10 μM LPA in the presence, or absence of 2 μg/ml actinomycin D, 24 h prior to determining cell viability (Murph, Hurst-Kennedy et al. 2007). Actinomycin D reduced the percentage of viable cells by 52% compared to control cells. In contrast, co-incubation of actinomycin D and LPA only resulted in a 25% reduction in viable cells. Thus, LPA partially protected cells from actinomycin D-induced cell death. The LPA-induced attenuation of p53 signaling provides an additional mechanism that can contribute to LPA-dependent cell survival.

CHAPTER 5

DISCUSSION

In this study, we describe a novel target of LPA-induced cell signaling. Namely, that LPA through stimulation of its receptors reduces the abundance of the p53 tumor suppressor, which in turn decreases p53-dependent transcription. The LPA-dependent reduction of p53 is caused by enhanced degradation since inhibition of proteasomal action prevented the loss of p53. The signaling pathway leading from LPA receptors to p53 degradation involves PI_3K and Akt since decreasing the activity of either of these proteins prevented the reduction of p53. This novel action of LPA contributes to the cell survival and growth promoting effects of LPA. It helps to explain how increased LPA action promotes tumor progression and protects tumors against chemotherapy.

p53 is degraded both in the cytoplasm and in the nucleus through a proteosomal pathway (Shirangi, Zaika et al. 2002; Kashuba, Mattsson et al. 2003; O'Keefe, Li et al. 2003), which we now show to be activated by LPA. We found that LPA stimulation of A549 lung carcinoma cells led to a rapid loss of p53 protein from the nucleus of these cells and a concurrent decrease in the cellular levels of total p53. p53 contains several nuclear import signals (NLS) as well as multiple nuclear export signals (NES) (Middeler, Zerf et al. 1997; Zhang and Xiong 2001; O'Keefe, Li et al. 2003). In non-tumorigenic cells, association of p53 with the E3 ubiquitin ligase, mdm2, promotes ubiquitination and nuclear export of p53 with subsequent degradation of the protein by cytoplasmic proteasomes (Wu, Bayle et al. 1993; Haupt, Maya et al. 1997; Kubbutat, Jones et al. 1997). However, p53 can also be degraded in the nucleus by nuclear proteasomes (Shirangi, Zaika et al. 2002; Joseph, Zaika et al. 2003; Rockel, Stuhlmann et al. 2005).

Our data demonstrate that LPA stimulation leads to a dose-dependent, LPA-induced decrease of nuclear p53. Furthermore, the proteasomal inhibitor, lactacystin, prevented the LPA-induced loss of nuclear p53. Given that lactacystin inhibits both nuclear and cytoplasmic degradation (Rockel 2002), the accumulation of p53 in the nucleus in the presence of LPA and lactacystin suggests that LPA may also stimulate p53 degradation in the nucleus.

Consistent with the LPA-induced loss of nuclear p53 protein, we observed a corresponding decrease in the expression of the endogenous gene target, $p21^{CIP1}$. One of the consequences of p53 mobilization is to stimulate the transcription of the cyclin inhibitor, $p21^{CIP1}$, which in turn promotes G_1 cell-cycle arrest (Zuo, Dean et al. 1998). The concurrent loss of p21 suggested that the LPA-induced loss of p53 led to decreased p53-dependent transcription. In support of this hypothesis, we found that LPA signaling, either by endogenous or overexpressed LPA receptors inhibited the expression of a p53-dependent luciferase transcriptional reporter gene.

It was intriguing that over-expression of LPA receptors alone was sufficient to promote the inhibition of p53-dependent transcription. However, several lines of evidence support the specificity of this response. First and most important, stimulation of native, untransfected A549 cells with LPA alone inhibited p53-dependent transcription. This demonstrates that ligand stimulation of endogenous LPA receptors is sufficient to reduce p53. Second, treatment of native A549 cells with the LPA_3-selective agonist, OMPT (Hasegawa, Erickson et al. 2003), also potently reduced nuclear p53 localization. Third, treatment of cells with the LPA_1/LPA_3-selective antagonist, VPC32183(S), suppressed the LPA-induced loss of p53 from the nucleus of A549 cells. Finally, we

observed that the inhibition of p53-dependent transcription in cells by LPA receptor overexpression is prevented by incubation with exogenous phospholipase B, which degrades extracellular LPA that is likely generated by secreted autotaxin, and thus inhibits LPA signaling (Stracke, Krutzsch et al. 1992; Valet, Pages et al. 1998; Umezu-Goto, Kishi et al. 2002; Hama, Aoki et al. 2004). Thus, we propose that an autocrine mechanism involving a combination of the intracellular production of LPA and the conversion of extracellular LPC to LPA by autotaxin is sufficient to partially activate the endogenous LPA receptors in A549 cells and the over-expressed receptors in HepG2 cells.

What are the LPA signaling pathways that are responsible for the reduction of p53? LPA receptors stimulate multiple G protein-mediated signaling pathways (Fukushima, Kimura et al. 1998; Ishii, Contos et al. 2000; Anliker and Chun 2004). Our data indicate that PI3K and the protein kinase, Akt, are involved in the LPA-dependent reduction of cellular p53. The PI_3K/Akt signaling pathway is important for cell survival of normal and tumorigenic cells (Weiner and Chun 1999; Sautin, Crawford et al. 2001; Deng, Wang et al. 2003; Peres, Yart et al. 2003). Akt phosphorylates the E3 ubiquitin ligase, mdm2, which enhances both mdm2's nuclear import and its ubiquitin ligase activity. Thus, LPA stimulation of Akt may lead to increased p53 degradation via enhanced mdm2 activity (Fig. 5.1).

What are the consequences of LPA-induced attenuation of p53? LPA promotes the proliferation of a variety of normal and tumorigenic cells and it stimulates both cell proliferative and anti-apoptotic signaling pathways (Mills and Moolenaar 2003). Studies of the cellular mechanisms involved in mediating the growth promoting effects of LPA

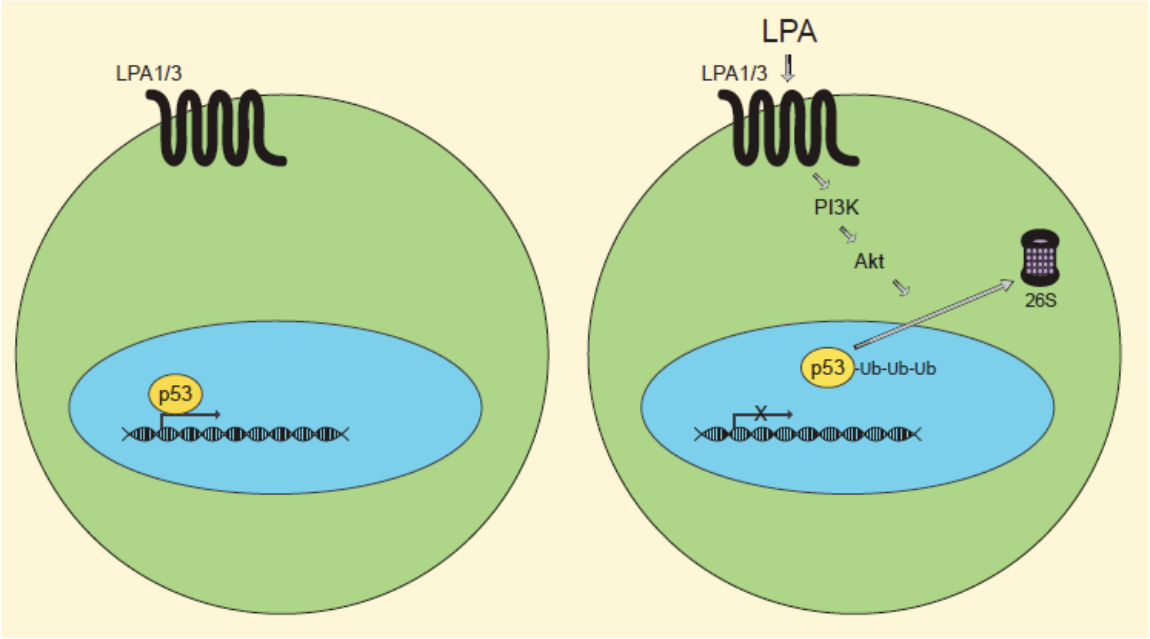

FIGURE 5.1. Proposed mechanism of LPA-mediated inhibition of p53 signaling in A459 cells. The PI_3K/Akt signaling cascade is activated in response to ligand stimulation of the LPA receptor resulting in increased activity of the E3-ubiquitin ligase, mdm2. Activated mdm2 mediates the nuclear export and polyubiquitnation of p53 leading to its proteosomal degradation. A decrease in p53 protein abundance results in a decrease in p53-mediated transcription.

have focused on the activation of Ras/MAP kinase and Rho GTPase signaling pathways as well as stimulation of the serine/threonine kinase, Akt. However, relatively little is known about the effects of LPA signaling on cell cycle regulators such as the p53 tumor suppressor. Our studies highlight a previously unappreciated effect of LPA signaling, namely that it decreases p53 concentrations and potently inhibits transcriptional activity of p53. In normal cells, activated p53 promotes G_1/S cell cycle arrest and/or apoptosis in response to DNA damage (Hofseth, Hussain et al. 2004). p53 is either mutated, or inactivated in 50% of all cancers, which enhances the ability of cancer cells to evade cell cycle arrest and apoptosis. In many tumor cells that contain wild type p53, key down-stream effectors such as the cyclin kinase inhibitor, $p21^{CIP1}$, are often mutationally inactivated.

Our results show that LPA receptor-mediated inhibition of p53 activity can enhance cancer cell survival by preventing the induction of apoptosis. We showed that LPA can partially protect A549 cells from actinomycin D-induced cell death (Murph, Hurst-Kennedy et al. 2007). Actinomycin D inhibits RNA polymerase activity (Wadkins, Jares-Erijman et al. 1996), which in turn activates p53 (Ashcroft, Taya et al. 2000). This action could also contribute to the observed effects of LPA in protecting cancer cells from cell death caused by chemotherapeutic agents (Frankel and Mills 1996). The action of LPA in increasing cyclin D1 expression (Hu, Albanese et al. 2003) would favor S-phase entry when LPA also decreases p53 expression. This would decrease the normal effects of p53 in blocking the transition at G1/S and cell cycle progression. This unscheduled (by increased cyclin D) and unrestricted (by decreased p53) S-phase entry

could lead to the accumulation of DNA damage after many cell generations and ultimately favor cell transformation.

Furthermore, several recent studies have demonstrated the transcription-independent induction of apoptosis by cytosolic p53 and p53 localized to mitochondria (Mihara, Erster et al. 2003; Chipuk, Kuwana et al. 2004; Erster, Mihara et al. 2004; Leu, Dumont et al. 2004). Cytosolic p53 directly binds and activates the pro-apoptotic protein, Bax, which induces the permeabilization of the outer mitochondrial membrane, cytochrome c release, and caspase-3 activation (Chipuk, Kuwana et al. 2004). Alternatively, a fraction of the total cellular p53 is translocated to the outer mitochondrial membrane in response to apoptotic stimuli (Mihara, Erster et al. 2003; Leu, Dumont et al. 2004). At the mitochondrial membrane, p53 interacts with Bcl-2 family members to promote cytochrome c release and caspase-3 activation. Thus, the LPA-mediated reduction of cellular p53 is expected to also diminish the ability of p53 to directly promote apoptosis in cancer cells through its interactions with pro-apoptotic and anti-apoptotic Bcl-2 family proteins. Indeed, this may contribute to the observed protection of A549 cells from actinomycin D-induced apoptosis by LPA. Taken together, these actions of LPA on p53 along with other proliferative signals through the Ras/Raf/ERK pathway can contribute to the actions of LPA in promoting tumor growth. The effect of LPA on p53 expression will be particularly critical in many tumor cells that already display decreased p53 levels.

Our work, therefore, identifies LPA as a physiological agonist that decreases p53 expression and thus demonstrates a further dimension whereby LPA can both decrease apoptosis and increase cell division. The present results provide further insights into the role of LPA in promoting cell division versus apoptosis and thereby cell proliferation.

The LPA-induced degradation of p53, and its consequent decreased control of cell cycle progression and cell death provide a novel dimension for understanding how LPA promotes tumor progression and protects tumor against chemotherapy.

PART II: LPA SIGNALING IN CELL DEATH, MATURATION, AND PROLIFERATION IN THE GROWTH PLATE

CHAPTER 6

INTRODUCTION

Lysophosphatidic acid (LPA 18:1; 1-oleoyl-2-hydroxy-sn-glycero-3-phosphate) is a bioactive lysophospholipid that consists of a single fatty acid chain and is produced by activated platelets and cancer cell types (Borges, Davanzo et al. 1998; Eder, Sasagawa et al. 2000). LPA is derived from a number of precursor lipids including phosphatidic acid (PA) which is generated by the metabolism of phosphatidyl choline (PC) by phospholipase D (PLD) (Aoki 2004). LPA exerts its effects on cells by activating the cell surface G-protein coupled receptors (GPCRs) LPA1/Edg2, LPA2/Edg4, LPA3/Edg7, LPA4/GPR23, and LPA5/GPR92 (Chun 1999; Goetzl and An 1999; Noguchi, Ishii et al. 2003; Lee, Rivera et al. 2006). These receptors collectively stimulate the $G_{\alpha i}$, $G_{\alpha q}$, $G_{\alpha s,}$ and $G_{\alpha 12/13}$ signaling pathways (Fukushima, Kimura et al. 1998; Chun 1999; Anliker and Chun 2004; Lee, Rivera et al. 2006; Lee, Rivera et al. 2007). In addition to G-protein coupled receptors, LPA has been shown to activate the nuclear fatty acid receptor peroxisome proliferator-activated receptor gamma (PPAR-γ) (McIntyre, Pontsler et al. 2003). LPA signaling has been implicated in a wide array of cellular processes including wound healing and smooth muscle contraction as well as cell proliferation, survival, and migration (van Corven, Groenink et al. 1989; Deng, Balazs et al. 2002; Fang, Schummer et al. 2002; Mills and Moolenaar 2003; Hama, Aoki et al. 2004; Ren, Xiao et al. 2006). These latter functions support a role for LPA signaling in cancer progression, where LPA has been shown to promote tumorigenesis by enhancing adhesion, migration, and invasion (Xu, Shen et al. 1998; Fang, Schummer et al. 2002; Ren, Xiao et al. 2006).

LPA appears to be involved in regulation of bone and cartilage. LPA has been shown to regulate osteoblasts (Caverzasio, Palmer et al. 2000; Panupinthu, Zhao et al. 2007) and chondrocytes are also sensitive to the lipid mediator (Kim, Lee et al. 2005) These studies suggest that LPA may also be involved in endochondral ossification, a process involving the formation of bone upon a cartilage template and the mechanism by which long bones in children and adolescents lengthen (Rauch 2005). This template is the result of growth, maturation, and calcification of growth plate cartilage (Ballock and O'Keefe 2003), which is regulated in part by the vitamin D metabolites 1,25-dihydroxy vitamin D3 [1α,25$(OH)_2D_3$] and 24,25-dihydroxy vitamin D3 [24R,25$(OH)_2D_3$] (Boyan, Schwartz et al. 1992). LPA acts synergistically with 1α,25$(OH)_2D_3$ to promote osteoblast differentiation (Gidley, Openshaw et al. 2006), providing evidence of a relationship between this metabolite and LPA signaling, but it is not known if there is a relationship between LPA and 24R,25$(OH)_2D_3$.

The resting zone of the growth plate provides a reservoir of chondrocytes that will eventually undergo terminal differentiation, hypertrophy, and apoptosis as the growth plate matures. The cells in the resting zone are surrounded by a proteoglycan-rich extracellular matrix and apoptosis is a relatively infrequent event (Aizawa, Kokubun et al. 1997; Erenpreisa and Roach 1999). These cells respond in particular to the 24R,25$(OH)_2D_3$, resulting in increased cell maturation, matrix synthesis, and cell survival (Sylvia, Schwartz et al. 1997; Boyan, Sylvia et al. 2001; Dean, Boyan et al. 2001; Schwartz, Ehland et al. 2002). 24R,25$(OH)_2D_3$ acts on resting zone chondrocytes via a PLD-dependent mechanism (Sylvia, Schwartz et al. 2001) and many 24R,25$(OH)_2D_3$-

mediated effects in resting zone chondrocytes have been shown to be dependent upon PLD activation (Boyan, Sylvia et al. 2001).

These observations implicate LPA as a second messenger during the promotion of cell maturation and survival in chondrocytes by 24R,25$(OH)_2D_3$. However, the downstream targets by which LPA exerts its effect on growth plate chondrocytes are unknown. One possibility is that LPA acts by modulating the abundance of the tumor-suppressor p53. LPA has been shown to promote the degradation of p53 in several cancer cell types (Murph, Hurst-Kennedy et al. 2007; Song, Wilkins et al. 2007), resulting in increased cell survival. Reduction of p53 protein abundance is necessary in osteoblast maturation (Lengner, Steinman et al. 2006; Tataria, Quarto et al. 2006) suggesting that LPA-mediated decreases in p53 may be important in the maintenance of cartilage tissue as well.

The purpose of this study was to investigate the role of LPA signaling in the maintenance of the growth plate resting zone. Specifically, we assessed how LPA regulates proliferation, maturation, and apoptotic cell death in growth plate chondrocytes using resting zone cells isolated from adult rat costochondral growth plate cartilage as our model system. We found that LPA enhances two markers of chondrocyte maturation: alkaline phosphatase enzymatic activity and [^{35}S]-sulfate incorporation. In addition, LPA was found to be a potent stimulator of proliferation. Lastly, LPA protects resting zone chondrocytes from apoptotic cell death by decreasing the abundance of the tumor suppressor p53 to alter p53 target gene expression and protein abundance. Collectively, these data suggest that LPA signaling promotes cellular proliferation, maturation and survival in resting zone chondrocytes demonstrating a novel physiological function of

LPA signaling and providing evidence that LPA produced by the cells in response to 24R,25$(OH)_2D_3$ stimulation may act to mediate its effects on resting zone chondrocytes.

CHAPTER 7

MATERIALS AND METHODS

7.1 Reagents

18:1 LPA (1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphate), OMPT ((2S)-1-oleoyl-2-O-methyl-glycero-3-phosphothionate), and VPC32183(S) ((S)-phosphoric acid mono-{2-octadec-9-enoylamino-3-[4-(pyridine-2-ylmethoxy)-phenyl]-propyl} ester) were purchased from Avanti Polar Lipids (Alabaster, AL). All lipid species were reconstituted in 1% charcoal-stripped bovine serum albumin (BSA) prior to treatment of cells. Unless otherwise stated, all other reagents were acquired from VWR International (West Chester, PA).

7.2 Cell Culture

The culture system used in this study has been previously described in detail (Boyan, Schwartz et al. 1988). Chondrocytes were obtained from the resting zone (reserve zone) of costochondral cartilage from 125-g male Sprague-Dawley rats and cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS), 1% antibiotics, and 50 μg/ml ascorbic acid (GIBCO-BRL, Gaithersburg, MD). Primary cells were cultured until fourth passage prior to experimental analysis.

7.3 LPA Isoforms

The abundance of LPA isoforms in lysates and media collected from the resting zone chondrocyte cultures was determined by liquid chromatography electrospray ionization tandem mass spectrometry (LC ESI MS/MS). Resting zone chondrocytes were cultured in T75 flasks and grown until confluence. Cells were then treated for 30 minutes

with starvation media (1% FBS) containing 1% bovine serum albumin (BSA) and 24R,25$(OH)_2D_3$ (10^{-9},10^{-8}, or 10^{-7}M) (BioMol, Plymouth Meeting, PA) or vehicle alone. After the treatment period, 1ml of conditioned media was collected and cell monolayers were harvested using 0.05M sodium hydroxide (NaOH). Media and lysate samples were spiked with 1 pmol of 17:0 LPA (internal standard) prior to lipid extraction via the Bligh-Dyer method (Bligh 1959). LC ESI MS/MS analysis was conducted at the Georgia Institute of Technology Bioanalytical Mass Spectrometry Facility using a Shimadzu HPLC pump and a Q-TRAP 4000 (Applied Biosystems, Foster City, CA). Reverse phase chromatography was performed using a Supelco C18 column with a flow rate of 500µl/min and an injection volume of 10µl. The isocratic buffers were 75:25 methanol:water (v/v) and methanol each with 5mM TEAA. Eluted samples were then analyzed on the QTRAP 4000 and LPA isoforms were compared to LPA standards supplied by Avanti Polar Lipids. Peak areas of LPA isoforms were normalized to 17:0 peaks to account for differences in lipid extraction efficiency.

7.4 LPA Receptor, Bax, Bcl-2, and p53 Expression

mRNA was harvested from resting zone chondrocytes using Trizol (Invitrogen, Carlsburg, CA) and reverse transcriptase polymerase chain reaction (RT-PCR) was used to identify the presence of the LPA receptors LPA1-5 and PPAR-γ. The following sequence specific primers were used: LPA1 sense: 5'-GGTTCTCTACGCTCACATC-3', LPA1 antisense 5'-GCAGTAGCAAGACCAATCC-3', LPA2 sense: 5'-CACCACCTCACAGCCATCC-3', LPA 2 antisense: 5'-AGACATCCACAGCACTCAGC-3', LPA3 sense: 5'-CTACAACAGGAGCAACAC-3', LPA3 antisense: 5'-CCAGCAGGTAGTAGAAGG-3', LPA4 sense: 5'-

ACAACTTTAACCGCCACTGG-3', LPA4 antisense: 5'-ATTCCTCCTGGTC CTGATGG-3', LPA5 sense: 5'-ACCTTGGTGTTCCCTATAATGC-3', LPA5 antisense: 5'-AGCCAGAGCGTTGAGAGG-3', PPAR-γ sense: 5'-CCGAAGAACCATCCGATTGAAG-3', and PPAR-γ antisense: 5'-CTCCGCCAACAGCTTCTCC-3'. In order to determine the effect of LPA on p53, Bax, and Bcl-2 mRNA expression, cells were treated with 0, 0.01, 0.1, and 1μM LPA for 6 h prior to harvesting the mRNA with Trizol. The following primers were used to amplify p53, Bax, and Bcl-2: p53 sense: 5'-CCGTCCCAGAAGGTTGCC-3', p53 antisense: 5'-CGC TGC TCC GAA GGT GAT-3', Bax sense: 5'-TTTGTTACAGGGTTTCATCC-3', Bax antisense: 5'-CCAGTTCATCTCCAATTCG-3', Bcl-2 sense: 5'-CTCGTGGCTGTCTCTGAAG-3', Bcl-2 antisense: 5'-TCTGCTGACCTCACTTGTG-3'. Glyceraldehyde-3-phosphate dehydrogenase (GADPH) was amplified as a control in each experiment: GAPDH sense: 5'-ATGCAGGGATGATGTTC-3', GAPDH antisense: 5'-TGCACCA CCAACTGCTTAG-3'.

7.5 Chondrocyte Maturation Assays

Confluent cultures were treated with LPA (0.01nM to 1μM) for the times indicated below. To determine if the LPA1/3 receptor was involved, cultures were also treated with LPA in the presence or absence of the LPA1/3-selective agonist (2S)-1-oleoyl-2-O-methyl-glycero-3-phosphothionate (OMPT) (Avanti Lipids, Alabaster, AL) (0.1nM to 1μM) (Hasegawa, Erickson et al. 2003) or the LPA1/3-selective antagonist (S)-phosphoric acid mono-(2-octadec-9-enoylamino-3-[4-(pyridine-2-ylmethoxy)-phenyl]-propyl) ester (VPC32183(S)) (Avanti Lipids) (0.01μM to 1μM) (Chang, Kim et

al. 2007). Chondrocyte maturation was assessed by examining alkaline phosphatase specific activity and [^{35}S]-sulfate incorporation.

To determine that 24R,25(OH)$_2$D$_3$ elicited its effects via an LPA-dependent mechanism, confluent cultures of resting zone cells were cultured for 24 hours in the presence and absence of the LPA1/3 selective antagonist VPC32183(S). Alkaline phosphatase specific activity was measured as described below.

Alkaline Phosphatase Specific Activity: Initial experiments determined the optimal time course by treating confluent cultures with 1μM LPA for 3, 6, 12, 18, and 24 hours. Subsequent experiments were performed after treating the cells for 24 hours. Following treatment, cell monolayers were lysed using 0.1% Triton X followed by sonication of each sample for 30 seconds. Alkaline phosphate activity was measured in cell layer lysates as a function of release of *para*-nitrophenol from *para*-nitrophenylphosphate at pH 10.2. Activity was normalized to the protein concentration of the lysates, determined using the macro-BCA assay (Macro BCA, Pierce Chemical Co., Rockford, IL).

[^{35}S]-Sulfate Incorporation: Mature chondrocytes produce a proteoglycan-rich extracellular matrix that is characterized by sulfated glycosaminoglycans. To assess the effects of LPA on chondrocyte maturation, confluent cells were labeled with [^{35}S]-sulfate 3 hours prior to harvest. At harvest, the conditioned media were removed, the cell layers (cells and matrix) were collected, and the amount of [^{35}S]-sulfate incorporated was determined as a function of protein in the cell layer (Martin, Schwartz et al. 1995).

7.6 DNA Synthesis

To determine if LPA regulated chondrocyte proliferation, DNA synthesis was assessed by measuring the incorporation of radio-labeled thymidine. Cells were grown to

sub confluence and treated with DMEM containing 1% FBS for 48 hours to induce quiescence. Cells were then treated with LPA (0.1nM to 1μM) in the presence or absence of OMPT (0.1nM to 1μM) or VPC32183(S) (0.01μM to 1μM) for 24 hours. Prior to harvest, cells were labeled for 3 hours with [^{3}H]-thymidine. The monolayers were washed three times with phosphate buffer solution (PBS) to remove unincorporated [^{3}H]. Cells were then fixed with cold 5% trichloroacetic acid followed by lysis with 1% sodium dodecyl sulfate. The amount of [^{3}H] activity was determined in each sample to determine the total amount of incorporated radio-labeled thymidine.

7.7 Apoptosis Assays

The role of LPA in chondrocyte survival was assessed by examining its ability to reduce apoptosis induced by two apoptogens: inorganic phosphate and chelerythrine (Teixeira, Mansfield et al. 2001; Zhong, Carney et al. 2008). Apoptotic cell death was determined by measuring caspase-3 activity, TUNEL staining, and DNA fragmentation. Confluent cultures of resting zone chondrocytes were treated with either 10^{-5}M chelerythrine or 7.5mM monobasic sodium phosphate to induce apoptosis. LPA (0.01μM, 0.1 μM, or 1 μM), VPC32183(S) (0.01μM, 0.1 μM, or 1 μM), or the vehicle was added to the cultures.

Caspase-3 Activity: Caspase-3 activity was determined using the Colorimetric CaspACE™ Assay System from Promega (Madison, WI). Cells were harvested 24h post treatment with 200μl cell lysis buffer followed by two 10 s periods of sonication. After harvest, 2μl of the caspase-3 selective substrate DEVD-pNA were added to each well containing 100μl of cell lysate and incubated at 37^{0}C for 4h. DEVD-pNA cleavage into

the colorimetric product pNA was measured at 405nm. Caspase-3 activity was normalized to protein content as determined by the Pierce Macro BCA Protein Assay Kit.

DNA Fragmentation: Cells were labeled with [^{3}H]-thymidine for 4 h prior to treatment. At the end of the treatment period, cell monolayers were washed with DMEM three times to remove unincorporated [^{3}H] and cells were lysed with TE buffer (10mM Tris-HCl, 1mM EDTA, 0.2% Triton X-100) for 30 minutes. Cell lysates were centrifuged at 13,000g for 15 minutes to separate intact DNA from fragmented DNA. The amount of incorporated [^{3}H]-thymidine was determined in each fraction to establish the total amount of fragmented DNA.

TUNEL Staining: DNA nicking was measured using the In Situ Cell Death Detection Kit (Roche Applied Science, Indianapolis, IN). After treatment, cells were fixed using 4% paraformaldehyde in PBS for 1hour. To detect nicks, cells were incubated with horse radish peroxidase-conjugated dUTP for 1hour at 37^{0}C. Nicks were visualized with DAB substrate (3,3-diaminobenzidinetetrahydrochloride) also purchased from Roche.

7.8 Regulation of p53

Confluent cultures in T75 flasks were treated for 6 h with 0, 0.01, 0.1, and 1μM LPA. After treatment, the cell monolayer was washed twice with PBS and harvested with RIPA buffer. mRNA for p53 was determined as described above. The abundance of p53 protein in both the whole cell lysate and nuclear and cytoplasmic fractions was determined by ELISA (p53 pan ELISA, Roche) and normalized to total cellular or total fraction protein as determined by Pierce Macro BCA Protein Assay Kit. Nuclear and cytoplasmic fractions were isolated by centrifuging whole cell lysates for 10 minutes at

13,000 rpm. The resulting supernatants (cytoplasmic fraction) were collected and the pellets (nuclear fraction) were resuspended in 500μL RIPA buffer.

To assess changes in p53-mediated transcription, luciferase reporter gene assays were conducted as previously described (Murph, Scaccia et al. 2003). Cells were transfected with two plasmids: one containing p53-controlled firefly luciferase (pp53_TA-Luc, Clonetech, Mountain View, CA) and the other carrying constitutively expressed *Renilla* luciferase (pLR-TK, Promega, Madison, WI). 24 h after transfection, cells were treated with 0, 0.01, 0.1, and 1μM LPA for 16 h and luciferase activity was measured using the Dual Luciferase Reporter Assay kit (Promega, Madison, WI).

7.9 Abundance of Bax, Bcl-2, and p21 Protein

Western blots were performed to determine the effect of LPA on the protein abundance of Bax, Bcl-2, and p21. Cell culture lysates were prepared from confluent resting zone cells and were resolved on 10% SDS-polyacrylamide gels. Blots of the gels were probed with monoclonal antibodies against Bax (Δ 21, Santa Cruz Biotechnology, Inc.), Bcl-2 (DC 21, Santa Cruz Biotechnology, Inc.), p21 (BD Pharmingen, San Jose, CA), or GAPDH (MAB374 Chemicon, Billerica, MA). Immunoreactive bands were detected using 1:5,000 dilutions of horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (Jackson Immunoresearch, West Grove, PA), and visualized using enhanced chemiluminescence (Super-Signal WestPico Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL). mRNA for Bax and Bcl-2 were performed as described above.

7.10 Statistical Analysis

Each experiment had six independent cultures per variable to ensure sufficient power to detect statistically significant differences. All experiments were conducted multiple times to validate the observations, but data from a single representative experiment are shown in the figures and are expressed as means $\pm$ SEM. Statistical analysis was conducted using ANOVA analysis followed by Student's T-test with a Bonferroni modification. Differences in means were considered to be statistically significant if the p value was less than 0.05.

CHAPTER 8

RESULTS

8.1 Resting Zone Chondrocytes Produce LPA and Express LPA Receptors

LC ESI MS/MS showed that three LPA isoforms, 16:0 LPA, 18:1 LPA, and 18:0 LPA, were present in both the cell monolayer (Fig. 8.1A) and conditioned media (Fig. 5.1B), indicating the existence of both intracellular and extracellular LPA in cultures of RC cells. $24R,25(OH)_2D_3$ increased the abundance of 16:0 and 18:1 LPA in the media, but not in the cell monolayer. The effect of $24R,25(OH)_2D_3$ on both LPA forms was dose-dependent and was greatest in cultures treated with 10^{-8} M. RT-PCR using sequence-specific primers detected the presence of the five cell surface G-protein coupled receptors, LPA1-5, and the nuclear receptor PPAR-gamma (Fig. 8.1C). Distinct bands for LPA1, LPA3, and LPA4 were observed. Bands for LPA2 and LPA5 were present but to a lesser extent.

$24R,25(OH)_2D_3$ exerted its effects on chondrocyte maturation via an LPA1/3 dependent mechanism. The LPA1/3-selective antagonist VPC32183(S) attenuated 24R,25(OH)2D3-mediated increases in alkaline phosphatase activity (Fig. 8.1D).

8.2 Exogenous LPA Enhances Chondrocyte Maturation

Initial time course experiments showed that LPA increased alkaline phosphatase specific activity at 24 hours, but no LPA effect was observed prior to this time point (data not shown). For this reason, all future maturation experiments were conducted following a 24 hour exposure to the lipid mediator. Both LPA and the LPA1/3-selective agonist OMPT increased alkaline phosphatase activity in the resting zone chondrocytes in a dose

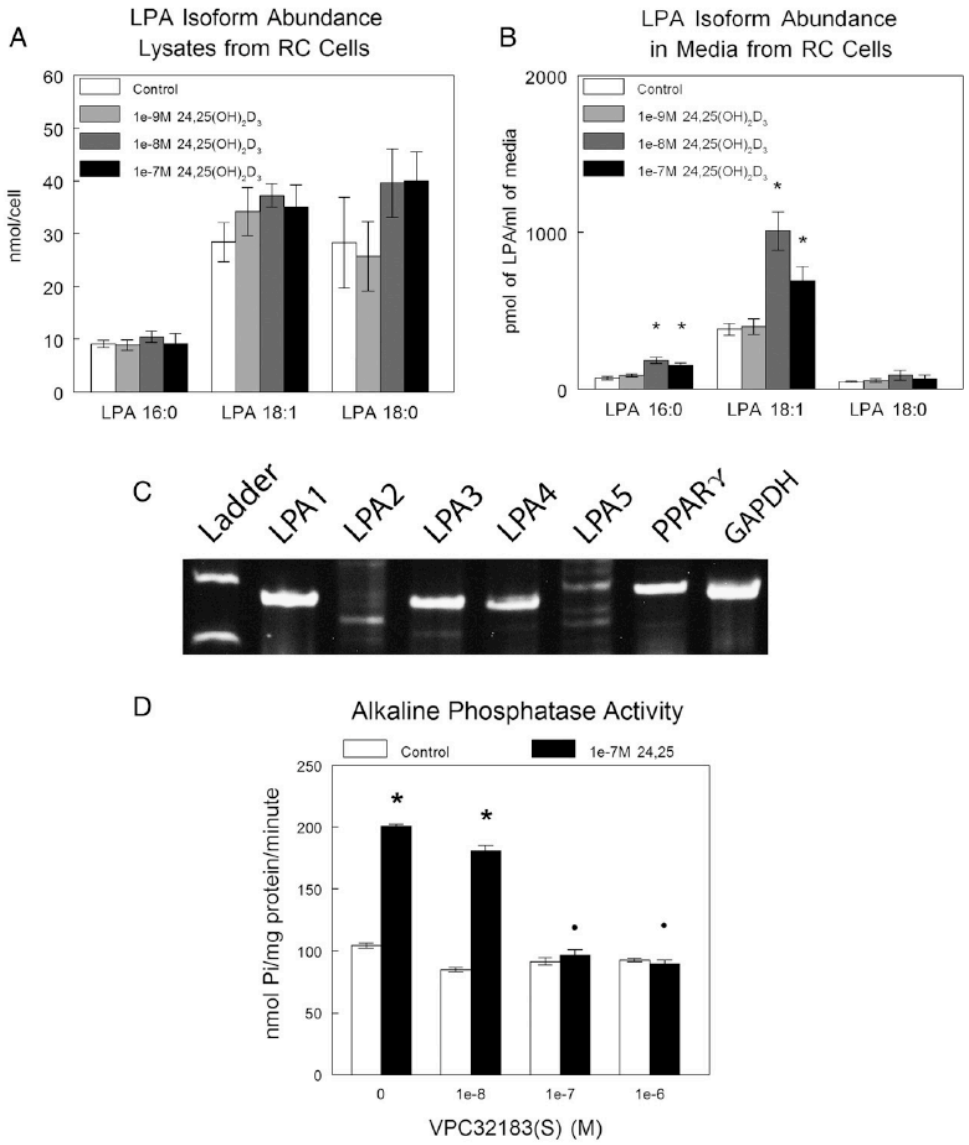

FIGURE 8.1. Resting zone chondrocytes produce LPA and express LPA receptors. LPA isoform abundance was determined by LC ESI MS/MS from cell layer lysates (A) and media (B) collected from resting zone chondrocytes treated with media containing vehicle alone or 10^{-9} to 10^{-7} M 24R,25$(OH)_2D_3$. (C) Total RNA was isolated from confluent cultures of male rat resting zone chondrocytes using Trizol. LPA receptor cDNA was generated using specific reverse primers for LPA1-5, PPAR-γ, and GADPH and Omniscript Reverse Transcriptase (Qiagen). LPA receptor fragments were amplified via polymerase chain reaction using sequence specific primers. (D) Male rat resting zone chondrocytes were treated with complete media or 24R,25$(OH)_2D_3$ in the presence or absence of the LPA1/3 selective antagonist VPC3218(S) for 24 h. Alkaline phosphatase specific activity was measured in cell layer lysates as a function of release of *para*-nitrophenol from *para*-nitrophenylphosphate at pH 10.2. (* =significant relative to untreated control, • = significant relative to 1e-7M 24R,25$(OH)_2D_3$ alone).

dependent manner at concentrations ranging from 0.01μM to 1μM (Fig. 8.2A and 8.2C). The same concentrations of LPA and OMPT also increased [^{35}S]-sulfate incorporation (Fig. 8.2B and 8.2D). Furthermore, VPC32183(S) attenuated LPA-mediated increases in both alkaline phosphatase activity and [^{35}S]-sulfate incorporation in a dose dependent manner (Fig 8.2E and 8.2F), indicating that the effects of LPA stimulation are dependent upon activation of LPA1 and/or LPA3.

8.3 LPA Increases DNA Synthesis in a Dose-Dependent Manner

Treatment of pre-confluent cells culture with 1μM LPA or OMPT enhanced DNA synthesis 100% over control cultures (Fig. 8.3A and 8.3B). Inhibition of LPA1 and LPA3 with VPC321283(S) inhibited LPA-mediated increases in proliferation (Fig. 8.3C). These data demonstrate LPA promotes proliferation in resting zone chondrocytes through activation of LPA1 and/or LPA3.

8.4 LPA Reduces the Stimulatory Effects of Phosphate and Chelerythrine Apoptosis

Both phosphate and chelerythrine increased DNA fragmentation relative to the control in a dose-dependent manner (Fig. 8.4A and 8.4B). LPA doses ranging from 0.01μM to 1μM completely and partially rescued phosphate and chelerythrine-induced DNA fragmentation, respectively. Similarly, LPA reduced DNA nicking induced by both apoptogens, evidenced by reduced TUNEL staining (Fig 8.4C). Inorganic phosphate and chelerythrine also increased caspase-3 activity and LPA reduced this marker of apoptosis as well (Fig. 8.4D, 8.4E). The rescue of Pi-induced caspase-3 activity by LPA was attenuated by VPC32183(S) (Fig. 8.4F).

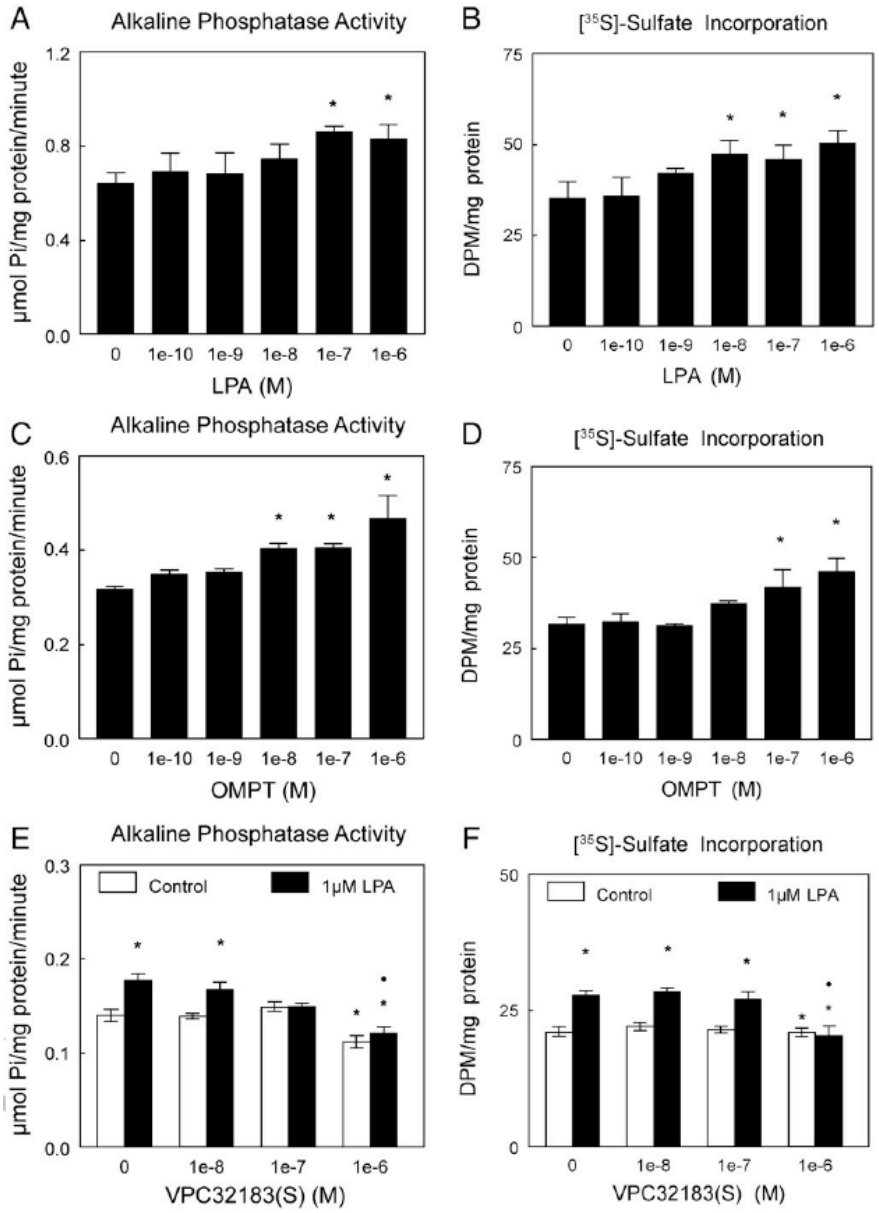

FIGURE 8.2. LPA increases maturation in resting zone chondrocytes. Chondrocyte maturation was determined by measuring alkaline phosphatase specific activity (A, C, E) and [^{35}S]-incorporation (B, D, F). Male rat resting zone chondrocytes were treated with complete media or varying concentrations of LPA (A, B) or the LPA1/3 selective agonist OMPT (C,D) for 24 h prior to harvesting the cell monolayer. (E, F) Additionally, cells were treated with complete media or 1µM LPA in the presence or absence of the LPA1/3 selective antagonist VPC3218(S) for 24 h. Alkaline phosphatase specific activity was measured in cell layer lysates as a function of release of *para*-nitrophenol from *para*-nitrophenylphosphate at pH 10.2. In order to measure sulfate incorporation, cells were labeled with [^{35}S]-sulfate 4 h prior to harvest. At harvest, the conditioned media was removed, the cell layers (cells and matrix) were collected, and the amount of [^{35}S]-sulfate incorporated determined as a function of protein in the cell layer. (* =significant relative to untreated control, • = significant relative to 1µM LPA alone).

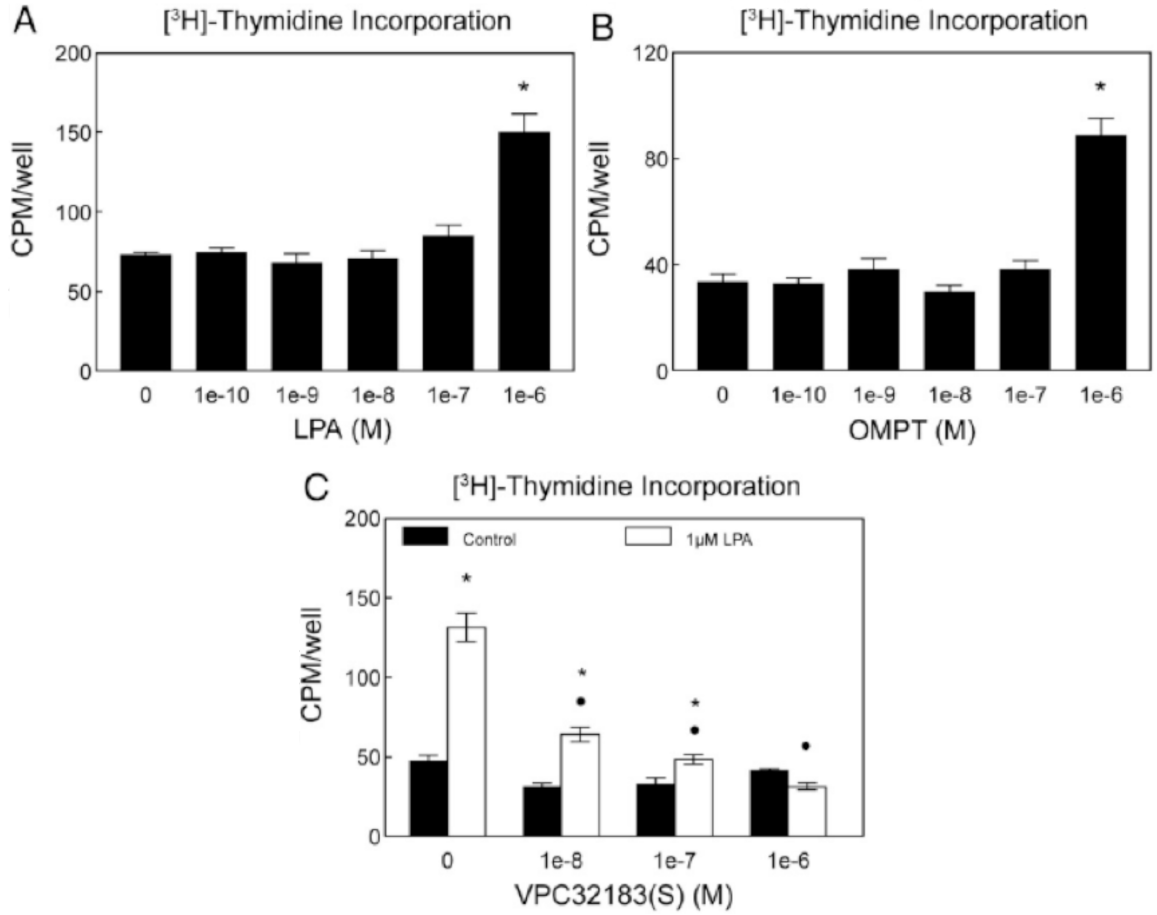

FIGURE 8.3. LPA enhances proliferation. DNA synthesis was determined by measuring the incorporation of radio-labeled thymidine. Cells were grown to sub confluence and treated with DMEM containing 1% FBS for 48 hours to induce quiescence. Male rat resting zone chondrocytes were treated with complete media or varying concentrations of LPA (A) or the LPA1/3 selective agonist OMPT (B) for 24 h prior to harvesting the cell monolayer. Cells were alternatively treated with complete media or 1μM LPA in the presence or absence of the LPA1/3 selective antagonist VPC3218(S) for 24 h (C). Prior to harvest, cells were labeled for 3 h with [^{3}H]-thymidine. The monolayers were washed three times with PBS to remove unincorporated [^{3}H]. Cells were then fixed with cold 5% trichloroacetic acid followed by lysis with 1% sodium dodecyl sulfate. The amount of [^{3}H] activity was determined in each sample to determine the total amount of incorporated labeled thymidine. (* =significant relative to untreated control, • = significant relative to 1μM LPA alone).

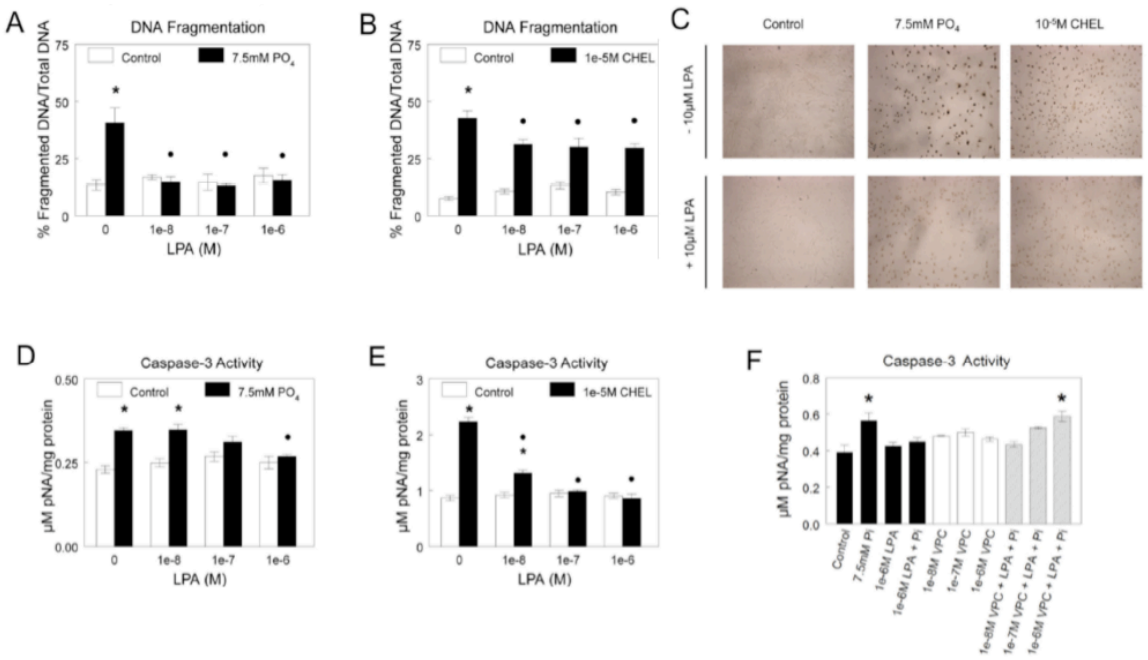

FIGURE 8.4. LPA protects cells from phosphate and chelerythrine-induced apoptosis. *DNA fragmentation:* Resting zone chondrocytes were labeled with [^{3}H]-thymidine for 4 h prior to treatment with the PKC inhibitor chelerythrine (A) or 7.5mM monobasic sodium phosphate (B) in the presence and absence of LPA for 24 h. At the end of the treatment period, cell monolayers were washed with DMEM three times to remove unincorporated [^{3}H] and cell were lysed with TE buffer (10mM Tris-HCL, 1mM EDTA, 0.2% Triton X-100) for 30 minutes. Cell lysates were centrifuged at 13,000g for 15 minutes to separate intact DNA from fragmented DNA. The amount of incorporated [^{3}H]-thymidine was determined in each fraction to establish the total amount of fragmented DNA. *TUNEL*: (C) Resting zone chondrocytes were treated with complete media (control), chelerythrine or 7.5mM monobasic sodium phosphate in the presence and absence of LPA for 24 h. Cells were fixed using 4% paraformaldehyde in PBS for 1 h. DNA nicking was assessed using the In situ Cell Death Detection Kit (Roche) followed by analysis with light microscopy. All images were obtained using an exposure time of 1.5 seconds. *Caspase-3 activity:* Caspase-3 activity was measured using the Colorimetric CaspACE™ Assay System (Promega) (D, E, F). Resting zone chondrocytes were harvested after treatment with complete media (control), chelerythrine or 7.5mM monobasic sodium phosphate in the presence and absence of LPA and/or VPC32183(S) for 24 h. Cell lysates were incubated with the caspase-3 substrate DEVD-pNA. Cleavage of DEVD-pNA into pNA was measured at 405nm and normalized to total protein. (* =significant relative to untreated control, • = significant relative to 7.5mM PO_4 alone or 10^{-5}M CHEL alone).

8.5 LPA Promotes Cell Survival via p53 Signaling

Control cultures of resting zone chondrocytes expressed p53 mRNA (Fig. 8.5A) and protein (Fig. 8.5B). Treatment with LPA had no effect on p53 mRNA at 6 hours, but there was a decrease in p53 protein at this time point. Nuclear p53 protein was decreased by LPA, whereas, cytoplasmic p53 did not change in response to the treatment (Fig 8.5C). Both p53-mediated transcription (Fig. 8.5D) and the abundance of the p53-target gene p21 (data not shown) were decreased by LPA. Moreover, LPA decreased both the mRNA expression (Fig. 8.6A) and protein abundance (Fig. 8.6B) of Bax. Conversely, both Bcl-2 mRNA and protein abundance were increased by LPA (Fig. 8.6A, 8.6B).

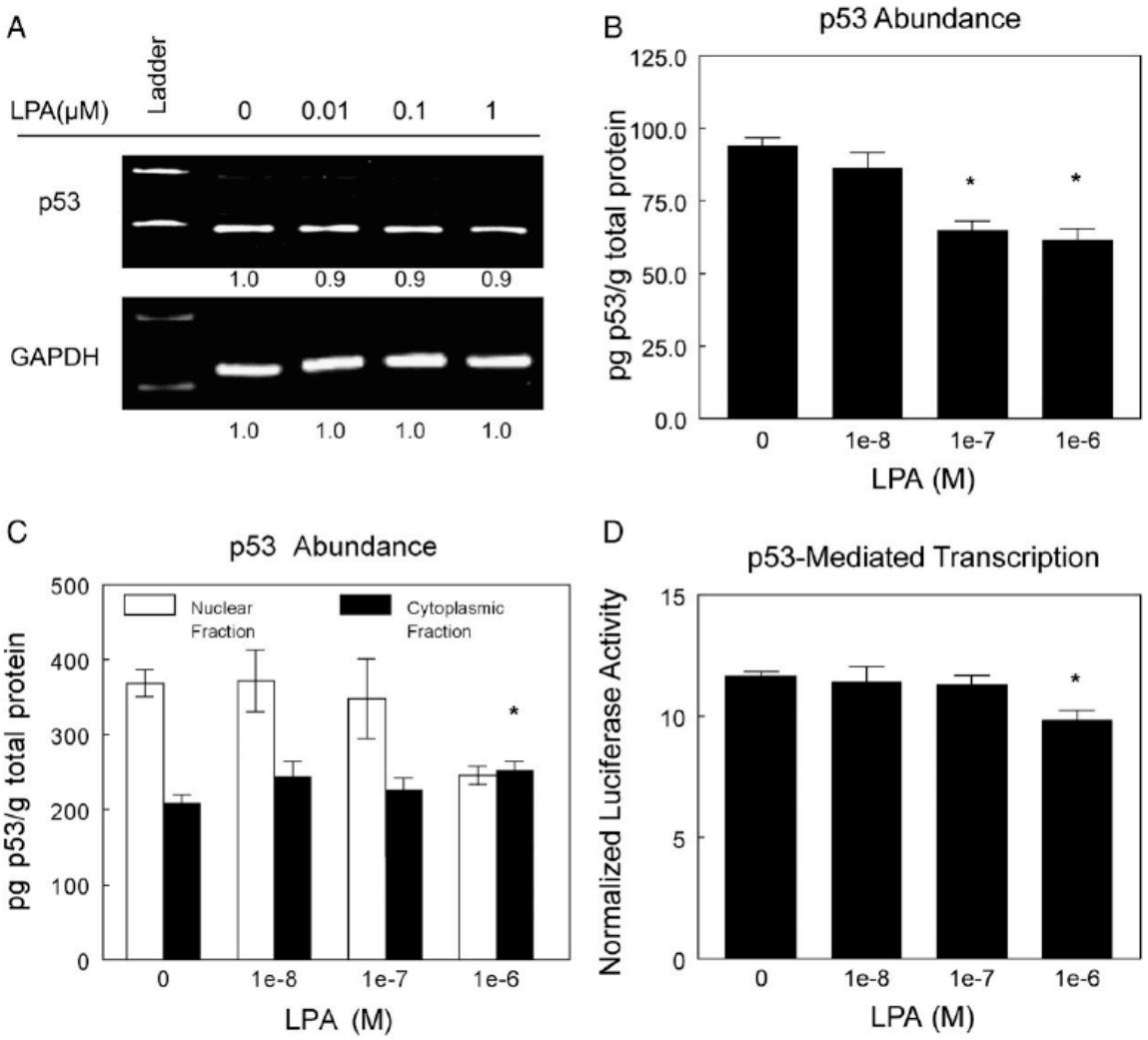

FIGURE 8.5. LPA reduces p53 at the translational level but not at the transcriptional level. RNA from resting zone chondrocyte cultures treated with 0, 0.01, 0.1, or 1μM LPA for 6 h was collected using Trizol (A). p53 and GAPDH cDNA were generated using specific reverse primers and Omniscript Reverse Transcriptase (Qiagen). Gene fragments were then amplified via polymerase chain reaction using sequence specific primers. Resting zone chondrocyte cultures were treated with complete media or with LPA for 6 hours prior harvest. The abundance of p53 was measured in both the whole cell lysate (B) and the nuclear and cytoplasmic fractions (C) by p53 pan ELISA (Roche). p53-mediated transcription was assessed as a function of normalized luciferase activity (D). Cells were transfected with p53-controlled firefly luciferase and constitutively active *Renilla* luciferase. 24 h post-transfection, cells were treated with LPA for 16 h prior to measuring luciferase activity.

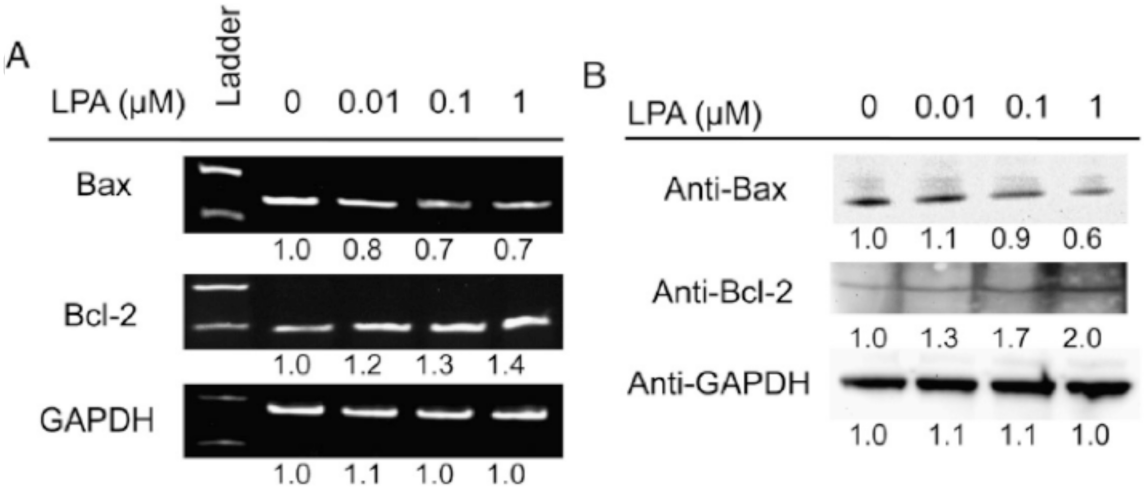

FIGURE 8.6. Bax and Bcl-2 mRNA and protein abundance are regulated by LPA. (A) Total RNA was isolated from male rat resting zone chondrocyte cultures using Trizol. Bax, Bcl-2, and GAPDH cDNA was generated using specific reverse primers and Omniscript Reverse Transcriptase (Qiagen). Gene fragments were amplified via polymerase chain reaction using sequence specific primers. (B) Resting zone chondrocyte cultures were treated with complete media or with LPA for 6 hours prior to immunoblotting of whole-cell extracts with mouse anti-Bcl-2, mouse anti-Bax, or mouse anti-GAPDH antibodies.

CHAPTER 9

DISCUSSION

Our results indicate that LPA is an autocrine regulator in the growth plate resting zone (Fig 9.1). Resting zone chondrocytes contain intracellular LPA and secrete extracellular LPA. The LPA isoforms identified, 16:0, 18:1, and 18:0, are the most biologically relevant of the LPA isoforms (Sando and Chertihin 1996; Pustilnik, Estrella et al. 1999; Sugiura, Nakane et al. 2002). In addition, LPA receptors are present in RC cells, demonstrating that they have the potential to respond to this phospholipid metabolite. Moreover, 24R,25$(OH)_2D_3$ increased the extracellular abundance of LPA 16:0 and LPA 18:1 and the LPA1/3-selective antagonist VPC32183(S) attenuated 24R,25$(OH)_2D_3$-mediated maturation, suggesting that LPA may act as a downstream mediator of vitamin D metabolite effects on resting zone cells.

This study demonstrates that LPA signaling plays a role in the maintenance of the resting zone cartilage by promoting the survival of the chondrocyte pool. At least two mechanisms are involved in the inhibition of apoptosis. LPA acts via reduced p53 and its downstream mediator p21, reduced Bax and increased Bcl-2. This is particularly important because resting zone cells serve as the pool for the growth zone and premature cell death in the resting zone could result in premature closure of the growth plate and limb shortening. In addition, LPA stimulated DNA synthesis, suggesting that LPA is involved in maintaining the pool of resting zone chondrocytes via proliferation. LPA has been shown to act as a mitogen in other systems (van Corven, Groenink et al. 1989; Sautin, Crawford et al. 2001; Kue, Taub et al. 2002; Radeff-Huang, Seasholtz et al. 2004; Kim, Lee et al. 2005), as well as in primary rat articular chondrocytes (Kim, Lee et al.

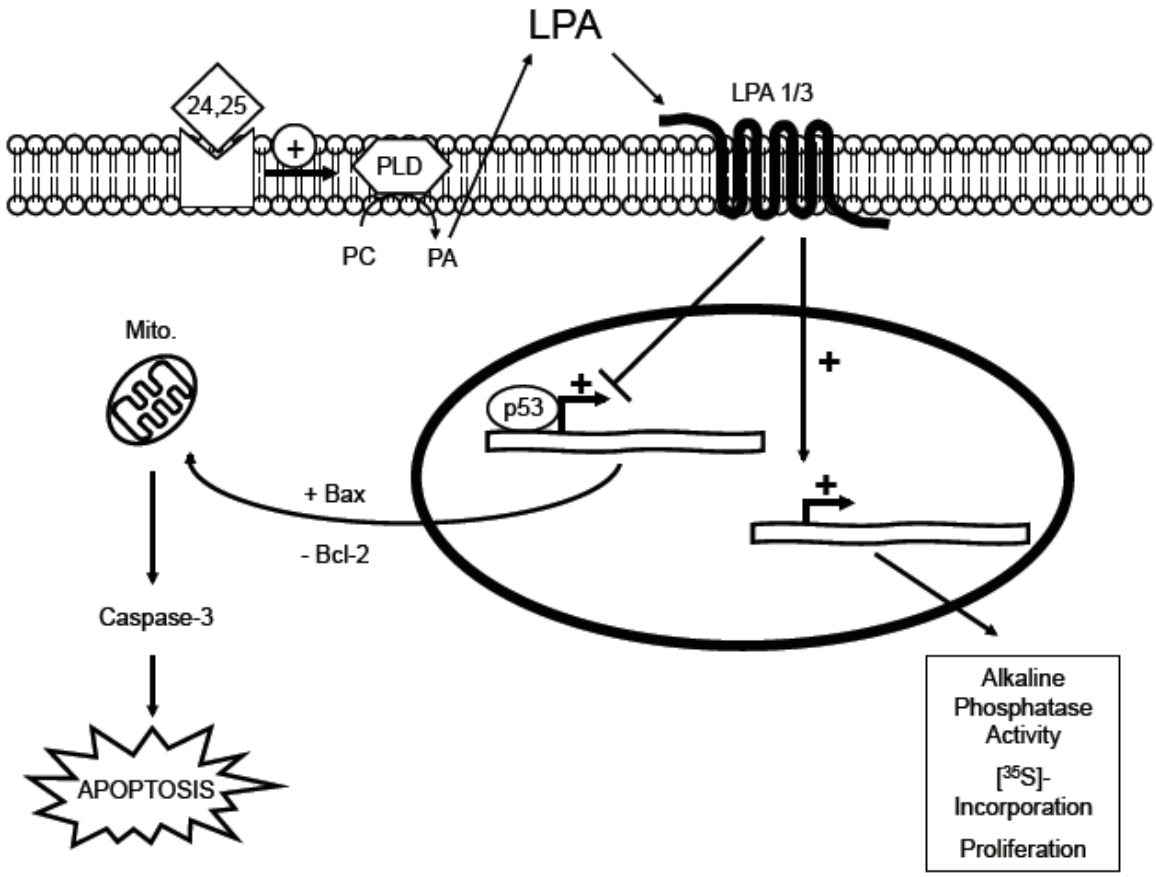

FIGURE 9.1. Proposed mechanism of LPA signaling in the resting zone of the growth plate. 24R,25$(OH)_2D_3$-mediated stimulation of phospholipase D (PLD) promotes the conversion of phosphatidyl choline (PC) to phosphatidic acid (PA) leading to LPA production. LPA stimulates LPA1 and/or LPA3, resulting in increases alkaline phosphatase activity, [^{35}S]-incorporation, and proliferation. LPA also decreases the abundance and transcriptional activity of the tumor suppressor p53 causing an increase in the Bcl-2:Bax ratio and a decrease in caspase-3 activity.

2005). However, it decreased proliferation of T/C-28a2 cells, a human articular chondrocyte-like cell line (Facchini, Borzi et al. 2005), raising the possibility that its effects are cell specific.

In addition to its stimulatory effects on DNA synthesis, LPA increased [^{35}S]-sulfate incorporation, suggesting an increase in the synthesis of a sulfated proteoglycan extracellular matrix around the newly generated chondrocytes. Alkaline phosphatase specific activity also increased, supporting the hypothesis that the chondrocytes were producing a mature matrix containing alkaline phosphatase-rich extracellular matrix vesicles (Boyan, Schwartz et al. 1990).

Our results strongly support a physiological role for LPA in promoting chondrocyte survival in the resting zone. We demonstrated by four different methods (DNA fragmentation, TUNEL staining, caspase-3 activity, and Bcl-2/Bax ratio) that LPA signaling reduced the induction of apoptosis by two agents shown previously to stimulate the apoptotic pathway in resting zone chondrocytes in vitro (Teixeira, Mansfield et al. 2001; Zhong, Carney et al. 2008). Moreover, the ability of LPA to rescue the apoptotic effect of Pi was attenuated by the LPA1/3 receptor antagonist, indicating that LPA1/3 signaling was responsible. The timing of programmed cell death is crucial in the maintenance of the growth plate. Inhibition of apoptotic signaling in hypertrophic chondrocytes prevents their terminal differentiation (Pucci, Adams et al. 2007), resulting in lengthening of the growth plate as is typically seen in the vitamin D-deficient rickets. Phosphate plays an important role in this process (Mansfield, Pucci et al. 2003), but it isn't known if the phosphate content is also a regulator of apoptosis in the resting zone.

Cell proliferation is associated with an increase in protein kinase C (PKC) in many cell types (Nishizuka 1984; Sylvia, Mackey et al. 1994), and LPA reduced the effects of PKC inhibition by chelerythrine. Previously we have showed that 24R,25$(OH)_2D_3$ stimulates PKC and cell proliferation in resting zone chondrocytes via a PLD-dependent mechanism (Schwartz, Sylvia et al. 2001). Our results suggest that LPA may mediate this response.

The LPA-induced decrease in the cellular abundance of the tumor suppressor p53 may be involved as well. The reduction of p53 correlates enhanced cell survival (Bartek, Iggo et al. 1990), indicating that the inhibition of p53 is the mechanism of LPA-mediated protection against cell death. The inhibition of p53 has also been implicated in the maturation of osteoblasts (Lengner, Steinman et al. 2006; Tataria, Quarto et al. 2006), suggesting that LPA-mediated decreases in p53 may be significant in chondrocyte maturation in addition to enhancing survival in these cells. LPA altered p53-mediated transcription and the expression of the p53-target genes p21, Bax and Bcl-2 at the transcriptional and translational level. The change in the cellular Bax to Bcl-2 ratio would result in the inhibition of the release of cytochrome c from the mitochondria (Jurgensmeier, Xie et al. 1998), halting the initiation of the apoptotic proteolytic caspase cascade. This is supported by our finding that LPA inhibits chelerythrine and phosphate-induced caspase-3 activity via an LPA1/3 mediated mechanism. Collectively, our results define a pathway for LPA-mediated enhancement of cell survival and chondrocyte maturation by which LPA decreases the abundance of p53 to alter p53-target gene expression resulting in the inhibition of caspase activity.

In summary, LPA was found to be a stimulator of resting zone chondrocyte proliferation and maturation and an inhibitor of chondrocyte apoptosis. This confirms a physiological role for LPA as a regulator of growth plate cartilage, and suggests that LPA produced via 24R,25$(OH)_2D_3$-stimulated PLD activity may mediate the actions of the seco-steroid in growth plate resting zone chondrocytes. Additionally, this establishes LPA as a potential therapeutic regulatory agent in controlling the processes of endochondral bone formation during long bone growth and development and during fracture repair.

PART III. 24R,25-DIHDROXYVITAMIN D3 PROTECTS CHONDROCYTES FROM APOPTOSIS THROUGH MODULATION OF LPA SIGNALING AND P53

CHAPTER 10

INTRODUCTION

Longitudinal bone growth in children and adolescents is mediated by growth plate cartilage, which is divided into four zones of maturation: the resting zone, the growth zone, the pre-hypertrophic/hypertrophic zone, and the calcifying cell zone. Inorganic phosphate (Pi), a physiological apoptogen, induces apoptosis in terminal growth plate chondrocytes at the epiphyseal-metaphyseal junction (Teixeira, Mansfield et al. 2001; Mansfield, Pucci et al. 2003; Zhong, Carney et al. 2008), allowing for the invasion of blood vessels and deposition of new bone. The resting zone of the growth plate supplies a pool of chondrocytes for the remainder of the growth plate. In response to growth stimuli, these cells undergo proliferation, terminal differentiation, hypertrophy, and apoptosis as they mature. Resting zone chondrocytes response preferentially to the vitamin D metabolite 24R,25-dihydroxyvitamin D3 [24R,25$(OH)_2D_3$], resulting in increased matrix production, maturation, and cell survival (Sylvia, Schwartz et al. 1997; Boyan, Sylvia et al. 2001; Dean, Boyan et al. 2001; Schwartz, Ehland et al. 2002). 24R,25$(OH)_2D_3$ exerts its effects through a membrane-associated vitamin D receptor (Boyan, Sylvia et al. 2003) resulting in activation of phospholipase D (PLD) and production of lysophosphatidic acid (LPA 18:1; 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphate), a bioactive lysophospholipid that regulates bone and cartilage (Sylvia, Schwartz et al. 2001; Hurst-Kennedy, Boyan et al. 2009).

LPA is the most simple of the glycerol lipids consisting of a single fatty acyl chain, a glycerol backbone, and a phosphate head group. LPA exerts its effects on cell through activation of the cell surface G-protein coupled receptors (GPCRs) LPA1/Edg2,

LPA2/Edg4, LPA3/Edg7, LPA4/GPR23, and LPA5/GPR92 and through the nuclear receptor peroxisome-proliferation-activation-receptor gamma (PPARγ) (Chun 1999; Goetzl and An 1999; McIntyre, Pontsler et al. 2003; Noguchi, Ishii et al. 2003; Lee, Rivera et al. 2006). Consequences of LPA signaling are broad and include neurite retraction, tumorigenesis, wound healing, proliferation, migration, and cell survival (van Corven, Groenink et al. 1989; Xu, Shen et al. 1998; Deng, Balazs et al. 2002; Fang, Schummer et al. 2002; Mills and Moolenaar 2003; Hama, Aoki et al. 2004; Ren, Xiao et al. 2006). Several recent studies have demonstrated that chondrocytes, osteoblasts, and osteocytes are sensitive to LPA, establishing LPA as a regulator of bone and cartilage (Facchini, Borzi et al. 2005; Kim, Lee et al. 2005; Karagiosis and Karin 2007; Panupinthu, Zhao et al. 2007; Panupinthu, Rogers et al. 2008).

Recently, we observed that 24R,25$(OH)_2D_3$ protects the resting zone chondrocyte-like ATDC5 cell line from Pi-induced apoptosis (Denison, Koch et al. 2009). Additionally, our group has reported that LPA is produced by resting zone chondrocytes in response to 24R,25$(OH)_2D_3$ and that the lipid mediator acts as an autocrine regulator to promote proliferation, cell survival, and maturation (Hurst-Kennedy, Boyan et al. 2009). LPA exerts these effects through activation of LPA1 and/or LPA3 and by modulating the abundance and transcriptional activity of the tumor suppressor p53. In cancer cells, LPA has been shown to regulate p53 in a similar manner through activation of the phosphoinositol 3-kinase (PI_3K) signaling cascade to promote nuclear export and subsequent degradation of p53 (Murph, Hurst-Kennedy et al. 2007). Collectively, these findings have led us to hypothesize that 24R,25$(OH)_2D_3$ protects

resting zone chondrocytes from Pi-induced apoptosis through LPA-mediated inhibition of p53.

Our goal was to elucidate the mechanisms by which 24R,25$(OH)_2D_3$ and LPA promote cell survival in the resting zone of the growth plate in the presence of Pi. We found that 24R,25$(OH)_2D_3$-mediated inhibition of Pi-induced apoptosis is dependent upon $G_{\alpha i}$, PLD, phosphatidylinositol-dependent phospholipase C (PI-PLC), and Ca^{++}-dependent signaling and through the inhibition of p53. $G_{\alpha i}$, PI_3K, nuclear export, and mdm2 signaling are necessary for the attenuation of Pi-induced cell death by LPA. Moreover, 24R,25$(OH)_2D_3$ attenuates Pi-induced cytochrome c translocation. Therefore, we have determined that 24R,25$(OH)_2D_3$ protects resting zone chondrocytes from Pi-induced apoptosis by producing LPA, which subsequently activates LPA1/3. This results in $G_{\alpha i}$-mediated stimulation of PI_3K signaling leading to mdm-2-directed degradation of p53. Additionally, we found that 24R,25$(OH)_2D_3$ promotes $G_{\alpha i}$-associated $G_{\beta\gamma}$ signaling to induce PI-PLC-mediated calcium release from the endoplasmic reticulum. Collectively, these pathways act to enhance cell survival in the resting zone in the presence of Pi.

CHAPTER 11

MATERIALS AND METHODS

11.1 Reagents

18:1 LPA (1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphate) was purchased from Avanti Polar Lipids (Alabaster, AL) and was reconstituted in 1% charcoal-stripped bovine serum albumin (BSA) prior to treatment of cells. Unless otherwise stated, all other reagents were acquired from VWR International (West Chester, PA).

11.2 Cell Culture

The culture system used in this study has been previously described in detail (Boyan, Schwartz et al. 1988). Chondrocytes were obtained from the resting zone (reserve zone) of costochondral cartilage from 125-g male Sprague-Dawley rats and cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS), 1% antibiotics, and 50μg/ml ascorbic acid (GIBCO-BRL, Gaithersburg, MD). Primary cells were cultured until fourth passage prior to experimental analysis.

11.3 Regulation of LPA Receptor Expression

Confluent cultures of resting zone chondrocytes were treated for 6 hrs with vehicle alone (control), 7.5mM sodium phosphate, 1e-6M LPA, or 1e-7M 24R,25$(OH)_2D_3$. After treatment, mRNA was harvested using Trizol (Invitrogen, Carlsburg, CA). Quantitative real-time polymerase chain reaction (QRT-PCR) was used to identify the presence of the LPA receptors LPA1-5 and PPAR-γ. The following sequence specific primers were used: LPA1 sense: 5'-GGTTCTCTACGCTCACATC-3', LPA1 antisense 5'-GCAGTAGCAAGACCAATCC-3', LPA2 sense: 5'-

CACCACCTCACAGCCATCC-3', LPA 2 antisense: 5'-AGACATCCACAGCACTCAGC-3', LPA3 sense: 5'-CTACAACAGGAGCAACAC-3', LPA3 antisense: 5'-CCAGCAGGTAGTAGAAGG-3', LPA4 sense: 5'-ACAACTTTAACCGCCACTGG-3', LPA4 antisense: 5'-ATTCCTCCTGGTC CTGATGG-3', LPA5 sense: 5'-ACCTTGGTGTTCCCTATAATGC-3', LPA5 antisense: 5'-AGCCAGAGCGTTGAGAGG-3', PPAR-γ sense: 5'-CCGAAGAACCATCCGATTGAAG-3', and PPAR-γ antisense: 5'-CTCCGCCAACAGCTTCTCC-3'. Glyceraldehyde-3-phosphate dehydrogenase (GADPH) was amplified as a control in each experiment: GAPDH sense: 5'-ATGCAGGGATGATGTTC-3', GAPDH antisense: 5'-TGCACCA CCAACTGCTTAG-3'.

11.4 Cell Viability

We used the LPA1/3-selective antagonist (S)-phosphoric acid mono-(2-octadec-9-enoylamino-3-[4-(pyridine-2-ylmethoxy)-phenyl]-propyl) ester (VPC32183(S), Avanti Polar Lipids, Alabaster, AL) (Chang, Kim et al. 2007) to assess the role of LPA receptor signaling in 24R,25$(OH)_2D_3$-mediated rescue of Pi-induced apoptosis. Confluent cultures of the resting zone chondrocytes were treated with 7.5mM monobasic sodium phosphate, 10^{-7}M 24R,25$(OH)_2D_3$, VPC32183(S) (0.01μM-1μM), or a combination of the aforementioned. Cell viability was assessed using the MTT (3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide, Sigma, St. Louis, MO) dye. The conversion of tetrazolium salt to formazan salt was measured at 570nm.

11.5 Caspase-3 Activity

The roles of 24R,25$(OH)_2D_3$ and LPA in chondrocyte survival were assessed by examining its ability to reduce caspase-3 activity induced by inorganic phosphate (Teixeira, Mansfield et al. 2001; Zhong, Carney et al. 2008). Confluent cultures of resting zone chondrocytes were treated for 24h with 7.5mM monobasic sodium phosphate (Pi) to induce apoptosis. At the same time, cells were treated with 24R,25$(OH)_2D_3$ (1e-7M) or LPA (1e-6M). VPC32183(S) was used to inhibit LPA1/3 signaling (1e-8M – 1e-6M, Avanti Polar Lipids, Alabaster, AL); pertussis toxin was used to inhibit $G_{\alpha i}$ signaling (PTX, 25ng/ml-0.25ng/ml, Sigma, St. Louis, MO). Cholera toxin was used to stimulate $G_{\alpha s}$ signaling (CTX, 100ng/ml-1ng/ml, Calbiochem, Gibbstown, NJ), while LY294002 was used to inhibit PI_3K activity (LY, 10μM-0.1μM, Cayman Chemicals, Ann Arbor, MI). D609 (50μM, Calbiochem, Gibbstown, NJ) and U73122 (10μM, Sigma, St. Louis, MO) were used to inhibit phosphatidylcholine-specific PLC (PC-PLC) and PI-PLC, respectively. Thapsigargin was used to block release of calcium from the endoplasmic reticulum (3μM, Sigma, St. Louis, MO). Wortmannin was used to inhibit PLD and PI_3K signaling (10μM, Calbiochem, Gibbstown, NJ); leptomycin B was used to block nuclear export (LMB, 50ng/ml-0.5ng/ml, Biomol, Plymouth Meeting, PA). Caspase-3 activity was determined using the colorimetric CaspACE™ Assay System from Promega (Madison, WI). Cells were harvested 24h post treatment with 200μl cell lysis buffer followed by two 10 s periods of sonication. After harvest, 2μl of the caspase-3 selective substrate DEVD-pNA were added to each well containing 100μl of cell lysate and incubated at 37^0C for 4h. DEVD-pNA cleavage into the colorimetric product pNA was measured at 405nm. Caspase-3 activity was normalized to protein content as determined by the Pierce Macro BCA Protein Assay Kit.

11.6 Modulation of p53 Abundance and p53-Mediated Transcription

Confluent cultures of resting zone chondrocytes were treated for 6 h with 0, 10^{-9}M, 10^{-8}M, 10^{-7}M 24R,25$(OH)_2D_3$. After treatment, the cell monolayer was washed twice with PBS and harvested with RIPA buffer. The abundance of p53 protein in both the whole cell lysate and nuclear and cytoplasmic fractions was determined by ELISA (p53 pan ELISA, Roche) and normalized to total cellular or total fraction protein as determined by Pierce Macro BCA Protein Assay Kit.

To assess changes in p53-mediated transcription, luciferase reporter gene assays were conducted as previously described (Murph, Scaccia et al. 2003). Cells were transfected with two plasmids: one containing p53-controlled firefly luciferase (pp53_TA-Luc, Clonetech, Mountain View, CA) and the other carrying constitutively expressed *Renilla* luciferase (pLR-TK, Promega, Madison, WI). 24 h after transfection, cells were treated with 0, 10^{-9}M, 10^{-8}M, 10^{-7}M 24R,25$(OH)_2D_3$ for 16 h and luciferase activity was measured using the Dual Luciferase Reporter Assay kit (Promega, Madison, WI).

11.7 Abundance of mdm2 and Phospho-mdm2 Protein

Western blots were performed to determine the effects of 24R,25$(OH)_2D_3$ and LPA on the protein abundance of mdm2 and phosphorylated mdm2. Cell culture lysates were prepared from confluent resting zone cells and were resolved on 10% SDS-polyacrylamide gels. Blots of the gels were probed with monoclonal antibodies against mdm2 (AbCam, Cambridge, MA), Phospho-mdm2 (Ser 166, Cell Signaling, Boston, MA), or GAPDH (MAB374 Chemicon, Billerica, MA). Immunoreactive bands were detected using 1:5,000 dilutions of horseradish peroxidase-conjugated goat anti-rabbit or

goat anti-mouse IgG (Jackson Immunoresearch, West Grove, PA), and visualized using enhanced chemiluminescence (Super-Signal WestPico Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL). Densitometry measurements were collected using Quantity One image analysis software (BioRad, Hercules, CA).

11.8 Cytochrome C Translocation

Confluent cultures of resting zone chondrocytes were mock-treated (control) or treated with 7.5mM Pi, 1e-7M 24R,25$(OH)_2D_3$, or a combination of the afore mentioned for 24 h. At harvest, cell lysates were fractionated into mitochondrial and cytoplasmic fractions using the Cytochrome C Apoptosis Assay Kit (BioVision, Mountain View, CA). Immunoblotting was conducted as described previously using a mouse monoclonal anti-cytochrome c antibody (BioVision).

11.9 Statistical Analysis

Each experiment had six independent cultures per variable to ensure sufficient power to detect statistically significant differences. All experiments were conducted multiple times to validate the observations, but data from a single representative experiment are shown in the figures and are expressed as means $\pm$ SEM. Statistical analysis was conducted using ANOVA analysis followed by Student's T-test with a Bonferroni modification. Differences in means were considered to be statistically significant if the p value was less than 0.05.

CHAPTER 12

RESULTS

12.1 LPA Receptor Expression is Regulated by LPA, Phosphate, and 24R,25(OH)$_2$D$_3$

Resting zone chondrocytes express the LPA receptors LPA1-5 and peroxisome-proliferation activation receptor gamma (PPAR-γ). Both 1e-7M 24R,25(OH)$_2$D$_3$ and 1e-6M LPA increased the mRNA expression of LPA1 (Fig 12.1A, 12.1B). Expression of LPA3, LPA4, and LPA5 was decreased after treatment with 7.5mM phosphate (Fig 12.1B).

12.2 24R,25(OH)$_2$D$_3$ Inhibits Apoptosis Through LPA1/3 Signaling

24R,25(OH)$_2$D$_3$ inhibited Pi-induced apoptosis as assessed by MTT cell viability assay (Fig. 12.2A) and caspase-3 activity assay (Fig. 12.2B). The LPA1/3-selective antagonist VPC32183(S) abolished the ability of 24R,25(OH)$_2$D$_3$ to reduce the increase in caspase-3 activity caused by Pi.

12.3 24R,25(OH)$_2$D$_3$ and LPA Inhibit Phosphate-Induced Caspase-3 Activity

24R,25(OH)$_2$D$_3$ reduced Pi-induced caspase-3 activity (Fig. 12.3). The $G_{\alpha i}$ inhibitor pertussis toxin (PTX) (Fig. 12.3A) attenuated 24R,25(OH)$_2$D$_3$-mediated rescue, whereas the $G_{\alpha s}$ stimulator cholera toxin (CTX) (Fig. 12.3B), the PI$_3$K inhibitor LY294002 (Fig. 12.3C), and the PC-PLC inhibitor D609 (Fig. 12.3D) did not. The PI-PLC inhibitor U73122, calcium ATPase inhibitor thapsigargin, and the PLD/PI$_3$K inhibitor wortmannin also inhibited this effect (Fig. 12.3E, 12.3F, 12.3G).

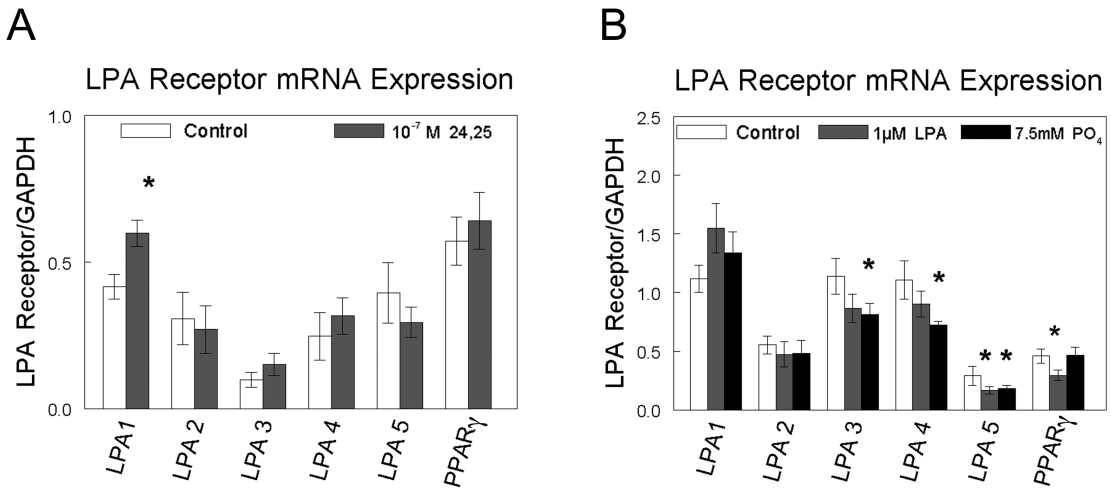

FIGURE 12.1. LPA receptor expression is regulated by LPA, phosphate, and 24R,25(OH)$_2$D$_3$. RNA was isolated via Trizol extraction from cultures of resting zone chondrocytes the were mock-treated (Control C,D) or treated with 1e-7M 24R,25(OH)$_2$D$_3$, 7.5mM Pi, or 1e-6M LPA. LPA receptor mRNA abundance was determined using receptor-specific Real-Time PCR primers.

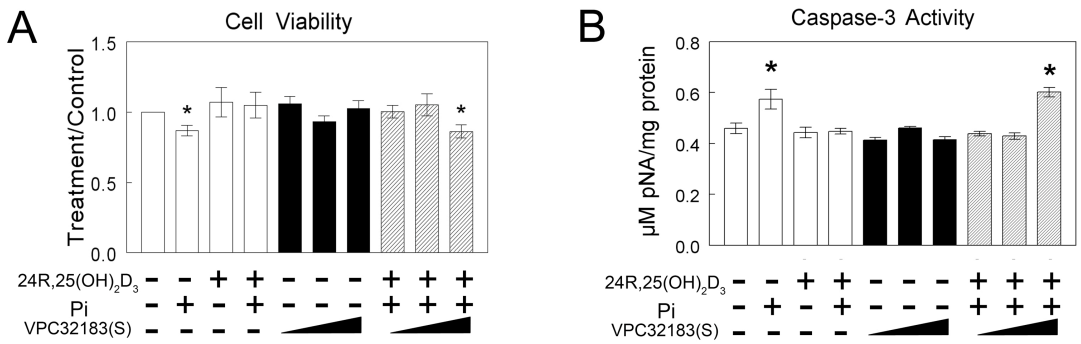

FIGURE 12.2 24R,25(OH)$_2$D$_3$ inhibits apoptosis through LPA1/3 signaling. Confluent cultures of resting zone chondrocytes were treated with 1e-7M 24R,25(OH)$_2$D$_3$, 7.5mM Pi, VPC32183(S) (1e-8, 1e-7, 1e-6M), or a combination of the aforementioned for 24 hours. After the treatment period, cell viability was assessed by MTT assay (A) and caspase-3 activity was measured (B, Colorimetric CaspACE™ Assay System, Promega

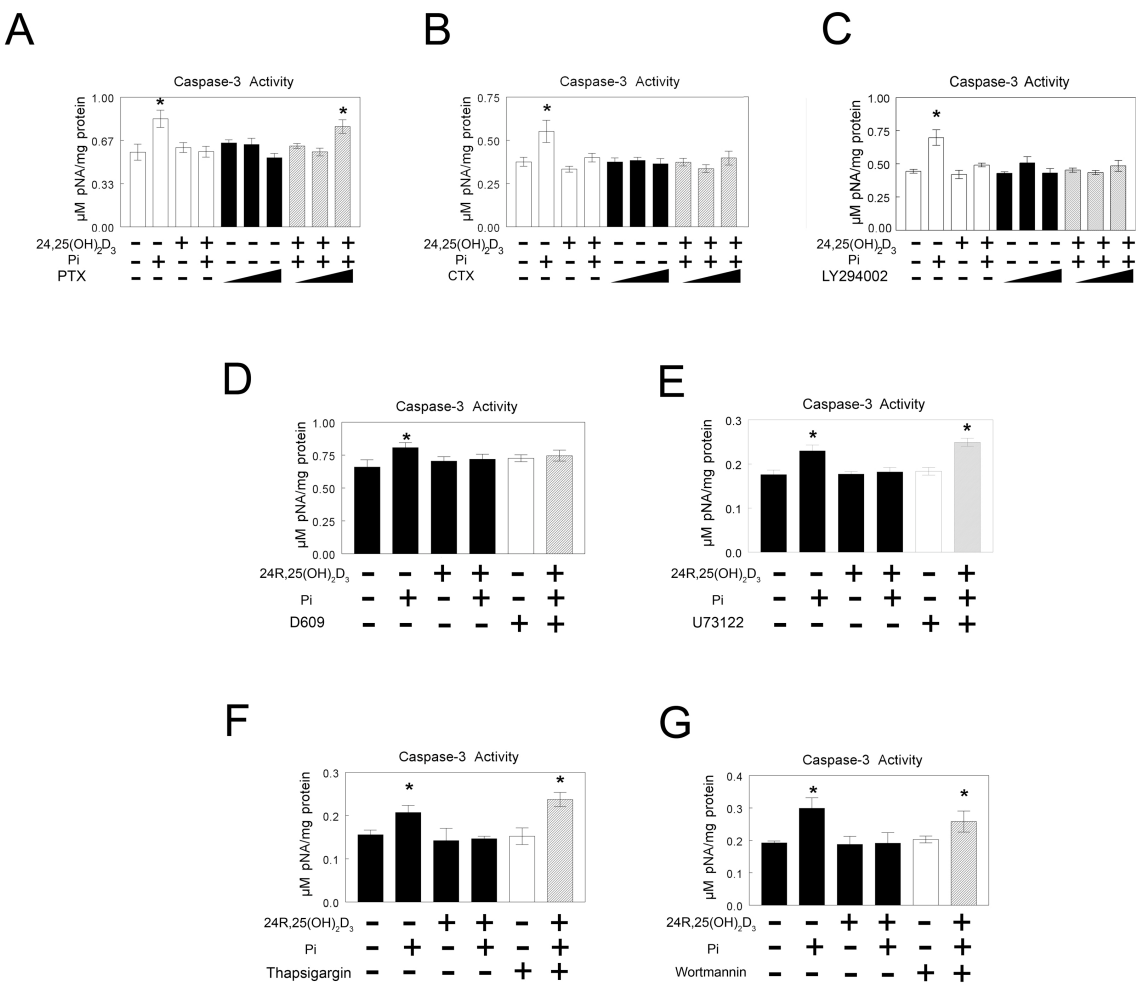

FIGURE 12.3. 24R,25(OH)$_2$D$_3$ inhibits Pi-induced caspase-3 activity through $G_{\alpha}i$, PLD, and Ca^{++}-dependent signaling. Confluent cultures of resting zone chondrocytes were treated for 24h with 7.5mM monobasic sodium phosphate, 24R,25(OH)$_2$D$_3$ (10^{-7}M), pertussis toxin (A, PTX, 25ng/ml-0.25ng/ml), cholera toxin (B, CTX, 100ng/ml-1ng/ml), LY294002 (C, 10µM-0.1µM), D609 (D, 50µM), U73122 (E, 10µM), thapsigargin (F, 3µM), Wortmannin (G, 10µM) or a combination of the aforementioned. Caspase-3 activity was determined using the Colorimetric CaspACE™ Assay System from Promega and normalized to total protein.

LPA treatment also attenuated the increase in caspase-3 activity induced by Pi (Fig 12.4). This rescue of Pi-induced apoptosis by LPA was inhibited by PTX, but not by CTX (Fig. 12.4A, 12.4B). LY294002 and the nuclear export inhibitor leptomycin B (LMB) also blocked LPA-mediated inhibition of Pi cell death (Fig. 12.4C, 12.4D).

12.4 24R,25(OH)$_2$D$_3$ Modulates p53 Levels and Activity

24R,25(OH)$_2$D$_3$ treatment resulted in a decrease in p53 abundance (Fig. 12.5A) and p53-mediated transcription (Fig. 12.5B) as assessed by ELISA and luciferase reporter gene assay, respectively. 24R,25(OH)$_2$D$_3$ did not alter the abundance of active, phosphorylated or total mdm2, an E3 ubiquitin ligase that negatively regulates p53 (Fig. 12.6A). LPA, however, did increase the phosphorylation mdm2 (Fig. 12.6B).

12.5 Pi-Induced Cytochrome C Translocation is Attenuated by 24R,25(OH)$_2$D$_3$

Cytochrome c is primarily localized in the mitochondria in control and 24R,25(OH)$_2$D$_3$ treated lysates (Data not shown, Ming Zhong). Pi treatment induced an increase in the abundance of cytochrome c in the cytoplasmic fraction, while decreasing it in the mitochondrial fraction.

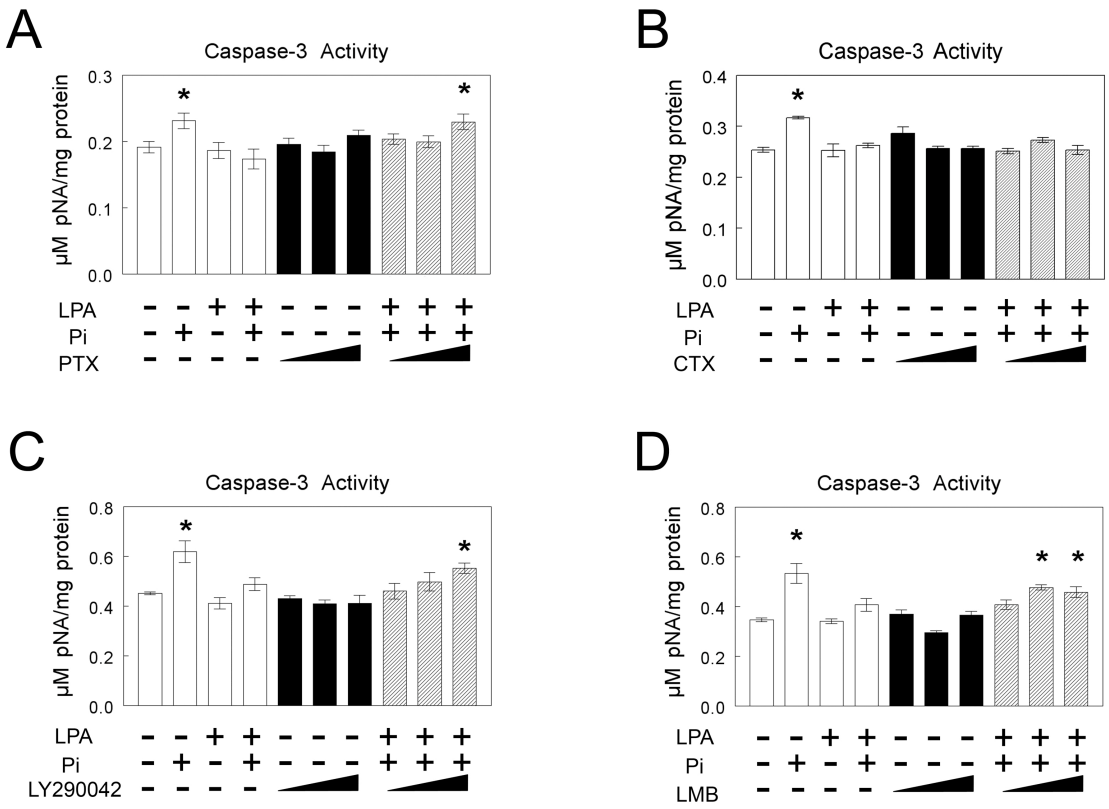

FIGURE 12.4. LPA inhibits Pi-induced caspase-3 activity through $G_{\alpha i}$, PI_3K, and nuclear export-dependent signaling. Confluent cultures of resting zone chondrocytes were treated for 24h with 7.5mM monobasic sodium phosphate, LPA (1 µM), pertussis toxin (3A, PTX, 25ng/ml-0.25ng/ml), cholera toxin (3B, CTX, 100ng/ml-1ng/ml), LY294002 (3C, 10µM-0.1µM), leptomycin B (3D, LMB, 50ng/ml-0.5ng/ml), or a combination of the aforementioned. Caspase-3 activity was determined using the Colorimetric CaspACE™ Assay System from Promega and normalized to total protein.

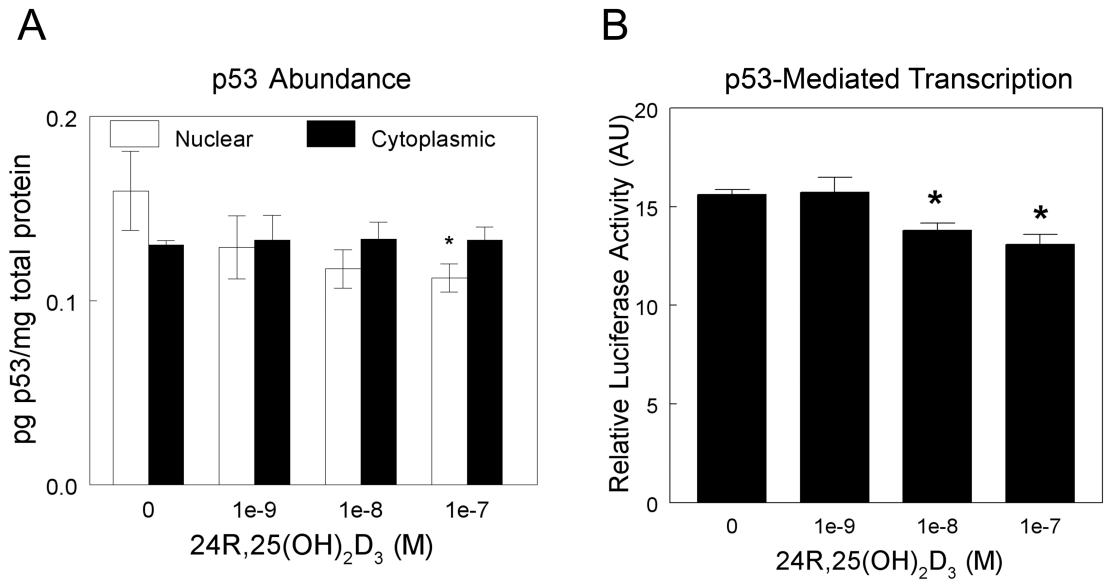

FIGURE 12. 5. 24R,25(OH)$_2$D$_3$ reduces p53 abundance and p53-mediated transcription. (A) Resting zone chondrocytes were treated with 0, 1e-7M, 1e-8M, or 1e-9M 24R,25(OH)$_2$D$_3$. The abundance of p53 protein in both the nuclear and cytoplasmic fractions was determined by ELISA (p53 pan ELISA, Roche) and normalized to total fraction protein. (B) Cells were transfected with two plasmids: one containing p53-controlled firefly luciferase and the other carrying constitutively expressed *Renilla* luciferase. After 24 h, cells were treated with 0, 1e-7M, 1e-8M, or 1e-9M 24R,25(OH)$_2$D$_3$ for 16 h and luciferase activity was measured using the Dual Luciferase Reporter Assay kit (Promega). Firefly luciferase activity was normalized to *Renilla* luciferase activity to determine p53-mediated transcription.

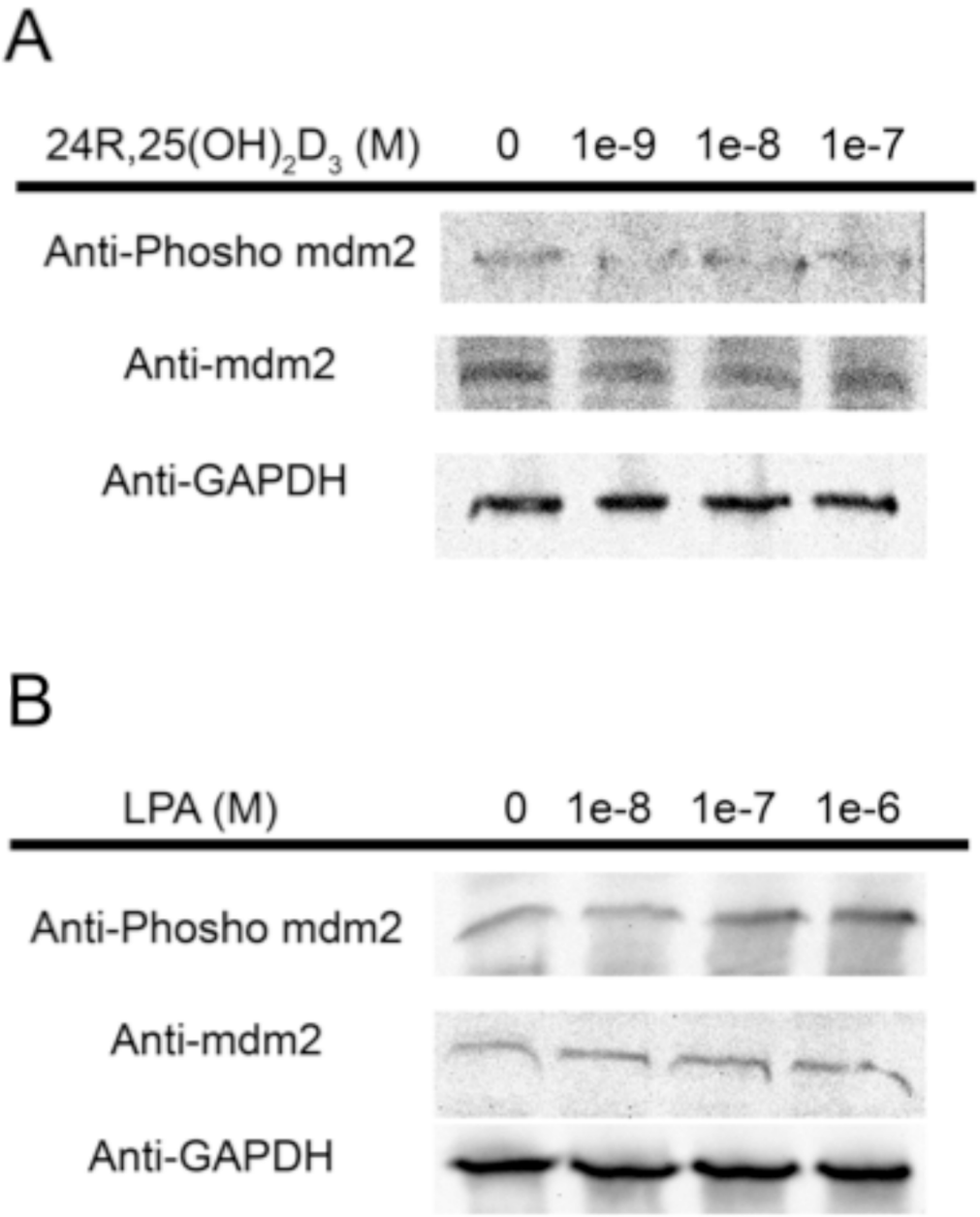

FIGURE 12.6. LPA and 24R,25(OH)$_2$D$_3$ regulate mdm2 phosphorylation differently. Confluent cultures of resting zone chondrocytes were treated with 24R,25(OH)2D3 (A, 0, 1e-9, 1e-8, or 1e-7M) or LPA (B, 0, 1e-8, 1e-7, or 1e-6M) for 6 hours prior to immunoblotting with anti-Phospho-mdm2, anti-mdm2, and anti-GAPDH antibodies.

CHAPTER 13

DISCUSSION

Our data demonstrate that 24R,25$(OH)_2D_3$ inhibits Pi-induced apoptosis in resting zone chondrocytes through LPA signaling. Previously, we have shown that 24R,25$(OH)_2D_3$ increases the abundance of extracellular LPA and that 24R,25$(OH)_2D_3$-stimulated alkaline phosphatase activity is dependent upon LPA1/3 receptor signaling (Hurst-Kennedy, Boyan et al. 2009). In this study, we demonstrate that 24R,25$(OH)_2D_3$ also increases the expression of LPA1 mRNA and that 24R,25$(OH)_2D_3$-mediated inhibition of Pi-induced apoptosis is sensitive to the LPA1/3-selective antagonist VPC32183(S). Collectively, these observations implicate LPA as a second messenger during the promotion of cell maturation and survival in chondrocytes by 24R,25$(OH)_2D_3$. Recently, a single nucleotide polymorphism (SNP) in LPA1 has been indentified in osteoarthritic patients (Mototani, Iida et al. 2008). Moreover, the LPA1-/- mouse exhibits a smaller skeleton relative to wild-type mice (Contos, Ishii et al. 2002). These findings, in conjunction with our own demonstrating that 24R,25$(OH)_2D_3$ and LPA (Hurst-Kennedy, Boyan et al. 2009) exert their effects in chondrocytes via LPA1 and/or LPA3, indicate that LPA1 is significant in the regulation of cartilage.

We have found that LPA-mediated rescue of Pi-induced apoptosis is sensitive to pertussis toxin and LY294002, but not to cholera toxin. This indicates that LPA exerts its anti-apoptotic effects through $G_{\alpha i}$ and PI_3K signaling. A consequence of activation of this cascade is the activation of Akt/protein kinase B (PKB) (Downward 2004). Akt phosphorylates mdm2 inducing its translocation into the nucleus where it mono- and poly-ubiquitinates p53 (Li, Brooks et al. 2003; Milne, Kampanis et al. 2004). This results

in the nuclear export and subsequent proteosomal degradation of p53. Our data show that the reduction of caspase-3 by LPA is sensitive to the nuclear export inhibitor leptomycin B and that LPA increases the phosphorylation of mdm2, supporting the claim that LPA is modulating p53 through this pathway. Interestingly, this is the mechanism by which LPA inhibits p53 signaling in A549 lung carcinoma cells (Murph, Hurst-Kennedy et al. 2007), suggesting that this pathway is conserved amongst different cell types.

In our study, 24R,25$(OH)_2D_3$-mediated inhibition of Pi-induced caspase-3 activity was not inhibited by the PI_3K inhibitor LY294002. Moreover, 24R,25$(OH)_2D_3$ did not increase the abundance of phosphorylated mdm2 at 6hr post-treatment. This suggests that 24R,25$(OH)_2D_3$ is stimulating one or more additional pathways to inhibit apoptosis in addition to LPA-mediated inhibition of p53. Resting zone chondrocytes mature in response to 24R,25$(OH)_2D_3$ through increased PKC and PKA activity, resulting in MEK1/2 signaling (Schwartz, Ehland et al. 2002). It is possible that activation of these effectors also enhances cell survival. Another possible pathway is the PLC signaling cascade. 24R,25$(OH)_2D_3$-mediated inhibition of apoptosis was attenuated by pertussis toxin, a $G_{\alpha i}$ inhibitor. Pertussis toxin also inhibits $G_{\alpha i}$-associated Gβγ signaling, which can stimulate PLC activation (Smrcka and Sternweis 1993; Smrcka 2008). Our data show that LPA1/3, PI-PLC, and release of intracellular calcium from the endoplasmic reticulum are necessary for the inhibition of apoptosis by 24R,25$(OH)_2D_3$. Taken together, this suggests that 24R,25$(OH)_2D_3$ is initiating LPA-mediated stimulation of Gβγ-induced PLC activity, resulting in release of intracellular calcium.

Resting zone chondrocytes responds to 24R,25$(OH)_2D_3$ with increased proliferation, maturation, and matrix production (Helm, Sylvia et al. 1996; Sylvia,

Schwartz et al. 1997). In this study we demonstrate that 24R,25$(OH)_2D_3$ also protects chondrocytes from apoptosis induced by Pi in their microenvironment. Together, this suggests that 24R,25$(OH)_2D_3$ stabilizes chondrocytes in the resting zone by inhibiting degradation characteristic of apoptotic hypertrophic chondrocytes. This implies that 24R,25$(OH)_2D_3$ modulates growth plate development by controlling the rate and extent of chondrocyte transition from resting zone to growth zone phenotype.

In summary, our data identify a new signaling axis in the inhibition of apoptosis in the growth plate (Fig. 13.1). Chondrocytes respond to 24R,25$(OH)_2D_3$, which stimulates the signaling of its second messenger, LPA, resulting in inhibition of p53 signaling and enhanced cell survival. Information gleaned from this study provides new understanding into the maintenance of the pool of chondrocytes in the resting zone of the growth plate. Therefore, we identify this signaling axis as a potential therapeutic target for the treatment of bone fracture repair and as a regulatory agent of endochondral ossification during skeletal development and long bone growth.

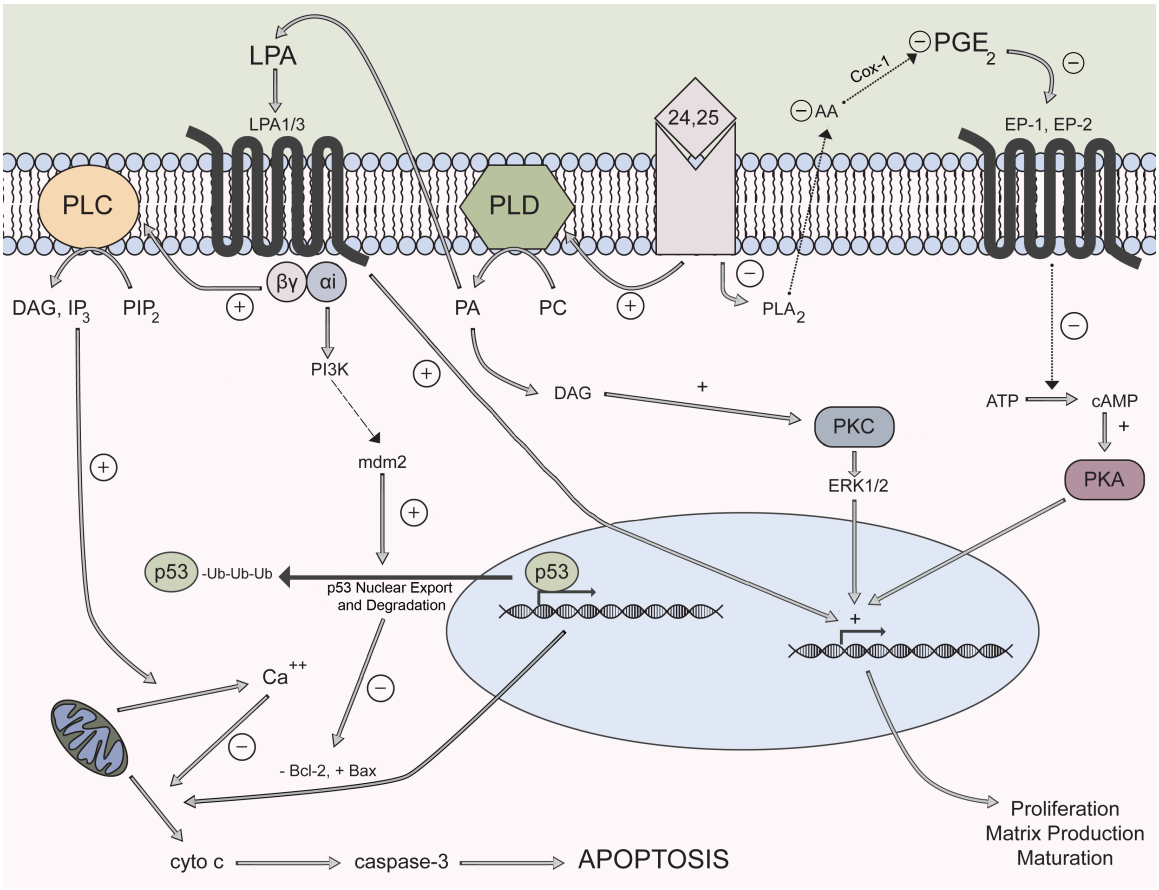

FIGURE 13.1. Mechanisms of 24R,25(OH)$_2$D$_3$ and LPA-mediated inhibition of apoptosis in resting zone chondrocytes. Resting zone chondrocytes respond to 24R,25(OH)$_2$D$_3$, resulting in increased phospholipase D (PLD) activity. Activation of PLD stimulates the production of lysophosphatidic acid (LPA). Stimulation of LPA1/3 receptor(s) by LPA leads to activation of $G_{\alpha i}$ and phoshoinositol-3 kinase (PI_3K) signaling. This results in increased phosphorylation of the E3-ubiquitin ligase murine double minute 2 (mdm2). Phospho-mdm2 translocates to the nucleus where it can mono- or poly-ubiquitinate p53 to promote its nuclear export and proteosomal degradation. A decrease in p53 protein abundance and nuclear localization yields reduced p53-mediated transcription and decreased caspase-3 activity, resulting in increased cell survival, maturation, and proliferation. 24R,25(OH)$_2$D$_3$ also stimulates PKC and PKA signaling and modulates intracellular calcium levels through $G_{\beta\gamma}$-mediated PLC activation, which may also pay a role in 24R,25(OH)$_2$D$_3$'s anti-apoptotic effects.

CHAPTER 14

CONCLUSIONS AND FUTURE DIRECTIONS

14.1 Summary

The overall goal of this study was to determine the mechanism by which LPA regulates cell death. In the first study, we found that LPA enhances resistance to chemotherapy-induced apoptosis in A549 lung carcinoma cells by stimulating the degradation of the tumor suppressor p53. This results in decreased p53-mediated transcription and enhanced cell survival. In the second study, we found that LPA-directed regulation of cell death has a physiologically relevant role in the maintenance of the growth plate. The vitamin D metabolite 24R,25$(OH)_2D_3$ stimulates PLD-mediated LPA production resulting in increased proliferation, maturation, and inhibition of apoptosis. LPA exerts many of these effects through modulation of p53 signaling. In the final study, we investigated the mechanisms behind the inhibition of cell death by 24R,25$(OH)_2D_3$ and LPA in the growth plate. We found that LPA is a second messenger in 24R,25$(OH)_2D_3$-mediated inhibition of apoptosis. Consequences of LPA signaling include $G_{\alpha}i$, PI_3K, mdm2-induced degradation of p53. $G_{\alpha}i$-associated $G_{\beta\gamma}$ signaling also plays a role by initiating PLC-directed calcium release from the endoplasmic reticulum.

The 24R,25$(OH)_2D_3$, LPA, p53 signaling axis has the potential to affect human health in both negative and positive manners. On the one hand, the inhibition of p53 by LPA has the capacity to promote tumorigenesis. Conversely, stimulating 24R,25$(OH)_2D_3$ and/or LPA signaling may be effective in improving bone fracture healing and in treating

endochondral ossification-related disorders. The applications of this work will be discussed as followed.

14.2 Modulation of LPA Signaling in the Treatment of Cancer

Our data show that LPA enhances the resistance of A549 lung carcinoma cells to the chemotherapeutic drug actinomycin D. This suggests that LPA signaling *in vivo* may help cancer cells evade apoptosis, especially in breast, prostate, and ovarian cancers where LPA signaling is known to enhance tumorigenesis. Therefore, administrating inhibitors of LPA signaling in conjunction with chemotherapeutics may improve chemotherapeutic-induced tumor cell death. The inhibitors could include LPA receptor antagonists to decrease LPA-mediated signaling or inhibitors of enzymes that generate LPA such as ATX or AGK.

Since LPA regulates a wide array of cellular functions, it is important to consider systemic effects resulting from modulation of LPA signaling. A general decrease in LPA signaling could result in reduced blood clotting and inflammatory responses, impairing the body's ability to heal. Recently, a group has found that administration of proteasome inhibitor to children with cancer causes irreversible growth retardation (Zaman, Menendez-Benito et al. 2007). This phenomenon is attributed to increased levels of p53 in growth plate chondrocytes. Given our data demonstrating the importance of LPA-mediated inhibition of p53 in growth plate maintenance, inhibition of LPA signaling in children with cancer may also cause growth retardation. Therefore, delivery vehicles that administer drugs locally should be considered while developing LPA inhibitors as anti-cancer therapeutic agents.

The vitamin D metabolite $1,25(OH)_2D_3$ is an established inhibitor of tumor formation in skin (Chida, Hashiba et al. 1985; Pence, Richard et al. 1991). Recently, an inverse relationship has been identified between breast cancer occurrence and sunlight exposure, suggesting a role for this metabolite in other types of cancer (Millen, Pettinger et al. 2009). Moreover, $1,25(OH)_2D_3$ is an inducer of p53 and the downstream p53 target BRCA1 (Campbell, Gombart et al. 2000; Gupta, Dixon et al. 2007). Together, these data implicate $1,25(OH)_2D_3$ as an anti-tumor agent. Our findings, however, suggest that the metabolite $24R,25(OH)_2D_3$ may promote tumorigenesis by stimulating LPA-mediated inhibition of p53. Therefore, it may be interesting to examine the levels of $1,25(OH)_2D_3$ and $24R,25(OH)_2D_3$ in the tumor microenvironment as cancer progresses. It may be that tumors enrich their environment preferentially with $24R,25(OH)_2D_3$ in order to evade chemotherapeutic-induced cell death. Consequently, including inhibitors of 24-hydrolyase and/or $1,25(OH)_2D_3$ analogues in an anti-cancer drug regiment may inhibit tumor progression.

14.3 LPA as a Regulator of Fracture Repair and Skeletal Development and Growth

Disruptions in embryonic growth plates severely impair or abolish skeletal formation. Moreover, premature closure of post-natal growth plates halts long bone growth, resulting in shorter stature. Our findings demonstrate that resting zone growth plate chondrocytes respond to LPA with increased proliferation, maturation, and cell survival. Additionally, $24R,25(OH)_2D_3$ increases both LPA1 receptor expression and extracellular LPA abundance, resulting in decreased Pi-induced apoptosis. Collectively, these observations strongly suggest that LPA is significant in maintaining the pool of chondrocytes in the resting zone of the growth plate and subsequently is important during

endochondral ossification. Consequently, these findings make LPA an attractant therapeutic target for controlling skeletal development and growth.

Due to the structural and biological similarities between growth plates and fracture calluses, our data also suggest that LPA may enhance bone fracture healing. Others have found that LPA signaling promotes osteoblast-mediated mineralization and osteocyte reconnectivity (Gidley, Openshaw et al. 2006; Karagiosis and Karin 2007; Panupinthu, Rogers et al. 2008), implicating LPA in the later stages of fracture healing. Based on our results, it is possible that LPA signaling also enhances the viability of chondrocytes in the fracture callus during the intermediate stage of healing. Collectively, this work suggests that administration of LPA at a fracture site after injury could enhance the healing process. *In vivo* animal studies examining the role of LPA in fracture repair are needed to confirm this hypothesis.

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