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PARAOXONASE 2 IS CRITICAL FOR NON-SMALL CELL LUNG CARCINOMA PROLIFERATION

By

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B.S., Morehead State University, 2010

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ABSTRACT

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Non-small cell lung carcinoma (NSCLC) comprises 85% of lung cancer diagnoses and is plagued by drug resistance. Thus, elucidating the underlying mechanisms of NSCLC is paramount to expand future treatment options. Paraoxonase 2 (PON2), an intracellular enzyme with arylesterase and lactonase functions, has well-established anti-atherosclerotic activity. Recent studies show PON2 is overexpressed in a variety of tumors and confers drug resistance, although these interactions have not been thoroughly examined in NSCLC. Thus, we sought to investigate the role of PON2 in cellular proliferation using PON2-knockout mice, primary mouse cells, and NSCLC cell lines. Using these approaches, we demonstrate that PON2 is required for NSCLC proliferation, but dispensable for normal mouse development and non-transformed cell proliferation. These observations highlight PON2 as a potential therapeutic target against NSCLC.

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CHAPTER 1: INTRODUCTION

1. Paraoxonases

The paraoxonases (PONs) comprise 3 members (PON1, PON2, and PON3) of an antioxidant enzyme family encoded in a gene cluster on the long arm of chromosome 7 (7q21.3-22.1) [1]. These enzymes are evolutionarily ancient, first appearing in invertebrates, and are highly conserved in mammals, displaying 80-95% sequence identity at both the amino acid and nucleotide level [2]. PON2 is presumed to be the oldest, eventually giving rise to PON1 and PON3 through gene duplication events [3]. All PONs exhibit lactonase and arylesterase activity, but are otherwise functionally diverse [4]. For instance, PON1 was first discovered and named for its ability to hydrolyze paraoxon, the toxic metabolite of the organophosphate insecticide, parathion [5]. Although their name would suggest the contrary, PON2 and PON3 lack this paraoxon-detoxifying activity but retain the designation based on genetic homology. Intriguingly, no endogenous substrates have been identified for this class of enzymes.

1.1 PON1

PON1 is the most characterized of the PONs. Early work on PON1 focused on its xenobiotic metabolizing capability, specifically in the context of organophosphate detoxification, [6], [7]. Recent and emerging PON1 research,

however, has shifted to exploring its role in atherosclerosis, immunity, and cancer [8]. The PON1 gene encodes a 43 kDa protein consisting of 354 amino acids [9] which is produced by the liver and released into circulation where it associates with high-density lipoprotein (HDL) particles and is distributed throughout the body [10]. Once in the serum, PON1 inhibits and reverses the oxidization of low-density lipoproteins (LDL), an initiating step in the development of atherosclerosis [11]. This effect has been extensively demonstrated in cell-based systems and in PON1-knockout mice[11]–[14]. Additionally, a number of human studies have suggested a relationship between polymorphisms in the coding and promoter regions of the human PON1 gene and susceptibility to cardiovascular disease [15]. The sheer abundance of research linking PON1 status to atherogenesis has sparked much interest in PON1 as a biomarker and/or therapeutic target against oxidative vascular damage [16].

PON1 has also been shown to mediate bacterial infection. Through its lactonase activity, PON1 can cleave a class of bacterial quorum-sensing compounds, the acyl-homoserine lactones [17]. The hydrolysis of the lactone ring effectively inactivates acyl-homoserine lactones and disrupts bacterial intercellular communication and colonization of host tissues.

The antioxidant and anti-inflammatory properties of PON1 have prompted researchers to investigate its role in cancer. Indeed, several published reports confirm a link between PON1 and cancer. An investigation conducted by Marchesani et al. monitored prostate cancer development in a population of Finnish men and found a specific PON1 single nucleotide polymorphism (SNP),

I102V, was associated with increased incidence [18]. Similarly, Stevens et al. discovered the L55M SNP correlated to an increased risk of breast cancer in a cohort of post-menopausal women [19]. More recently, Aldonza et al. used data from the cancer genome atlas (TCGA), lung cancer patient samples, and lung cancer cell lines to show that PON1 provides an anti-apoptotic function to stimulate tumor growth and progression [20]. While these observations are promising, the underlying mechanisms remain undetermined.

1.2 PON3

In contrast to PON1, PON3 is the most recently-identified and least-studied member of this family. PON3 is a 40 kDa protein synthesized mainly by the liver and, to a lesser extent, the kidney which associates with circulating plasma HDLs [21]. In circulation, PON3 is capable of detoxifying oxidized LDLs (oxLDLs) at a higher capacity than PON1, although circulating levels of PON3 are much lower than PON1 [21]. PON3 has also been detected intracellularly, localized to endoplasmic reticulum (ER) and mitochondria [22], [23]. Similar to its other family members, PON3 elicits anti-inflammatory, anti-oxidant responses *in vitro* and *in vivo* which have been connected to atherosclerosis, diabetes, neurodegenerative disorders, and cancer [2], [5], [24].

1.3 PON2

PON2, unlike the other PONs, is expressed intracellularly in nearly every tissue surveyed [25], where it localizes to mitochondria, ER, and nuclear lamina

[26] as a transmembrane protein with a molecular mass of 43 kDa [27]. PON2 shares anti-oxidant and anti-inflammatory characteristics with other PONs, such as preventing and reversing oxidative damage to LDL [25]. In addition, PON2 also exerts intracellular influence, generally by mitigating oxidative stress in mitochondria and ER. Its widespread tissue distribution and subcellular localization implicate PON2 in a variety of physiological processes

1.3.1 PON2 and Atherosclerosis

PON2 is perhaps most thoroughly characterized as a mitigating factor in the development of atheromatous plaques. Although it is not known to bind to HDL particles directly, PON2 exerts a protective effect against vascular damage. Indeed, this was expertly demonstrated in separate studies using PON2-deficient mice challenged with a high-fat diet [28]–[30]. The first of these studies, carried out by Ng et al., reported that mice deficient in PON2 fed on a high-fat diet developed larger atherosclerotic lesions than their wild type counterparts [30]. Paradoxically, PON2-deficient mice had lower serum levels of very low-density lipoprotein (VLDL) and LDL, which are known to contribute to atherogenesis. However, VLDL/LDL isolated from PON2-deficient mice were significantly more inflammatory to monocytes *in vitro* than the same lipoproteins isolated from wild type mice [30]. Later studies conducted by Devarajan et al. expanded these findings using PON2-deficient mice crossed to hyperlipidemic ApoE$^{-/-}$ mice to demonstrate that PON2 protected against atherosclerotic plaque formation by reducing the mitochondrial formation of reactive oxygen species (ROS) at complex III of the electron transport

chain (ETC) [29]. A further study by the same group showed that PON2 modulates ER stress by stabilizing calcium homeostasis in macrophages, ultimately promoting cellular survival [28]. These animal studies, taken in conjunction with abundant *in vitro* evidence, demonstrate that PON2 is major component of atherosclerosis pathophysiology.

1.3.2 PON2 and Type II Diabetes Mellitus

The link between PON2 and type II diabetes mellitus (T2DM) is largely speculative and is grounded in PON1's influence on T2DM and the larger contribution of systemic inflammation and oxidative stress on T2DM development and severity. Despite numerous reports of an association between the A148G PON2 variant and T2DM, a recently published meta-analysis failed to reach the same conclusion [31]. However, a study conducted on an Iranian population of healthy and diabetic patients reported a positive correlation with another PON2 variant, S311C [32]. Based on the lack of consensus in this regard, more research into PON2's impact on T2DM is required.

1.3.3 PON2 and Cancer

Research efforts in the last decade have broadened their focus to include PON2's role in cancer. During this period, a clearer picture of how PON2 activity influences neoplastic tissue has begun to develop. Generally, PON2 has been shown to contribute to apoptotic escape and chemotherapeutic resistance through its ability to scavenge ROS in mitochondria and mitigate ER stress [8], [24], [33].

However, these effects have only been observed in a limited variety of cancers. Further work is required to understand the full pro-tumorigenic mechanisms of PON2. Nonetheless, work up to this point has provided a substantial springboard for future studies.

The first recorded implication of PON2 in the context of cancer was conducted by Frank et al. who investigated gene expression patterns in patients with Philadelphia chromosome-positive (Ph^+) chronic myelogenous leukemia (CML) that failed to respond to the kinase inhibitor imatinib [34]. At the time, acquired resistance to imatinib was well-documented, so this group sought to uncover genes that mediate primary resistance. Leukocyte RNA was isolated from Ph^+ patients with CML that were classified as either responders or non-responders, converted to cDNA, and analyzed using microarray hybridization technology. In non-responders, genes involved in oxidative stress, apoptosis, and DNA repair were identified as being significantly up- or down-regulated [34]. Among proteins with the highest fold change was PON2. Overall, the researchers identified over 100 genes that contributed to primary imatinib resistance in Ph^+ patients with CML. To our knowledge, this is the first report drawing an association between PON2 and cancer.

Shortly thereafter, Altenhofer et al. hypothesized that superoxide (O_2^-) scavenging activity of PON2 at the mitochondrial inner membrane would promote tumor survival [35]. O_2^- dismutates (via superoxide dismutase, SOD; or spontaneously) to H_2O_2 which leads to the release of cytochrome c from mitochondria and ultimately activates the intrinsic apoptotic cascade, resulting in

cell death [36]. In the study, investigators sought to determine whether PON2's anti-oxidant activity was reliant on its lactonase activity. Using a site-directed mutagenesis approach, the researchers showed that PON2 retains its anti-oxidant functions independent of its lactonase activity [35]. Although not directly a cancer-related study, the authors were among the first to suggest a pro-tumorigenic role for PON2.

Another early study implicating PON2 in human cancer was conducted by Kang et al. [37]. Investigators measured relapse-free survival (RFS) of patients with high-risk pediatric acute lymphoblastic leukemia (ALL) for a maximum of 6 years. The stated goal of this investigation was to strengthen predictions in patient outcomes and improve the existing risk classification system for patients with pediatric ALL. To this purpose, patients were separated into low- and high-RFS populations and DNA microarray data were integrated with cytometric measures to assemble a unique gene expression profile to predict RFS. Of the 38 genes most strongly correlated with RFS, PON2 expression was negatively correlated with RFS; i.e. patients with high PON2 expression relapsed earlier than patients with low PON2 expression [37]. Although indirectly, the results of this study further suggested that PON2 serves to promote carcinogenesis.

In a paper published by Witte et al., a dual role for PON2 in tumor cell pathophysiology was established [38]. The same group of researchers had, a year earlier, proposed that PON2's role in reducing oxidative stress may contribute to tumorigenesis [35]. This study focused on redox homeostasis and ER stress pathways in tumor cells, based on PON2's subcellular localization with

mitochondria and the ER, and cell death/stress pathways mediated by these organelles. First, the investigators compared PON2 expression in tumor vs. normal tissues isolated from over 400 patients with cancer. Using this approach, they found high relative expression (>2 fold) of PON2 in tumors from uterus, liver, kidney, lymphoid tissues, or urinary bladder; and moderate relative expression (~1.5 fold) of PON2 in tumors from the thyroid gland, prostate, pancreas, and testis [38]. One possible limitation of this methodology is that samples were pooled—all tumor samples of a given subtype were combined, and all normal tissue samples, likewise—making pairwise comparisons impossible. Thus, relative PON2 upregulation may have been missed due to inter-patient variability in baseline PON2 expression. Next, PON2 was either ectopically overexpressed or knocked down via RNA interference (RNAi) in a wide variety of healthy and malignant cell lines. The results of these experiments indicate that PON2 overexpression blunts the cytotoxic effects of chemotherapeutic agents, and that knockdown increases apoptotic sensitivity [38]. Finally, mechanistic insight was provided by a series of traditional biochemical experiments demonstrating that PON2 prevents the ER-stress mediated induction of pro-apoptotic CHOP (CCAAT/-enhancer-binding protein homologous protein) and inhibits intrinsic apoptotic signaling [38]. These observations confirmed an earlier hypothesis and paved the way for future PON2 studies in the context of cancer.

PON2's protective role in tumor cells was further demonstrated in the context of oral squamous cell carcinoma (OSCC). A report published in 2015 describes the stabilizing effect of PON2 expression on radiation-induced apoptosis

in OSCC cells [39]. The study explored activation of the intrinsic apoptotic signaling cascade following irradiation in various OSCC cell lines and correlated these responses with PON2 expression. In agreeance with previous studies, the OSCC cells that overexpress PON2 exhibit lower induction of cell death than those with lower PON2 expression—an effect which was reversible by RNAi-mediated knockdown of PON2 [39]. A subsequent study reported the involvement of the Wnt/β-catenin pathway in regulating PON2 expression [40]. Researchers identified sites in the PON2 gene promoter that are bound by transcription factors involved in Wnt/β-catenin signaling using a computational approach, a liquid chromatography-mass spectrometry (LC-MS) based proteomics method, and a siRNA assay screen. Employing traditional biochemical approaches, it was then demonstrated that PON2 is directly regulated by ligands of Wnt and its upstream transcription factors [40]. This regulatory network was not previously explored and has yet to be validated in another tumor type. Nonetheless, this work enhances our current mechanistic understanding of the apoptotic resistance afforded by PON2.

Recently, PON2 was identified in an integrative genomics study of pancreatic ductal adenocarcinoma (PDAC) by Nagarajan et al [41]. Once identified *in silico* and validated in an initial screen, PON2 was knocked down via shRNA-mediated RNAi and subsequently diminished metastasis of PDAC cells in multiple *in vivo* models [41]. Further *in silico* analysis coupled with chromatin-immunoprecipitation (ChIP) assays also revealed transcriptional repression of PON2 by p53, which was previously undocumented. Intriguingly, this study also

demonstrated that PON2 expression may modulate glucose uptake by stimulating GLUT1-mediated glucose transport. This work reveals a regulatory pathway by which PON2 promotes tumor survival and metastasis, although, further work is required to determine whether these interactions are present in other cancer types, or if the observations described in this report are specific to PDAC alone.

Largely, this body of work provides a cohesive lens through which PON2's tumor-promoting role may be viewed. Although the complete mechanisms and interactions have not been fully established, PON2 confers apoptotic resistance to tumor cells by abating ER stress and reducing ROS formation in mitochondria. This protection enables various tumor cells to overcome the cytotoxic effects of chemotherapeutics [34], [38] and irradiation [39], [40], and promotes metastasis [41]. Additionally, PON2 receives and exerts regulatory control through commonly-dysregulated oncogenic networks, such as the Wnt/β-catenin pathway [40] and p53 [41]. In conjunction with large-scale studies that have demonstrated that PON2 is upregulated in numerous and diverse tumor types [38], [42], these considerations necessitate further study of PON2's oncogenic contribution in other neoplastic tissues and cells.

2. Non-small cell lung carcinoma

Lung cancer claims more lives each year than any other cancer in the US and globally [43]. Non-small cell lung carcinoma (NSCLC) is the dominant subtype of lung cancer, comprising 85% of all diagnoses [44]. Under the umbrella of NSCLC are three distinct histological subtypes: A*denocarcinoma* is the most

frequently indicated histology of NSCLC and accounts for 35-40% of lung cancer cases; *Squamous cell carcinoma* accounts for 25-30% of cases and is most common in smokers; The remaining NSCLC diagnoses are classified as *Large cell carcinoma*, which accounts for around 10% of all lung cancer diagnoses [45]. Although tumors of a given histological subtype share morphological characteristics, next generation sequencing technology has revealed a high degree of heterogeneity, complicating therapeutic intervention [44]. Indeed, the intra- and inter-tumoral variability may contribute to the near-inevitability that patients with NSCLC will develop drug resistance during treatment [44]. With these considerations in mind, it is perhaps unsurprising that 5-year survival in patients with NSCLC has only moderately improved [46].

RESEARCH OBJECTIVE

While numerous studies have implicated PON2 overexpression in a variety of tumor types with respect to chemotherapeutic resistance, tumorigenesis, disease progression, and metastasis, PON2's impact on NSCLC treatment is poorly understood. In the present study, we sought to explore the potential interplay between PON2 expression and NSCLC proliferation using knockout animals, human and mouse tissue samples, primary cells, NSCLC cell lines, and non-transformed human epithelial cells. These insights will broaden our understanding of NSCLC pathophysiology, which will enhance future treatment options and improve outcomes for patients with NSCLC.

CHAPTER 2: METHODS

Reagents

Dulbecco's Modified Eagle's Medium (DMEM), penicillin/streptomycin, trypsin, and L-glutamine were obtained from Mediatech (Manassas, VA); fetal bovine serum was purchased from Gemini (Broderick, CA); Bronchial Epithelial Cell Growth Medium (BEGM) and SingleQuots were purchased from Lonza (Walkersville, MD); and propidium iodide and β-Mercaptoethanol were purchased from Invitrogen (Carlsbad, CA). N-(3-oxododecanoyl)-homoserine lactone (C12), polybrene and puromycin were purchased from Sigma-Aldrich (St. Louis, MO). Antibodies (Abs) for western blot were anti-β-actin mAb (A5441; Millipore Sigma; Burlington, MA), anti-Human PON2 mAb (ab183710; Abcam; Cambridge, England), anti-Mouse PON2 pAb (ABIN1573944; antibodies-online.com; Atlanta, GA), peroxidase-conjugated goat anti-rabbit IgG (65-6120; Thermo Fisher; Waltham, MA) and peroxidase-conjugated goat anti-mouse IgG (65-6520; Thermo Fisher).

Cell culture

HEK-293T, A549, and NCI-H1299 cell lines were purchased from ATCC (Manassas, VA). Human bronchia/tracheal epithelial (HBE) immortalized by telomerase and SV40 large T antigen were obtained from Professor Barrett Rollins

(Harvard Medical School). HBE cells were cultured in BEGM supplemented with SingleQuots. All other cells were cultured in DMEM containing 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin. All cell lines were cultured in a 5% CO_2 humidified incubator at 37°C. The cells were passaged at 1:5–1:10 dilutions and were continuously cultured no longer than 3 weeks. All stocks come from thawed vials that were frozen at passage two after receipt from ATCC and were authenticated by ATCC cell bank using the Short Tandem Repeat (STR) profiling.

Plasmids

Control double nickase plasmid (SC-437281) and PON2 CRISPR/Cas9 plasmid (SC-403181-NIC) were purchase from Santa Cruz (Santa Cruz, CA). Control double nickase plasmid comprises a pair of plasmids, each with D10A mutant Cas9 nuclease and a non-targeting 20nt scramble gRNA. While one plasmid in the pair contains a puromycin-resistance gene, the other plasmid in the pair contains a GFP marker to visually confirm transfection. PON2 CRISPR/Cas9 knockout plasmid comprises 2 pooled plasmids, each encoding the Cas9 nuclease, a PON2-specific 20nt gRNA, and a selection marker. One Plasmid contains a puromycin-resistance gene and the other plasmid has a GFP marker. gRNA sequences direct the Cas9 protein to induce a site-specific double strand break (DSB) in exon 3 of PON2 genomic DNA. Control shRNA plasmid-A (SC108060) and PON2-shRNA plasmids (SC62838) were purchased from Santa Cruz. Control shRNA plasmid-A encodes a non-specific scramble shRNA; PON2-

shRNA plasmid comprises 3 lentiviral vector plasmids with 19-25nt shRNAs targeted to PON2. Each shRNA plasmid contains a gene for puromycin resistance as a selection marker. Lentiviral helper plasmids pMDLg/pRRE (12251), pRSV.Rev (12253) and pMD2.G (12259) were purchased from Addgene (Watertown, MA). The identities of each plasmid were verified by sequencing.

Generation of cells with reduced PON2 expression by shRNA

HEK-293T cells ($1.5*10^6$) were plated in 6-cm tissue culture plates and cultured for 24 hours. To produce lentivirus, HEK293T cells were transfected with control shRNA plasmid-A (SC108060) or PON2-shRNA plasmids (SC62838) along with the lentiviral helper plasmids pMDLg/pRRE, pRSV.Rev and pMD2.G using Lipofectamine2000® transfection reagent (Thermo Fisher) as a lipid transport milieu. Tissue culture medium containing lentiviral particles was collected 48- and 72-hours following transfection and was filtered through a sterile syringe filter with 0.4 μM polyethersulfone membrane (VWR; Radnor, CA). Twenty-four hours prior to lentiviral infection, A549, HEK-293T and HBE cells ($0.2*10\text{^}6$) were seeded in 6-well tissue culture plates. To infect cells, the culture medium of plated cells were replaced with the medium containing lentiviral particles supplemented with 10 μg/mL polybrene. The infection was repeated 24 hours later. Twenty-four hours after two rounds of infection, puromycin (5 μg/mL) was added to the medium to kill uninfected cells. PON2 expression in the bulk population of infected cells was examined by western blot analysis. Bulk populations of infected cells were cultured in the medium supplemented with 1 μg/mL puromycin.

Generation of cells deficient in PON2 expression by CRISPR/Cas9

NCI-H1299 cells ($2*10^6$) were plated in 10-cm tissue culture plates and cultured for 24 hours. Control double nickase plasmid (SC-437281) and PON2 CRISPR/Cas9 plasmid (SC-403181-NIC) were transfected into NCI-H1299 cells using Lipofectamine2000® transfection reagent (Thermo Fisher) following manufacturer's protocol. Twenty-four hours after transfection, puromycin (5 μg/mL) was added to the cell to eliminate untransfected cells. NCI-H1299 cells were grown in culture medium supplemented with 5 μg/mL puromycin for 20 days until stably transfected cells grew up. Single clones of stably transfected cells were established by seeding single GFP-expressing cells into one well of 96-well tissue culture plates using fluorescence-activated cell sorting (FACS; MoFlo, Beckman Coulter, Brea, CA). Once GFP-positive cell clones grew up in the medium containing 5 μg/mL puromycin, PON2 expression in cloned cell lines were examined by western blot analysis. To ensure stable plasmid expression, cells were grown in culture medium supplemented with 1 μg/mL puromycin.

Western Blot analysis

Tissue lysates were prepared using human lung tumor samples from patients with NSCLC and organ tissue derived from wild type or PON2-knockout mice as follows: Frozen tissue samples were resuspended in tissue protein extraction reagent (Thermo Fisher) supplemented with protease inhibitors (cOmplete; Roche; Indianapolis, IN) and phosphatase inhibitors (PhosSTOP;

Roche). Following homogenization and centrifugation, protein concentration was determined by bicinchoninic acid (BCA assay) (Thermo Fisher). Whole cell extracts of cultured mammalian cells were acquired using RIPA buffer (Thermo Fisher) supplemented with protease inhibitors (cOmplete; Roche). For human tissue, 30 μg of total protein was electrophoresed; for mouse organ tissue, 10 μg of total protein was used; for cultured cells, 30 μg of total protein was analyzed. All of the samples were electrophoresed in 4–12% Bis/Tris gels (Bio-Rad; Hercules, CA). Separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore) and incubated with respective primary or secondary antibodies diluted with 10% (w/v) nonfat dry milk (Bio-Rad) in blotting buffer (1X phosphate-buffered saline [PBS] with 0.2% Tween-20). The enhanced chemiluminescence detection system (Thermo Fisher) was used to detect proteins as previously described [47]. ImageJ software (NIH, Bethesda, MA) was used to quantify the Optical density (OD) values of western blot signals.

Cell death assays

The indicated MEF cells were plated in a 48-well tissue culture plate with 10,000 cells in each well and cultured for 24 hours. Unless otherwise stated, medium containing 0.2% DMSO with or without C12 was incubated with the cells. Following treatment with DMSO or C12, cells were harvested in the presence of 1 μg/ml propidium iodide (PI). Cell viability was measured by PI exclusion using flow cytometry (FACScalibur, Beckon Dickinson, San Jose, CA). The percentage of cell death was determined as100 minus the value of cell viability measurement.

Cellular proliferation experiments

Proliferation was assessed by a cell counting method using a hemocytometer. Equal numbers of cells were plated and cultured in 12-well plates with 3 replicates for 4 time points: 24, 48, 72, and 96 hours. Initial cell number was selected based on cell size and proliferation rate as follows: $1.5*10^4$ HBE cells (control- and PON2-shRNA); $1.5*10^4$ HEK-293T cells (control- and PON2-shRNA); $1.5*10^4$ A549 cells (control- and PON2-shRNA); $1.5*10^4$ NCI-H1299 cells (vector clones 2 and 3; CRISPR-PON2 clones 4 and 14).

Cell cycle analysis

A549 cells ($5*10^5$) were centrifuged at 300 x g for 5 minutes and washed twice with 500 µl 1X PBS. Cells were then fixed with 1 ml 70% ethanol in 1X PBS at 4^{o}C overnight. After centrifugation (300 x g for 5 minutes), cells were washed twice with 1X PBS and resuspended in 500 µl 1X PBS. 50 U RNase A (Qiagen; Valencia, CA) were added to samples followed by incubation at 37^{o}C for 1 hour. Then propidium iodide (5 µg) was added to samples which were incubated for 30 minutes at 37^{o}C before flow cytometric analysis (FACScalibur).

Liquid Chromatography-Mass Spectrometry

Lysates from mouse organ tissues and primary MEF cells were prepared according to a published method [48], with minor modifications. Mouse organs were harvested and thoroughly rinsed with 1X PBS, flash-frozen in liquid N_2, and

stored at -80°C. When needed, organ tissue was thawed on ice, to which 1 volume of lysis buffer (25 mM Tris/HCl containing 1 mM $CaCl_2$ and supplemented with protease inhibitors [cOmplete; Roche] and phosphatase inhibitors [PhosSTOP; Roche]) was added. Tissue was sonicated on ice for 1 min. Lysates were centrifuged at 14,000 x g and 4°C for 10 min. Supernatant protein concentration was determined using the BCA assay (Thermo Fisher). Final protein concentration was adjusted to 2 µg/µL in lysis buffer.

Primary MEF cells were seeded into 10 cm tissue culture plates at a concentration of 10^6 cells per plate and cultured overnight. Cells were collected by trypsinization and cell number was determined using a hemocytometer. 10^6 cells were centrifuged at 300 x g, supernatant was aspirated, and cells were resuspended in 1 mL ice-cold 1X PBS. Wash step was repeated once more, then cells were centrifuged at 5000 x g and 4°C for 5 min and supernatant was removed by aspiration. Cell pellets were stored at -80°C. When needed, cells were thawed on ice, to which 100 µL lysis buffer was added to resuspend cells. Cells were sonicated on ice for 10 sec. Lysates were centrifuged at 14,000 x g and 4°C for 10 min. Supernatant protein concentration was determined using the BCA assay (Thermo Fisher). Final protein concentration was adjusted to 2 µg/µL in lysis buffer.

10 µg of total protein from mouse organ tissue lysate or primary MEF cell lysate was diluted to a final volume of 100 µL in 5 mM Tris/HCl (ph 7.4) containing 1 mM $CaCl_2$. Reactions were initiated with either 1% MeOH or 1% 5 mM C12 (in Methanol; final concentration 50 µM C12) and incubated at 37°C for 30 min. The reaction was stopped with 70 µL of ice-cold acetonitrile and immediately analyzed

by liquid chromatography–mass spectrometry (LC-MS) without further manipulation. LC-MS experiments were carried out at the University of Louisville medicinal chemistry core facility. A sample volume of 15 µL was injected into an Agilent 1260 HPLC system (Agilent; Santa Clara, CA) equipped with an Agilent 6224 Accurate-Mass Time-of-Flight MS utilizing electrospray ionization. The HPLC system was equipped with an Zorbax Extend-C18 column (1.8 µm, 2.1 × 50 mm; Agilent) with a mobile phase consisting of mass spectrophotometry grade water (with 0.1% formic acid and 0.1% methanol) and acetonitrile (with 0.1% formic acid) and operated in positive ion mode (3500 V V-Cap, 750 V OctRF Vpp, 65 V skimmer, 135 V fragmentor, 40 psi Nebulizer gas, 12 L/min drying gas, and 325^{o}C gas temperature). Samples were eluted with a linear gradient of 5 to 100% acetonitrile at 0.3 mL/min over 10 min. Agilent software analysis suite was used to determine respective measured masses of spectrometry signals.

In vivo animal studies

Animals were housed in an AALAC- (Association for Assessment and Accreditation of Laboratory Animal Care) accredited pathogen-free barrier facility. All procedures were in accordance with the University of Louisville Institute for Animal Care and Use Committee (IACUC). Fertilized C57BL/6J-*Tyr*$^{c-2J}$ (i.e. B6 Albino; Jackson Laboratory; Bar Harbor, ME) embryos were co-injected with Cas9 mRNA and a sgRNA targeted to the mouse PON2 gene at exons 3. Treated mouse embryos were implanted into a pseudopregnant female mouse to resume gestation. Viable F_0 pups were weaned, and genotypes were determined by

sequencing at the PON2 locus. An F_0 pup was identified to be heterozygous for a single base-pair insertion in exon 3 that resulted in an early termination codon. Heterozygous mice were backcrossed to wild type C57BL/6J mice (Jackson) for 5 generations to minimize genetic heterogeneity and simultaneously retain the mutant PON2 allele. Backcrossed offspring were selectively bred to achieve desired genotypes to be used in body weight and organ weight experiments. Genotyping of mice was carried out at the University of Louisville genomics core facility by sequencing mouse tail DNA. Body weight of C57BL/6J wild type and homozygous PON2-knockout mice was recorded biweekly from 1 month of age until 3 months, at which time heart, lungs, liver, kidneys, and spleen were dissected and weighed.

Isolation of primary mouse embryonic fibroblast (MEF) cells

Primary MEF cells were harvested and cultured according to a published protocol [49]. Briefly, embryos were aseptically dissected from female C57BL/6J mice at 13 days after a copulation plug was observed. Following removal of embryonic brain, heart, and liver, remaining tissue was finely minced using sterile scissors and incubated in 0.25% trypsin-EDTA for 10 min at 37^{o}C. Solution was thoroughly agitated with a pipette and incubated for an additional 10 min at 37^{o}C. Primary MEF medium (DMEM containing 10% FBS, 100 units/mL penicillin, 100 µg/mL streptomycin, and 0.1 mM β-Mercaptoethanol) was added to deactivate trypsin and mixed using a pipette. Larger fragments were allowed to sink to tube bottom for 10 min, then cell suspension was plated in 15 cm tissue culture plates,

incubated at 37ºC overnight, and refreshed with warm medium the following day. After 2 days, primary MEF cells were collected by trypsinization and resuspended in cryopreservation medium (10% DMSO in FBS), whereupon cells were aliquoted and stored in 2 mL cryovial tubes at -140ºC for long-term storage.

Lung tissues from patients with non-small cell lung carcinoma

Tumor tissues along with corresponding adjacent normal tissues from patients with non-small cell lung carcinoma (NSCLC) were acquired from the James Graham Brown Cancer Center Bio-Repository at University of Louisville in accordance with an approved institutional review board (IRB) protocol. Tissue samples were stored at 37ºC before experiments.

Statistical analysis

Data are presented as mean ± standard deviation. Proliferation studies were performed independently in triplicate. Animal weight studies were carried out with 10 animals per sex per group. Organ weight results are 5 per sex per group. A Student's two-tailed unpaired t-test with p-value of < 0.05 was used to determine statistical significance.

CHAPTER 3: RESULTS

PON2 is overexpressed in lung tumor samples from patients with NSCLC

As discussed in the introduction, a number of studies have correlated PON2 overexpression in a wide variety of solid tumors and blood cancers with tumorigenesis, disease progression, and chemotherapeutic sensitivity [34], [37], [38], [41], [42]. Of these studies, a limited number have focused on lung cancer in general [24], [42], but have failed to interrogate PON2 expression in NSCLC specifically. To determine the relative expression level of PON2 in NSCLC, we acquired lung tumor samples from 11 patients with NSCLC and compared PON2 protein levels with corresponding adjacent normal lung tissue using western blot analysis (Figure 1A). Optical density (OD) values for PON2 and actin were quantified using ImageJ software (NIH). Next, the OD values of PON2 were normalized to corresponding actin OD values for both normal and lung tumor samples, then the ratio of relative PON2 expression of lung tumor tissue vs. normal tissue was calculated (Figure 1B). Using this analytical approach, we found that 8 of the 11 patients with NSCLC examined exhibited PON2 overexpression in lung tumor tissue, whereas PON2 expression was slightly decreased in the remaining 3 patients. While PON2 expression levels among different NSCLC tumor tissue samples were consistently high, PON2 expression in normal tissue samples varied markedly. This might be attributed to different precancerous stages bearing

various premalignant lesions in adjacent normal tissues [50]. These data highlight a correlation between PON2 expression and malignant transformation in human patients with NSCLC, consistent with a recent study revealing a negative correlation between PON2 expression and patient prognosis of many cancer types [42].

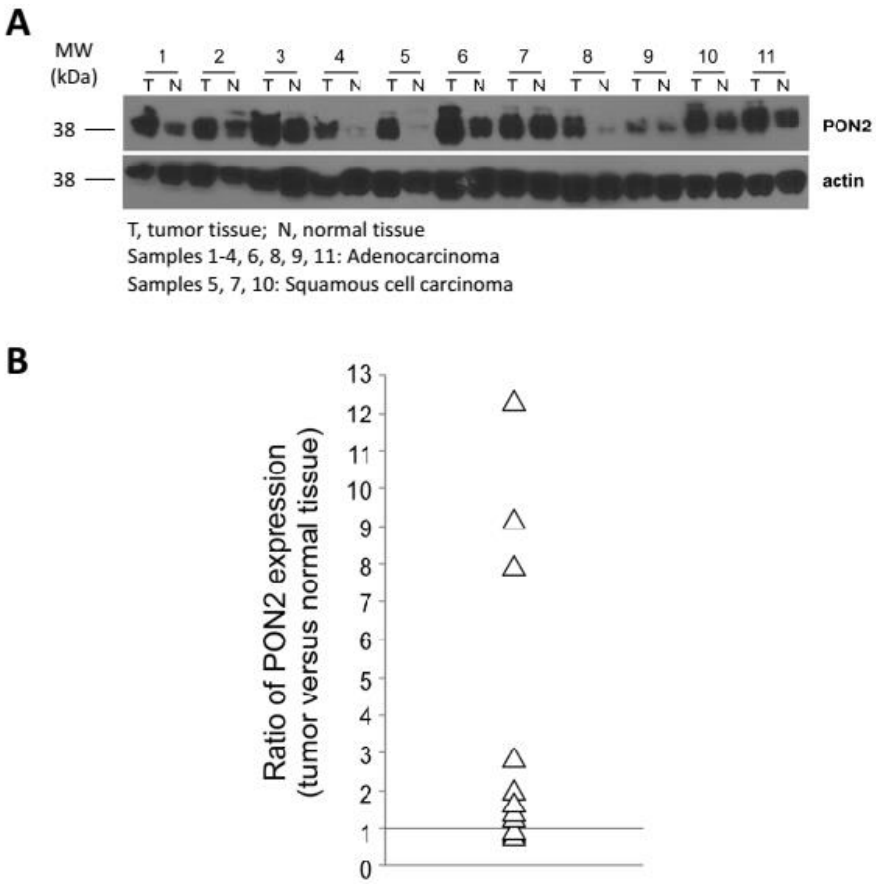


Figure 1. PON2 is upregulated in NSCLC tumor samples. **(A)** PON2 expression was evaluated in tumor (T) and adjacent normal lung (N) tissue samples derived from 11 patients with NSCLC. **(B)** Optical density values in (A) were determined using ImageJ software (NIH) and used to calculate the ratio of PON2 expression (normalized to actin) in tumor tissue compared with corresponding adjacent normal lung tissue. Values greater than 1 indicate PON2 overexpression in tumor vs. normal tissue; 8 of 11 patient samples exhibited increased PON2 expression in NSCLC tissue.

Generation of PON2-knockout mice using CRISPR/Cas9

Ng and colleagues first generated PON2-deficient mice using a gene-trapping method to investigate the impact of PON2 on atherogenesis [30]. While additional studies employing this mouse strain have further elucidated PON2's effect on atherosclerosis, no studies have used PON2-deficient mice in the context of cellular proliferation and tumorigenesis. Since the PON2-deficient mice are not available to us and gene-trapping has a potential pitfall of allowing leaky expression of the trapped gene [51]–[53], we collaborated with the University of Louisville's genomics core facility to employ a CRISPR/Cas9 approach to generate mice with systemic PON2 deletion. A single guide RNA (sgRNA) designed to target exon 3 of the *PON2* gene was co-injected with Cas9 mRNA into fertilized C57BL/6/TyrC2J mouse embryos, then implanted into pseudopregnant female mice. Genotyping was performed by PCR and Sanger sequencing, which revealed an individual with a single nucleotide insertion in exon 3 that resulted in an early termination codon (Figure 2). This individual mouse was then outcrossed with wild type C57BL/6J mice whose progeny were further selectively outcrossed to retain the mutant PON2 allele. Following 5 generations of outcrossing, mice were bred to generate wild type ($PON2^{+/+}$; WT), heterozygous ($PON2^{+/-}$), and homozygous knockout ($PON2^{-/-}$; PON2-KO) mice for further experimentation.

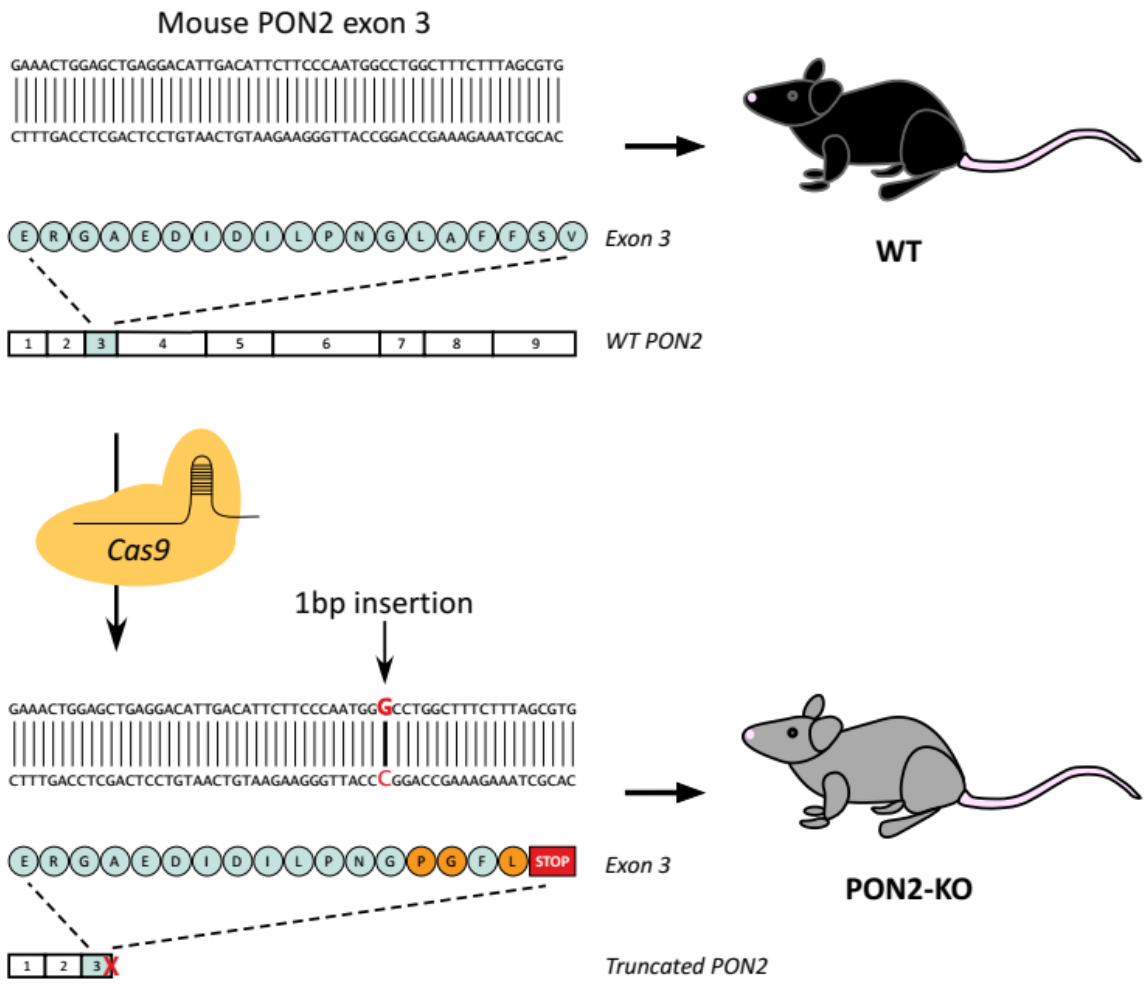


Figure 2. Generation of PON2-knockout mice using a CRISPR/Cas9 approach. Fertilized C57BL/6J/TyrC2J embryos were co-injected with sgRNAs targeted to exon 3 the PON2 gene and Cas9 mRNA, then implanted in pseudopregnant female C57BL/6 mice. The *PON2* locus was sequenced in each pup, revealing an individual with a single nucleotide insertion at exon 3 that resulted in an early termination codon. Mice were selectively bred to expand the population carrying the mutant PON2 allele.

Tissues of PON2-knockout mice are deficient in PON2 protein expression

To confirm that disrupting the genomic sequence of PON2 in mice leads to systemic deficiency in PON2 expression, we assessed PON2 expression in various organs. Although PON2 is a ubiquitously expressed protein, several major organs are known to produce relatively high levels of PON2 [25]. To this purpose, mouse tissues, including lung, stomach, small intestine and large intestine, were acquired from wild type ($PON2^{+/+}$), heterozygous knockout ($PON2^{+/-}$), and homozygous knockout ($PON2^{-/-}$) mice. The protein levels of PON2 in the collected tissues were evaluated by western blot analysis. As shown in Figure 3, PON2 protein was detected in all tissues isolated from wild type mice. Moreover, PON2 protein levels in the tissues of heterozygous knockout mice were lower than their counterparts from wild type mice, which is in agreement with the number of intact PON2 allele in the tissues. Importantly, no PON2 protein was detected in any of the tissues from the mice with PON2 homozygous knockout, indicating that there is a systemic deficiency in PON2 protein expression in PON2 homozygous knockout mice.

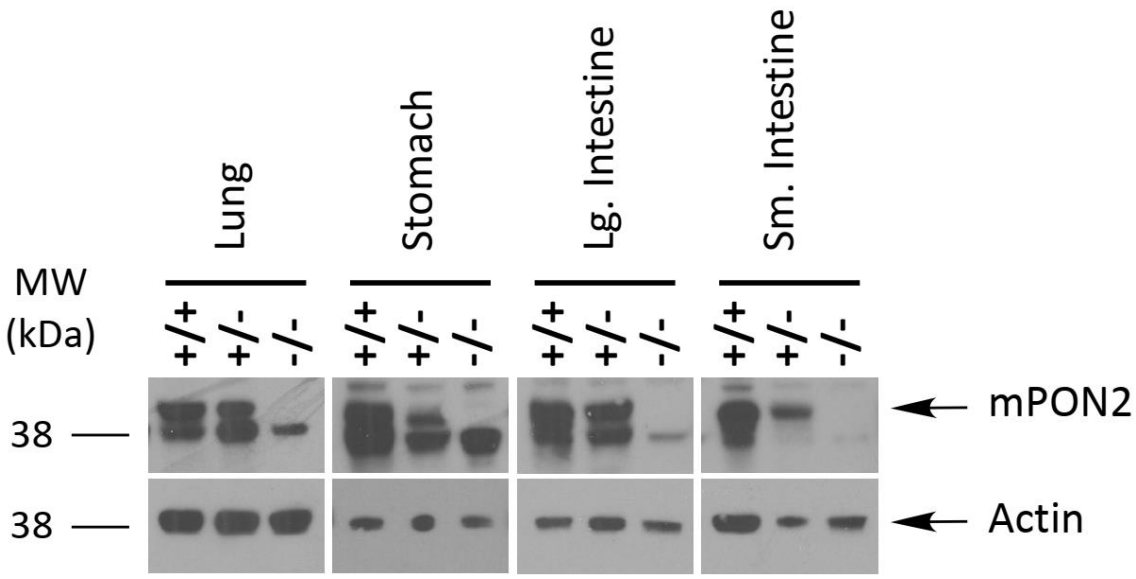


Figure 3. Tissues from PON2-knockout mice lack PON2 expression. Tissues known to express PON2 at high levels (lungs, stomach, large intestine, and small intestine) were collected from wild type (+/+), heterozygous (+/-), and homozygous knockout (-/-) C57BL/6J mice and used to prepare tissue lysates. Western blot analysis was performed to determine mouse PON2 (mPON2; upper band) expression. Actin was detected as a loading control. The molecular weight markers are labelled on the left.

C12-induced cell death in primary MEFs depends on PON2

PON2 is known to mediate mammalian cell death signaling triggered by various apoptotic stimuli, including *N*-(3-oxododecanoyl)-L-homoserine lactone (so called C12), a bacterial quorum-sensing molecule produced by *Pseudomonas aeruginosa* [1], [11]. Our previous studies indicate that PON2 is essential for apoptosis induced by C12 in a number of mammalian cell types, which is attributed to its lactonase activity [47], [54]. To further explore the biological consequences of PON2 expression deficiency in mice, we investigated how primary cells isolated from wild type or PON2-knockout mice respond to C12 exposure. To this purpose, we first isolated primary MEFs from 13 day-old embryos and examined PON2 expression by western blot analysis. As shown in Figure 4A, PON2 protein expression in MEFs generated from PON2-knockout mice was not detected, which is in agreement with the data acquired from tissue samples (Figure 3). Upon treatment with different doses of C12, wild type MEFs underwent cell death in a dose-dependent fashion (Figure 4B). In contrast, cytotoxicity of C12 was completely abrogated in MEFs lacking PON2 expression, consistent with our earlier findings about the vital role of PON2 in C12-induced cell death [47], [54]. Overall, these data provide more evidence that deleting PON2 gene expression in mice affects how cells respond to apoptotic stimuli.

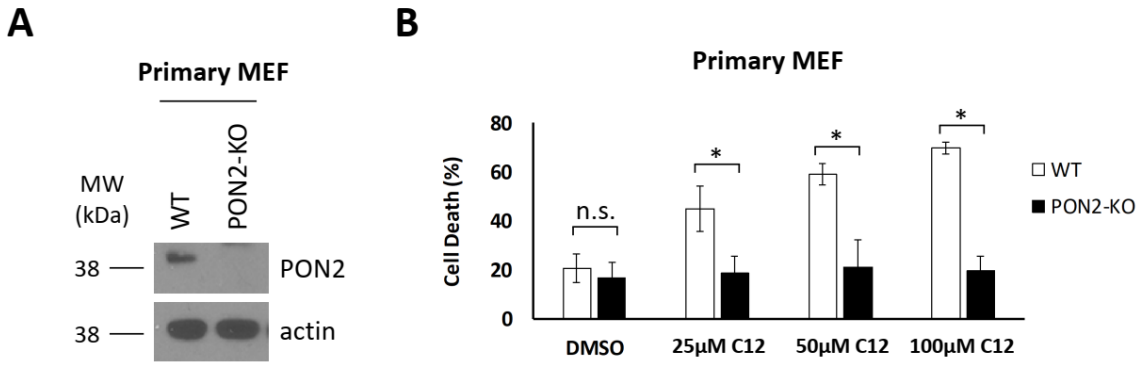
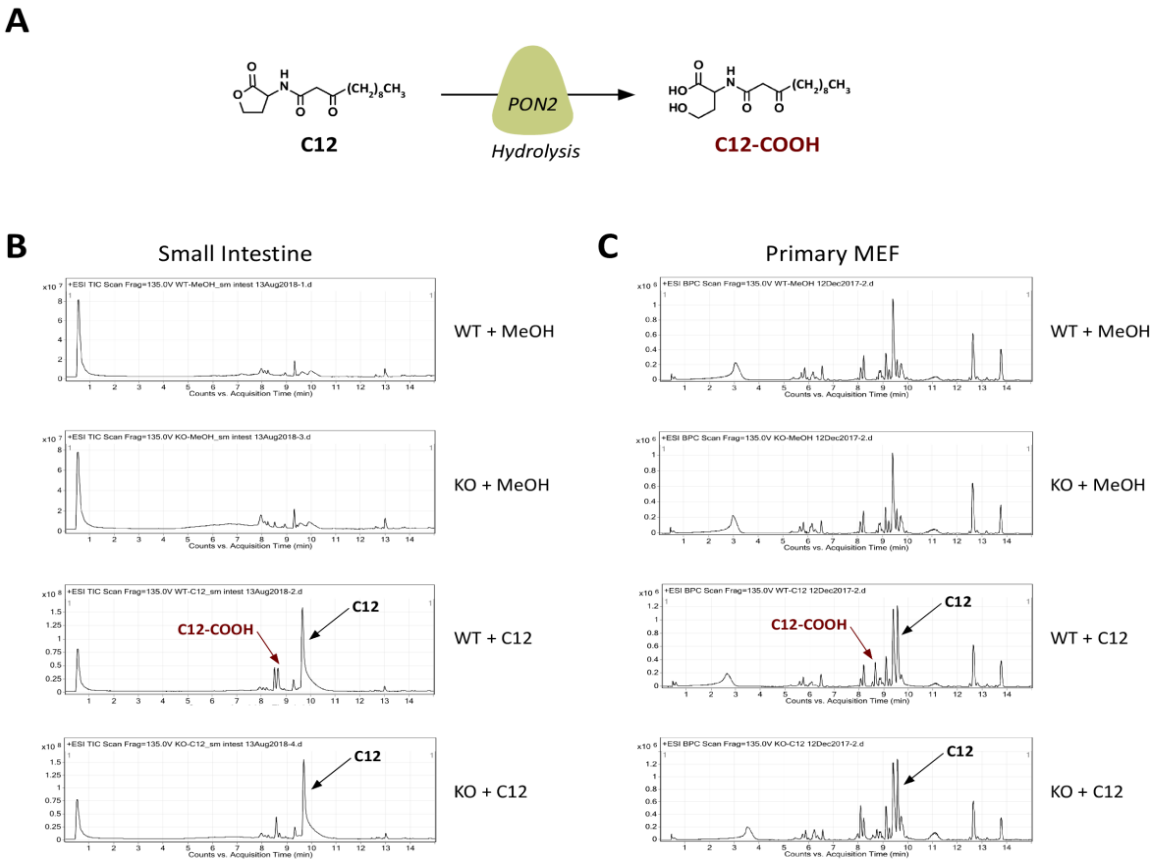


Figure 4. Primary MEFs are sensitive to C12-mediated cell death in a PON2-dependent manner. (A) Primary MEF cells were isolated from either wild type or PON2-knockout mice, and PON2 expression was assessed via western blot analysis. Actin was detected as a loading control. The molecular weight markers are labelled on the left. **(B)** PON2 is required for the cytotoxic effects of C12. To assess PON2 function in primary MEF cells, wild type and PON2-knockout MEFs were exposed to either DMSO or increasing concentrations of C12 for 24 hours and cell death was quantified using flow cytometry to detect propidium iodide uptake. Data are mean ± standard deviation of three independent experiments. n.s. = no significance. Asterisks indicate P values of < 0.05 (*) by student's unpaired t test.

Tissues and cells of PON2-knockout mice lack PON2 enzymatic activity

To functionally confirm PON2 deficiency in PON2-knockout mice, we investigated whether tissues and cells from PON2-knockout mice still possessed PON2 enzymatic activity. As a lactonase, the major enzymatic activity of PON2 is to hydrolyze lactones[55]. Among the substrates of PON2, C12 is a well-studied specific substrate. Via its lactonase activity, PON2 catalyzes the specific hydrolysis of the lactone ring of C12 to form a carboxylic acid derivative (C12-COOH; Figure 5A). The other members of the paraoxonase family, PON1 and PON3, exhibit lactonase activity, albeit at orders of magnitude lower than PON2 [1]. Thus, the presence of C12-COOH is a reliable indicator of PON2 lactonase activity. Therefore, we performed a series of LC-MS experiments in which various lysates from wild type and PON2-knockout mice were tested for their activity to hydrolyze C12. To detect PON2-mediated hydrolysis of C12, we collaborated with Dr. Burlison at the medicinal chemistry facility at the University of Louisville. Whole cell lysates from primary MEFs and small intestine tissue isolated from wild type and PON2-knockout mice were incubated with either methanol (MeOH) or C12 (in MeOH) and analyzed by LC-MS (Figures 5B and 5C). In both small intestine tissue and primary MEFs, hydrolysis of C12 (indicated by black arrows) to its carboxylic acid derivative C12-COOH (indicated by red arrows) was detected in wild type samples. Conversely, the lactonase-opening conversion of C12 to C12-COOH was undetectable in both PON2-knockout samples, indicating an absence of PON2 enzymatic activity. Because some signals in the chromatograms of the PON2-knockout samples may appear to be C12-COOH, the on-board Agilent

software analysis suite was used to determine respective measured masses (Figures 5D and 5E). In small intestine and primary MEF samples from PON2-knockout mice, the difference between measured mass of any detected molecule and the predicted mass of C12-COOH was orders of magnitude beyond the acceptable error value (<5 ppm is acceptable), indicating that C12-COOH was not present. The results of these experiments reveal that PON2 enzymatic function is absent in tissues and cells collected from mice deficient in PON2 expression.



D

C12-treated small intestine

	Compound	Formula	Calculated exact mass	Measured accurate mass	Error (ppm)
WT	C12	$C_{16}H_{28}NO_4$	298.2013	298.2024	**3.68878**
	C12-COOH	$C_{16}H_{30}NO_5$	316.2118	316.2122	**1.26497**
KO	C12	$C_{16}H_{28}NO_4$	298.2013	298.2024	**2.01206**
	C12-COOH	$C_{16}H_{30}NO_5$	316.2118	315.2048	**-3184.57**

E

C12-treated primary MEF

	Compound	Formula	Calculated exact mass	Measured accurate mass	Error (ppm)
WT	C12	$C_{16}H_{28}NO_4$	298.2013	298.2024	**3.68878**
	C12-COOH	$C_{16}H_{30}NO_5$	316.2118	316.2125	**1.26497**
KO	C12	$C_{16}H_{28}NO_4$	298.2013	298.2025	**3.68878**
	C12-COOH	$C_{16}H_{30}NO_5$	316.2118	316.3216	**347.236**

Figure 5. PON2 enzymatic activity is abrogated in tissues and primary cells from PON2-knockout mice. To functionally examine the enzymatic activity of PON2, lysates of small intestine tissue and primary MEFs were prepared from wild type and PON2-knockout mice, treated with either methanol (MeOH) or 50 µM C12 (in MeOH), and analyzed by liquid chromatography-mass spectrometry (LC-MS). **(A)** PON2 specifically catalyzes the lactone ring-opening hydrolysis of C12 to produce a carboxylic acid derivative (C12-COOH). **(B)** Mass spectra obtained from small intestinal tissue lysates exposed to either MeOH or C12. Black arrows indicate presence of C12; red arrows indicate presence of C12-COOH. **(C)** LC-MS experiment was repeated under the same conditions with lysates of MEFs from wild type and PON2-knockout animals. **(D)** The masses of identified molecules in chromatography spectra of small intestine tissue lysates shown in (B) were determined. The molecules whose masses are most similar to predicted C12 mass or predicted C12-COOH mass were listed. **(E)** The mass of identified molecules on chromatography spectra shown in (C) was calculated. The molecules with masses closest to predicted C12 mass or predicted C12-COOH mass were listed.

Systemic PON2 deficiency does not affect mouse growth and development

Organism-wide changes associated with PON2 deficiency have been previously explored, but only in the limited context of serum lipoproteins and vascular diseases [2], [29], [30]. To assess whether disruption of the PON2 gene expression influences mouse growth and development on a broad scale, we monitored mouse body weight biweekly from 4 until 12 weeks of age (Figure 6A), at which point organ weight was assessed (Figure 6B). Assessment of body weight revealed no significant difference between male wild type and PON2-knockout mice, as well as between female wild type and PON2-knockout mice (Figure 6A; n=10 per sex per genotype). At the conclusion of the body weight assessment, heart, lung, liver, kidney, and spleen organs were removed from a cohort of mice (n=5 per sex per genotype) and weighed (Figure 6B). Organ weight was normalized to body weight whereupon statistical analysis revealed no significant difference between wild type and PON2-knockout mice in respective sex groups. Taken together, these observations suggest that PON2 is dispensable for normal mouse growth and development.

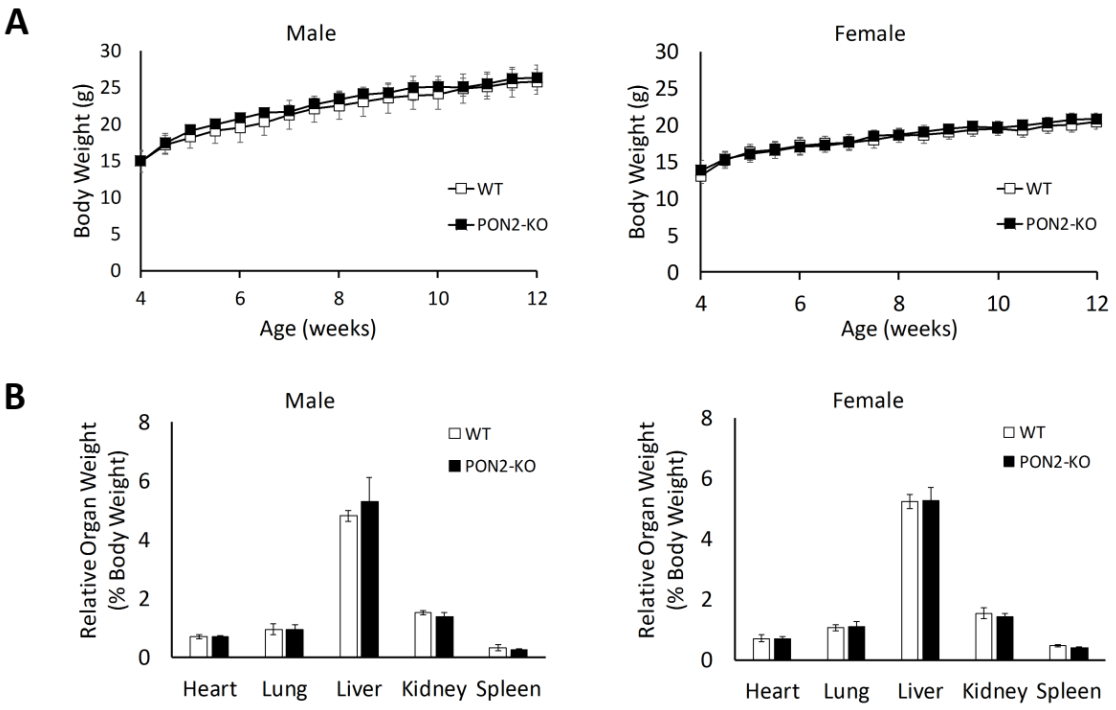


Figure 6. Normal mouse growth and development is unaffected by PON2 deficiency. (A) Wild type (WT) and homozygous PON2-knockout (PON2-KO) C57BL/6J male and female mice (n=10 per group) were weighed biweekly from 4 weeks until 12 weeks of age. **(B)** Heart, lung, liver, kidney, and spleen tissues were harvested at 12 weeks and weighed from indicated sex and genotype cohorts (n=5 per group). Data are mean ± standard deviation.

PON2 plays a vital role in the proliferation of NSCLC cells in vitro

Since PON2 expression is enhanced in human NSCLC tissues (Figure 1), we investigated whether altered PON2 expression would impact proliferation of NSCLC cells *in vitro*. To this purpose, we first generated NSCLC cells with decreased PON2 protein levels, accomplished by two approaches: PON2 knockdown in A549 cells via RNA interference (RNAi) and PON2 knockout in NCI-H1299 cells via CRISPR/Cas9 (Figure 7). Then we measured cell proliferation by monitoring cell number increase in NSCLC cell with different expression levels of PON2. A549 cells were infected with lentiviral particles derived from a vector expressing either a non-specific scrambled shRNA (control-shRNA) or a PON2-specific shRNA, and the effects of knockdown were verified by western blot analysis (Figure 7A). Equal numbers of both A549 cell types were plated and counted daily over 4 days. We observed a significant decrease in total cell number in the PON2-shRNA cells compared with the control-shRNA cells (Figure 7B).

To further address the interplay between PON2 and NSCLC proliferation, NCI-H1299 cells were transfected with a vector expressing either a nuclease-deficient Cas9 with a non-specific scrambled sgRNA, or Cas9 with a PON2-specific sgRNA. Single clones were established using fluorescence-activated cell sorting (FACS), then selected for proliferation experiments based on PON2 expression; vector clones with unaltered PON2 expression were chosen and CRISPR-PON2 clones with undetectable expression were selected (Figure 7C). Similar to the observed differences in cell proliferation of A549 cells (Figure 7B), NCI-H1299 cells deficient in PON2 expression exhibited a reduced proliferation

over 4 days (Figure 7D). Variations in proliferation of vector cell lines failed to reach statistical significance, as did differences in growth of CRISPR-PON2 clones. However, both CRISPR-PON2 clones grew at markedly reduced rates compared with both vector clones in a statistically significant manner. The outcomes of these experiments indicate that a decrease or elimination of PON2 expression negatively impacts NSCLC proliferation *in vitro*.

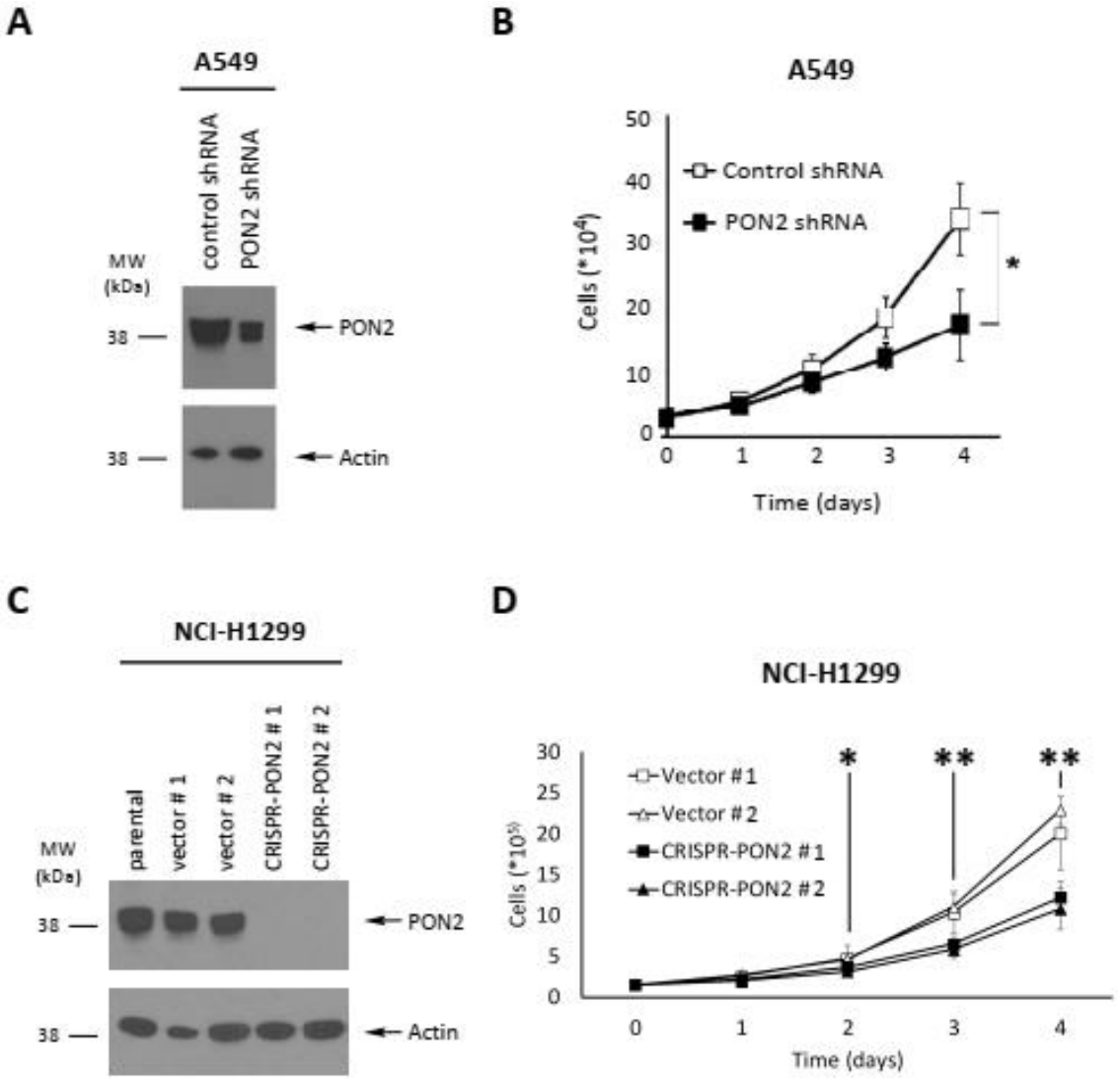


Figure 7. PON2 deficiency hinders proliferation of NSCLC cells. (A) PON2 expression in A549 cells was stably reduced by shRNA. The expression of PON2 was detected by western blot analysis. The molecular weight markers are labelled on the left**. (B)** Equal numbers of A549 control- and PON2-shRNA cells were plated in 12-well plates and cell number was determined using a hemocytometer daily over 4 days. Control-shRNA cells proliferated significantly faster than PON2-shRNA cells by day 4. Data are mean ± standard deviation (n=3); *p<0.05. student's unpaired t test. **(C)** PON2 expression was determined in parental NCI-H1299 cells, 2 clonal lines expressing a nuclease-deficient Cas9 and scrambled gRNA (vector clones #1 and #2), and 2 clonal lines expressing Cas9 and a gRNA specific to human PON2 (CRISPR-PON2 clones #1 and #2). The molecular weight markers are labelled on the left**. (D)** To assess proliferation, an equal number of cells from each line were seeded in 12-well plates and counted with a hemocytometer daily for 4 days. Data are mean ± standard deviation (n=3); *p<0.05 (both vector clones vs. both CRISPR-PON2 clones), **p<0.01 (both vector clones vs. both CRISPR-PON2 clones). student's unpaired t test.

PON2 expression is not essential for untransformed epithelial cell proliferation

Next, we sought to determine the effect of reduced PON2 expression on proliferation using non-malignant human epithelial cell lines. To accomplish this goal, we employed an experimental approach similar to that used in NSCLC proliferation experiments (Figure 7). Immortalized human bronchial epithelial (HBE) cells [56] or HEK-293Tcells were stably infected with either control shRNA lentivirus or PON2-specific shRNA lentivirus. PON2 knockdown was verified via western blot analysis (Figure 8A). The proliferation of the cells was evaluated for 4 days. In contrast to NSCLC cells, the proliferation of both HBE and HEK-293T cell lines was unaffected by diminished PON2 expression (Figure 8B). At the end of the 4-day experiment, the difference in cell number was not statistically significant for both HBE and HEK-293T cells. These data suggest that, unlike NSCLC cells, non-malignant human epithelial cells do not require PON2 for their proliferation. Furthermore, these results corroborate our *in vivo* observations that mice deficient in PON2 expression develop and grow normally (Figure 6).

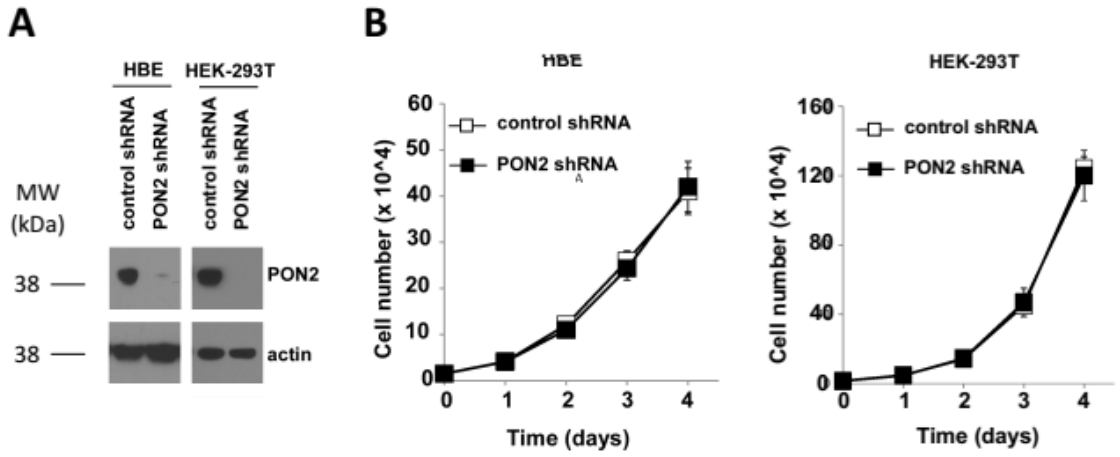


Figure 8. PON2 knockdown fails to affect untransformed human epithelial cell proliferation. Immortalized human bronchial epithelial (HBE) and human embryonic kidney-293T (HEK-293T) cells were stably infected with either a non-specific control shRNA lentivirus or a PON2-specific shRNA lentivirus. The molecular weight markers are labelled on the left. **(A)** PON2 expression was evaluated by western blot analysis. **(B)** Control- and PON2-shRNA expressing HBE and HEK-293T cells were plated at a density of $1.5*10^4$ cells in 12-well plates and cell number was counted daily for 4 days. Data are mean ± standard deviation of 3 independent experiments; n=3.

PON2 expression deficiency in NSCLC cells leads to G1 cell cycle arrest.

To further explore the biological effects of reducing PON2 expression on NSCLC cellular proliferation, we carried out cell cycle analysis using flow cytometry to evaluate the cell cycle profile of A549 cells expressing either PON2 shRNA or an empty vector (Figure 9A). We observed an increase in the percentage of PON2-deficient cells in the G1 phase of the cell cycle compared to the percentage of emtpy vector-expressing cells in the G1 phase. Additionally, there was a reduction in the percentage of cells in S phase of the cell cycle in PON2-shRNA expressing cells compared to their empty vector-expressing counterparts (Figure 9B). Based on these observations, we concluded that deficiency in PON2 expression causes cell cycle arrest at G1 phase in A549 cells.

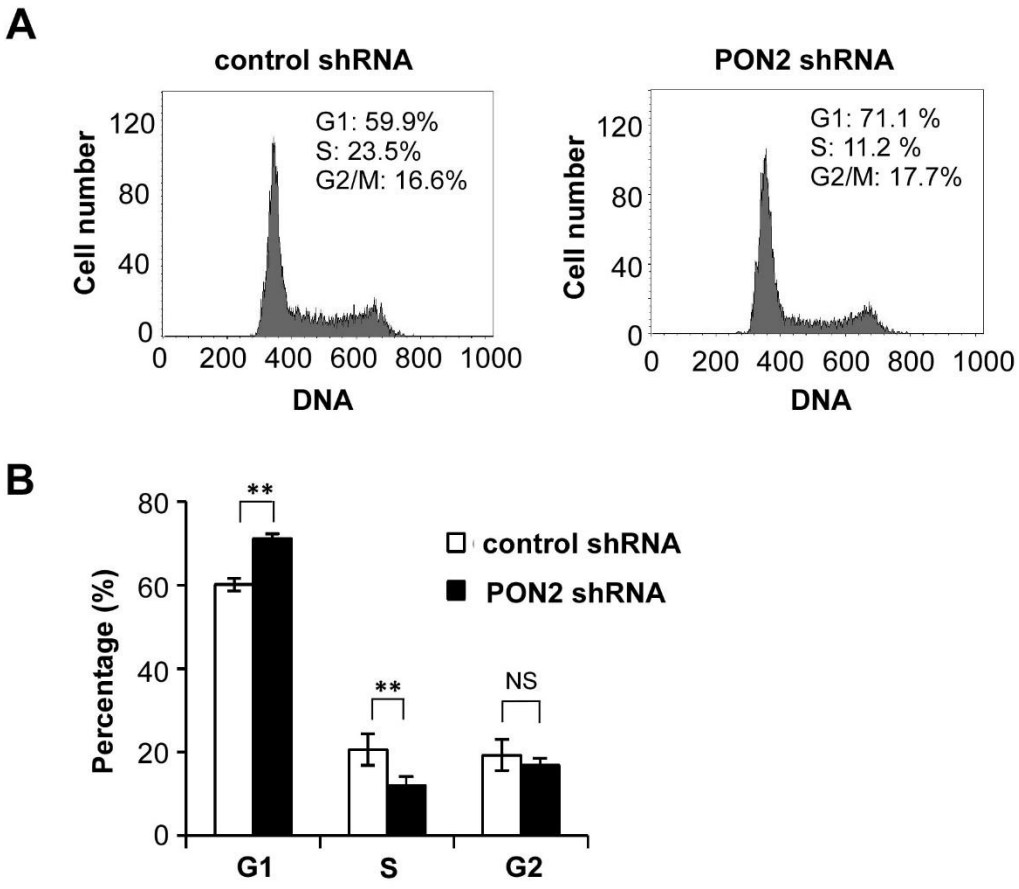


Figure 9. Deficiency in PON2 expression induces G1 cell cycle arrest of A549 cells. (A) Cell cycle profiles of A549 cells expressing normal or reduced levels of PON2 were evaluated. **(B)** The data shown in (A) were calculated and analyzed. Higher percentage of the cells in G1 phase of cell cycle was found in A549 cells with reduced PON2 expression. The data are presented as mean ± standard deviation of three independent experiments. Asterisks indicate P values of < 0.01 (**) by Student's unpaired t test. "ns", no significance.

CHAPTER 4: DISCUSSION

While previous research efforts have revealed pro-tumorigenic contributions of PON2 in a variety of cancer types [33], [38], little attention has been given to its role in NSCLC. In the present study, we investigated altered PON2 expression as it relates to non-malignant and NSCLC cellular growth using various experimental paradigms, such as genetically engineered mice, primary cells, as well as non-transformed and NSCLC cell lines with altered expression levels of PON2. We opted to use biological systems of variable complexity– cell-free systems, well-established cell lines, primary cells, mouse and human tissues, and whole-organism experiments– to thoroughly investigate PON2's potential utility as a selective target to impede NSCLC proliferation.

Previous studies have largely concluded that PON2 plays a pro-tumorigenic role through numerous proposed mechanisms. A recent large-scale study was performed by analyzing RNA- and DNA-sequencing data collected from over 10,000 patients with more than 30 types of cancer to interrogate whether PON2 mutations and expression impact patient outcomes [42]. Indeed, the researchers established a negative correlation between PON2 expression and patient prognosis across numerous types of neoplastic disease. Similarly, Witte et al. surveyed hundreds of patient samples from dozens of cancer types to discover upregulation of PON2 in numerous solid tumors, leukemias, and lymphomas [38].

Subsequent PON2 knockdown or overexpression experiments indicate that PON2 mitigates ER stress and prevents the activation of the intrinsic apoptotic pathway [54], [57] .

Despite the implication of PON2 in a multitude of cancers, research into specific tumor types has been limited. In the context of oral squamous cell carcinoma (OSCC), Kruger et al. described PON2 overexpression in tumors isolated from patients with OSCC and proposed that PON2's anti-apoptotic effects are regulated through the Wnt/β-catenin network [39], [40]. Interestingly, one published report examined the role of PON2 in pancreatic ductal adenocarcinoma and expanded its functions to preventing the cellular starvation response to promote tumor growth, anchorage-independence, and metastasis to distant sites [41]. Similarly, Bacchetti et al. characterized differences in PON2 expression using human bladder cancer tumors of various stages [58]. In addition to solid tumor studies, PON2 has also been identified in gene expression profiles of imatinib-resistant chronic myelogenous leukemia [34] and subsets of pediatric acute lymphoblastic leukemia [37]. In stark opposition to these reports, Devarajan et al. suggested a tumor suppressor function for PON2 using a xenograft model of ovarian cancer in mice [59]. While this is the only such finding of an anti-tumor effect of PON2 upregulation, it warrants further investigation.

PON2-knockout mice were previously generated by Ng and colleagues to study the protective role of PON2 during the development of atherosclerotic plaques [30]. These researchers used a gene-trapping method, which has been characterized as having the potential pitfall of allowing leaky expression of the

trapped gene [51]–[53]. Other groups have also utilized this particular mouse strain to investigate the influence of PON2 on vascular disease[28]–[30], [60]. To prevent confounding results due to aberrant PON2 expression *in vivo*, we employed a CRISPR/Cas9 approach to systemically disrupt the *PON2* locus in mice. In major organs known to harbor high levels of PON2 protein (heart, lung, small intestine, and large intestine), PON2 was undetectable by western blot in homozygous mutant mice, indicating a complete and robust knockout (Figure 3). Additionally, we assessed PON2 enzymatic activity in small intestine tissue and primary MEFs using LC-MS to detect the hydrolysis of C12 to its carboxylic acid derivative (Figure 5). The presence of C12-COOH was detected in lysates prepared from WT, but not PON2-knockout mice, which confirms that PON2 activity is lost in animals with homozygous PON2 mutations. To further determine PON2 biological function, we treated primary MEFs from wild type or PON2-knockout mice with increasing concentrations of C12, whose cytotoxicity depends on PON2 lactonase activity [1]. We observed a dose-dependent decrease in cell viability following treatment with C12 in wild type, but not PON2-knockout MEFs (Figure 4), correlating PON2 status with C12-mediated cell death.

In contrast to other reports which analyzed the physiological consequences of PON2 depletion in the presence of an atherogenic diet or other genetic manipulations [29], [30], the present study is the first to monitor mouse growth in the context of PON2 knockout *per se*. To this purpose, we found that mice lacking PON2 exhibit normal body and organ weight compared with wild type mice (Figure 3). While more extensive anatomical and physiological examinations may uncover

evidence to the contrary, these observations suggest that PON2 is not required for normal mouse growth and development.

The disposable nature of PON2 expression for non-malignant cell proliferation is further corroborated by RNAi experiments in which PON2 expression was decreased in non-transformed HBE and HEK-293T cells (Figure 8). In both cell lines, PON2 knockdown failed to affect cell proliferation *in vitro*. These observations, coupled with the knockout animal experiments (Figure 6), strengthen the notion that PON2 expression may be depleted with no disruption to normal tissue homeostasis.

In contrast with the aforementioned observations, PON2 depletion in NSCLC markedly reduced cellular proliferation (Figure 7). Using two separate approaches to disrupt PON2 expression– RNAi in A549 cells and CRISPR/Cas9-mediated knockout in NCI-H1299 cells– we observed significant reductions in cell number using both heterogeneous bulk populations (A549) and clonal cell lines (NCI-H1299). These observations indicate that PON2, while dispensable in non-transformed cells, is required for NSCLC cell proliferation.

In this study, using PON2-deficient mice, primary mouse cells, and NSCLC cell lines with altered PON2 expression, we have demonstrated that PON2 exhibits characteristics favorable for a potential therapeutic target against NSCLC: Its expression is not required for non-malignant cell proliferation, mouse growth and development is unaffected by PON2 deletion, and NSCLC cells require PON2 for their proliferation. Taken together, these properties highlight PON2 as a potentially important target in halting the proliferation of established NSCLC tumors

CHAPTER 5: CONCLUSION

In the present study, we highlighted PON2 as a unique vulnerability in NSCLC proliferation. This concept was demonstrated using PON2-knockout mice, tissue samples from patients with NSCLC, as well as normal and malignant cell lines with altered PON2 expression. The results of this study indicate that disruption of PON2, while inconsequential for non-transformed tissue, detrimentally alters NSCLC physiology in a manner that may be exploited for therapeutic benefit. Taken in conjunction with previous reports, PON2 represents an intriguing aspect of tumor biology that merits further investigation.

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ABSTRACTS

2018 **Whitt, A**., Neely, A., Jiu-Zhen, J., Li, C. Paraoxonase 2 plays a critical role in non-small cell lung carcinoma proliferation. *Research! Louisville.* 2018

2018 Meng, S., **Whitt, A.**, Eaton, J., Yaddanapudi, K., Li, C., Al-Rayyan, N., Tu, A. Exosomes from Embryonic Stem Cells against Lung Cancer as a Prophylactic Vaccine. *Research! Louisville*. 2018

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POSTERS

2018 **Whitt, A**., Neely, A., Jiu-Zhen, J., Li, C. Paraoxonase 2 plays a critical role in non-small cell lung carcinoma proliferation. *Research! Louisville.* 2018

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1. Schwarzer, C., Fu, Z., Morita, T., Whitt, A. G., Neely, A. M., Li, C., & Machen, T. E. (2015). Paraoxonase 2 serves a proapopotic function in mouse and human cells in response to the Pseudomonas aeruginosa quorum-sensing molecule N-(3-oxododecanoyl)-homoserine lactone. *Journal of Biological Chemistry, 290* (11), 7247-7258, doi:10.1074/jbc.m114.620039.

2. Zhao, G., Neely, A.M., Schwarzer, C., Lu, H., Whitt, A.G., Stivers, N.S., Burlison, J.A., White, C., Machen, T.E., Li, C. (2016). N-(3-oxo-acyl) homoserine lactone inhibits tumor growth independent of Bcl-2 proteins. *Oncotarget*, 7(5). doi:10.18632/Oncotarget.6827

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