Regulation of the Akt pathway by the aryl hydrocarbon receptor (AhR) in mouse lung fibroblasts

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March 2021

A thesis submitted to McGill University in partial fulfillment of the requirements of the Degree

Master of Science

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List of abbreviations

3'-UTR: three prime untranslated region
AhR: aryl hydrocarbon receptor
***Ahr*$^{-/-}$:** AhR knock-out
***Ahr*$^{+/-}$:** heterozygous AhR
***Ahr*$^{+/+}$:** AhR wild-type
AhRR: AhR repressor
AIP: AhR interacting protein
Akt: AKT serine/threonine kinase
ANOVA: analysis of variance
ARA9: AHR-associated protein 9
ARNT: AhR nuclear translocator
B[*a*]A: benzo[*a*]anthracene
B[*a*]P: benzo[*a*]pyrene
B[*b*]F: benzo[*b*]fluoranthene
BAD: Bcl2-associated agonist of cell death
BBP: benzyl butyl phthalate
BCA: bicinchoninic acid
bHLH: basic helix-loop-helix
BIM: Bcl-2-like protein 11
BNF: beta-naphthoflavone
BRN3: brain-specific homeobox/POU domain protein 3
CD: Crohn's disease
CH-223191: 1-methyl-N-[2-methyl-4-[2-(2-methylphenyl)diazenyl]phenyl]-1H-pyrazole-5-carboxamide
CK2: casein kinase 2
CLOCK: circadian locomotor output cycles protein kaput
CNS: central nervous system
Complete medium: minimum essential medium supplemented with 10% fetal bovine serum, 1X glutamine, 1X antibiotic-antimycotic solution, and 0.05 mg/ml gentamycin sulfate solution
COPD: chronic obstructive pulmonary disease
COUP-TFI: COUP transcription factor I
CS: cigarette smoke
CSE: cigarette smoke extract
CuSOD: copper-zinc SOD
CYP: cytochrome P450
CYP1A1: cytochrome P450 family 1 subfamily A member 1
DIM: 3,3'-diindolylmethane
DLX3: distal-less homeobox 3
DMSO: dimethyl sulfoxide
E2: 17β-estradiol
ECL: enhanced chemiluminescence
ECM: extracellular matrix
EGFR: epidermal growth factor receptors
ER: endoplasmic reticulum
ERRα1: estrogen-related receptor alpha1
ERα: estrogen receptor alpha
FAK: focal adhesion kinase
FBS: fetal bovine serum
FICZ: 6-formylindolo{3,2-b}carbazole
FOXO: forkhead box O family of transcription factor
GLS: glutathione synthetase
GPCR: G-protein-coupled receptor
GR: glutathione reductase
GSH-MEE: glutathione reduced ethyl ester
GSK-3β: glycogen synthase 3 beta
GSSG: glutathione disulfide
HAH: halogenated aromatic hydrocarbon
HEPES: 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
HIF-1α: hypoxia-inducible factor-α
HNF: hepatocyte nuclear factor
I3C: indole-3-carbinol
IAld: indole-3-aldehyde
IBD: inflammatory bowel disease
ICZ: indolo[3,2-b]carbazole
IDO: indoleamine2,3-dioxygenase

IL-1β: interleukin 1 beta
IL-22: interleukin 22
IL-8: interleukin 8
LC-MS-MS: liquid chromatography with tandem mass spectrometry
LHX3: LIM homeobox 3
MEF: mouse embryonic fibroblast
MEM: minimum essential medium
miRNA: microRNA
MLF: mouse lung fibroblasts
MNF: 3'-methoxy-4'-nitroflavone
MnSOD: manganese SOD
MS: mass spectrometry
mTOR: mammalian target of rapamycin
mTORC2: rapamycin complex 2
MTT: (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
NADH: nicotinamide adenine dinucleotide
ncRNA: non-coding RNA
NES: nuclear export signal
NF-κB: nuclear factor-κB
NLS: nuclear localization signal
NNK: 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
NPAS: neuronal PAS domain protein
NR: nuclear receptors
OD: optical density
PAH: polycyclic aromatic hydrocarbon
PARP: poly (ADP-ribose) polymerase
PAS: Per-ARNT-Sim
PBS: phosphate-buffered saline
PCB: polychlorinated biphenyl
PCDD: polychlorinated dibenzo-p-dioxin
PCDF: polychlorinated dibenzofuran
PDGF-BB: platelet-derived growth factor-BB
PDK1: phosphoinositide-dependent kinase-1
PGE2: prostaglandin E2
PHLPP: PH domain leucine-rich repeat protein phosphatase
PI3K: phosphoinositide 3-kinase
PIP3: phosphatidylinositol 3,4,5-triphosphate
PKA: protein cAMP-dependent protein kinase
PKC: protein kinase C
PKG: cGMP-dependent protein kinase
PLA2: phospholipase A2
PTEN: phosphatase and tensin homolog
RIPA: radioimmunoprecipitation
ROS: reactive oxygen species
RTK: receptor tyrosine kinase
SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis
siRNA: small interfering RNA
SNP: single nucleotide polymorphism
SOD: superoxide dismutase
Sp1: specificity protein-1
STAT6: signal transducer and activator of transcription 6
STRING: Search Tool for Retrieval of Interacting Genes
TAD: transactivation domain
TCDD: 2,3,7,8-tetrachlorodibenzo-p-dioxin
TCF/Lef: T-cell factor/lymphoid enhancer factor
TDO: trypthophan2,3-dioxygenase
TGF-β1: transforming growth factor β1
TiO_2: titanium dioxide
TiPARP: TCDD-inducible poly ADP-ribose polymerase
TMF: 6,2',4'-trimethoxyflavone
TMT: tandem mass tag
TNFα: tumor necrosis factor-alpha
TTP: tristetraprolin
UC: ulcerative colitis
UV: ultraviolet
XAP2: hepatitis B virus X-activating protein 2
XME: xenobiotic-metabolizing enzyme
XRE: xenobiotic responsive element
α-NF: α-naphthoflavone
β-NF: β-napthoflavone
γGLCL: γ-glutamyl-L-cysteine ligase

Abstract

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that is highly expressed in the lungs. While the role of AhR in mediating the toxic responses of dioxin has been extensively studied in the past decades, its importance in the regulation of normal cell physiology has only been appreciated recently. Studies have shown that AhR promoted cell cycle progression and suppressed apoptosis by upregulating essential pro-survival pathways in tumor cells. In this study, we hypothesized that AhR enhances the activation of the AKT serine/threonine kinase (Akt) pathway in mouse lung fibroblasts (MLFs) to promote cell survival. The aims of this study are (i) to determine if and how AhR regulates Akt activity in MLFs, and (ii) to determine how the regulation of Akt by AhR affects its downstream targets and cell viability. Contrary to our expectation, we found that serum-starved AhR knock-out (*Ahr*$^{-/-}$) MLFs exhibited significantly higher basal Akt phosphorylation than the wild-type MLFs. On the other hand, AhR did not affect Akt phosphorylation in MLFs exposed to growth factors and AhR ligands. By utilizing a phosphoinositide 3-kinase (PI3K) inhibitor, LY294002, we found that basal Akt phosphorylation in MLFs was dependent on PI3K. We then examined factors that regulate the PI3K/Akt pathway, such as oxidative stress, p85α and phosphatase and tensin homolog (PTEN) expression; however increased Akt phosphorylation in *Ahr*$^{-/-}$ MLFs was not likely caused by these factors. The reason for increased Akt phosphorylation in the *Ahr*$^{-/-}$ MLFs is unclear but could be a compensatory cell survival mechanism during serum starvation, as we observed significantly decreased cell viability in *Ahr*$^{-/-}$ MLFs treated with LY294002 but not in wild-type MLFs. Using a mass spectrometry (MS)-based approach, we identified several proteins that were differentially phosphorylated in the *Ahr*$^{-/-}$ MLFs compared with the wild-type. Many of these proteins are involved in the regulation of extracellular matrix (ECM), focal adhesion and cytoskeleton remodeling; some of them also

show potential interactions with the Akt. In conclusion, we found that *Ahr* ablation increased basal Akt phosphorylation in MLFs; however, more efforts are needed to understand the mechanism through which AhR regulates Akt phosphorylation. Our results also indicate that AhR may modulate the phosphorylation of a variety of proteins in MLFs. These findings will help advance our understanding of the physiological functions of AhR.

Résumé

Le récepteur d'aryl hydrocarbone (AhR) est un facteur de transcription activé par un ligand qui est fortement exprimé dans les poumons. Bien que le rôle de l'AhR dans la médiation des réponses toxiques de la dioxine a été largement étudié au cours des dernières décennies, son importance dans la régulation de la physiologie cellulaire normale n'a été appréciée que récemment. Des études ont montré que l'AhR favorisait la progression du cycle cellulaire et supprimait l'apoptose en régulant à la hausse les voies de survie essentielles dans les cellules tumorales. Dans cette étude, nous avons émis l'hypothèse que l'AhR améliore l'activation de la voie AKT sérine / thréonine kinase (Akt) dans les fibroblastes pulmonaires de souris (MLFs) pour favoriser la survie cellulaire. Les objectifs de cette étude sont (i) de déterminer si et comment l'AhR régule l'activité d'Akt dans les MLFs, et (ii) de déterminer comment la régulation d'Akt par l'AhR affecte ses cibles en aval et la viabilité cellulaire. Contrairement à nos attentes, nous avons constaté que les MLFs déficientes en l'AhR (*Ahr* $^{-/-}$) présentaient une phosphorylation d'Akt basale significativement plus élevée que les MLFs de type sauvage après la privation de sérum. En revanche, l'AhR n'a pas affecté la phosphorylation d'Akt dans les MLFs exposés à des facteurs de croissance et des ligands de l'AhR. En utilisant un inhibiteur de la phosphoinositide 3-kinase (PI3K), LY294002, nous avons découvert que la phosphorylation d'Akt basale dans les MLFs était dépendante de la PI3K. Nous avons ensuite examiné les facteurs qui régulent la voie PI3K / Akt, tels que le stress oxydatif et l'expression de le p85α et de la phosphatase et de homologue de la tensine (PTEN); cependant, l'augmentation de la phosphorylation d'Akt dans les MLFs *Ahr* $^{-/-}$ n'était probablement pas causée par ces facteurs. La raison de l'augmentation de la phosphorylation d'Akt dans les MLFs *Ahr* $^{-/-}$ n'est pas claire, mais pourrait être un mécanisme compensatoire de survie des cellules pendant la privation de sérum, car nous avons observé une diminution

significative de la viabilité cellulaire dans les MLFs *Ahr* $^{-/-}$ traités avec LY294002 mais pas dans les MLFs de type sauvage. En utilisant une approche basée sur la spectrométrie de masse (MS), nous avons identifié plusieurs protéines qui étaient différentiellement phosphorylées dans les MLFs *Ahr* $^{-/-}$ par rapport au type sauvage. Beaucoup de ces protéines sont impliquées dans la régulation de la matrice extracellulaire (ECM), l'adhésion focale et le remodelage du cytosquelette; certains d'entre eux présentent également des interactions potentielles avec l'Akt. En conclusion, nous avons constaté que l'ablation d'*Ahr* augmentait la phosphorylation basale d'Akt dans les MLFs; cependant, des efforts supplémentaires sont nécessaires pour comprendre le mécanisme par lequel l'AhR régule la phosphorylation d'Akt. Nos résultats indiquent également que l'AhR peut moduler la phosphorylation d'une variété de protéines dans les MLFs. Ces résultats aideront à faire progresser notre compréhension des fonctions physiologiques de l'AhR.

Acknowledgements

I would like to express my sincere thanks to my supervisor, Dr. Carolyn Baglole, for her constant guidance and support throughout my master's degree. I would also like to thank my advisors Dr. Carlos Telleria, Dr. Simon Rousseau and Dr. Sabah Hussain for providing useful advice and insightful comments on my project. Additionally, I would like to thank Dr. Edith Zorychta for her assistance with the editing of my thesis. I would also like to acknowledge Dr. Jean-Francois Trempe for his assistance with the mass spectrometry proteomics data analysis. Moreover, I would like to acknowledge faculty members of the Meakins-Christie Laboratories, particularly Dr. David Eidelman, for providing valuable feedback throughout my project. I would also like to show my gratitude to the Department of Pathology and the Meakins-Christie Laboratories for providing me financial support and various learning opportunities. I also wish to thank all the members of the Baglole laboratory who have given me meaningful feedback on my experimental design and provided me with technical assistance. Finally, I would like to offer my special thanks to my family for all the support they gave me during my graduate study.

CHAPTER 1: Introduction

1.1 The aryl hydrocarbon receptor (AhR)

1.1.1 AhR structure

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that belongs to the basic helix-loop-helix (bHLH)/Per-ARNT-Sim (PAS) superfamily (Hahn, 2002). The bHLH-PAS protein family is comprised of transcription factors that contain a bHLH domain followed by tandem PAS domains (Fribourgh and Partch, 2017). These transcription factors form heterodimers consisting of class I and class II subunits in order to function (Fribourgh and Partch, 2017). The expression or activity of the class I bHLH-PAS proteins are usually highly regulated by tissue- or environmental-specific factors whereas the class II proteins are ubiquitously-expressed (Fribourgh and Partch, 2017). The AhR is a class I bHLH-PAS protein (Fribourgh and Partch, 2017). Other bHLH-PAS class I proteins include hypoxia-inducible factor-α (HIF-1α, a master regulator of cellular responses to low oxygen levels), circadian locomotor output cycles protein kaput (CLOCK, a regulator of circadian rhythms) and neuronal PAS domain proteins (NPAS, a regulator of synapse development and synaptic plasticity) (Ploski *et al.*, 2011; Fribourgh and Partch, 2017). In the canonical AhR signaling pathway, activated AhR translocates into the nucleus where it dimerizes with AhR nuclear translocator (ARNT), a class II protein of the bHLH-PAS family, in order to bind to the xenobiotic responsive elements (XREs) on DNA (Monostory *et al.*, 2009).

The protein structure of AhR contains several functional domains, including a transactivation domain (TAD) near the C-terminal region, a bHLH domain near the N-terminus and a PAS domain in the middle (**Figure 1.1**) (Monostory *et al.*, 2009). The bHLH

region is a protein structural motif that is characterized by two α-helices connected by a loop (Jones, 2004). The bHLH domain of the AhR harbors the nuclear localization signal (NLS), the nuclear export signal (NES) and a basic region near the N-terminus that is required for DNA binding (Monostory *et al.*, 2009). Both the bHLH and the PAS domains mediate AhR/ARNT heterodimerization and the binding of the chaperone protein heat shock protein 90 (hsp90) to AhR in the cytoplasm (Monostory *et al.*, 2009). The PAS domain of the AhR is also involved in ligand binding, whereas the TAD contains binding sites for transcriptional coregulators (Monostory *et al.*, 2009).

Figure 1.1. Diagrammatic representation of the AhR protein structure. The AhR consists of a basic helix-loop-helix (bHLH) domain, a Per-ARNT-Sim (PAS) domain and a transactivation domain (TAD).

1.1.2 The AhR signaling pathways

1.1.2.1 AhR agonists and antagonists

The classical AhR signaling pathway (the AhR genomic pathway) can be activated by many exogenous and endogenous compounds that share similar structural and chemical properties, namely hydrophobicity, planarity and aromaticity (Flaveny *et al.*, 2009). Because most AhR ligands are hydrophobic and thus have very limited water solubility, they enter the cell across the plasma membrane by passive diffusion (Stevens, Mezrich and Bradfield, 2009). However, although AhR ligands have similar characteristics, they also exhibit structural diversity and differ in their binding affinities for the AhR, and different AhR ligands may

induce unique AhR conformational changes that can lead to various functional outcomes, depending on the context (Denison *et al.*, 2011). Therefore, it is important to know and understand the variety of substances that can act as AhR ligands. Based on their origin, AhR agonists can be divided into several classes, including endogenous, microorganism-derived, dietary and environmental.

1.1.2.1.1 Endogenous AhR agonists

Endogenous AhR agonists are chemicals that can be endogenously synthesized in higher organisms and act as AhR activators (Stejskalova, Dvorak and Pavek, 2011). These include indigoids, heme metabolites, arachidonic acid metabolites and tryptophan derivatives, among others (Nguyen and Bradfield, 2008; Stejskalova, Dvorak and Pavek, 2011). Indigo and indirubin have been identified in bovine serum and human urine (Adachi *et al.*, 2001). They are suggested to be potent AhR agonists and have EC_{50} values of 5 and 0.2 nM, respectively, in yeast (Adachi *et al.*, 2001). However, their role as physiological AhR ligands has been argued because the average levels of these indigoids in vertebrate tissue were below the level that can significantly upregulate AhR target gene expression (Guengerich *et al.*, 2004; Nguyen and Bradfield, 2008).

The heme metabolites bilirubin, biliverdin and hemin can induce activation of the AhR signaling pathway (Sinal and Bend, 1997). Bilirubin was the most potent AhR activator among the three and can interact with AhR directly, whereas biliverdin and hemin may need to be converted to bilirubin via heme metabolism pathways to activate AhR (Sinal and Bend, 1997). However, these heme derivatives are low-affinity AhR ligands as they require concentrations in the micromolar range to induce AhR activation (Sinal and Bend, 1997). Although normal human plasma contains 5 to 20 μM of bilirubin, 99% of it is bound to albumin and a small

amount of it is bound to the red blood cell membrane and other serum protein (Cashore, 1998). Therefore, the level of free bilirubin in the plasma is not likely to induce AhR-dependent gene expression under normal physiological conditions (Sinal and Bend, 1997; Cashore, 1998).

The ability of some arachidonic acid metabolites to activate AhR has also been studied. One of these, lipoxin A4, was suggested to be a high-affinity AhR agonist and is able to induce AhR activation at physiological nanomolar levels (Schaldach, Riby and Bjeldanes, 1999). Interestingly, lipoxin A4 does not contain aromatic rings as do classical AhR ligands and bears a negative charge at physiological pH, indicating that planarity and aromaticity are not the prerequisites for being AhR ligands and there are other considerations important in controlling the binding of molecules to the AhR (Nguyen and Bradfield, 2008). Lipoxin B4 is a structurally similar relative of lipoxin A4; however, lipoxin B4 exhibited no appreciable affinity for the AhR, suggesting very specific structural requirements for AhR ligands (Schaldach, Riby and Bjeldanes, 1999). Several prostaglandins have also been found to be AhR agonists (*i.e.* prostaglandin B_2, D_2, $F_{3\alpha}$, G_2, H_1, and H_2) (Seidel *et al.*, 2001). However, prostaglandins are relatively weak AhR ligands that require concentrations in the micromolar range to induce significant AhR activation and are unlikely to induce AhR activation at their biological levels in the blood (Seidel *et al.*, 2001). Nonetheless, prostaglandins secreted by non-parenchymal liver cells can reach a local concentration of 5-10 μM in the proximity of hepatocytes, which is possible to activate the AhR (Seidel *et al.*, 2001).

Another major class of endogenous AhR ligands is tryptophan derivatives, notably 6-formylindolo{3,2-b}carbazole (FICZ) and kynurenine (Rannug *et al.*, 1995; DiNatale *et al.*, 2010). FICZ can be produced by UV-induced or H_2O_2-mediated oxidation of tryptophan whereas kynurenine is a catabolic metabolite of tryptophan whose production is mediated by

the enzymes trypthophan2,3-dioxygenase (TDO) or indoleamine2,3-dioxygenase (IDO) (Rannug *et al.*, 1995; Mezrich *et al.*, 2010; Cella and Colonna, 2015). Both FICZ and kynurenine are high-affinity AhR ligands and can stimulate AhR activation within the nanomolar range, which is physiologically attainable (Rannug *et al.*, 1995; DiNatale *et al.*, 2010; Smirnova *et al.*, 2016).

1.1.2.1.2 Microorganism-derived AhR agonists

Some bacteria that are found in the soil and human gut, including strains of *Escherichia coli* and *Bacillus subtilis*, can produce tryptophan (Sarsero, Merino and Yanofsky, 2000; Hong *et al.*, 2009; Liu, Duan and Wu, 2016). Many bacteria and fungi can also metabolize tryptophan into AhR ligands (Cella and Colonna, 2015). For example, *Malassezia furfur*, a fungus that causes conditions such as pityriasis versicolor and seborrheic dermatitis (Thayikkannu, Kindo and Veeraraghavan, 2015), secretes tryptophan-derived AhR agonists including malassezin and indolo[3,2-b]carbazole (ICZ) (Gaitanis *et al.*, 2008). AhR activation induced by these substances is suspected to be involved in the pathogenesis of *M. furfur*-caused dermatoses (Gaitanis *et al.*, 2008). Evidence to support this includes the finding that malassezin-treated human melanocytes underwent apoptosis (Krämer *et al.*, 2005).

Moreover, increasing evidence suggests that the AhR pathway regulates the inflammatory response by sensing bacterial products. Several lactobacilli species, including *Lactobacillus bulgaricus* and *L. reuteri*, can activate the AhR, possibly by producing AhR ligands such as indole-3-aldehyde (IAld), that inhibits colitis by the induction of prostaglandin E_2 (PGE_2) and contributes to gut mucosal immune homeostasis via the induction of interleukin 22 (IL-22), respectively (Takamura *et al.*, 2011; Zelante *et al.*, 2013). AhR can also be activated by certain pathogen-produced pigmented virulence factors to mediate antibacterial

defense, such as the phenazines from *Pseudomonas aeruginosa* and the naphthoquinone phthiocol from *Mycobacterium tuberculosis* (Moura-Alves *et al.*, 2014). Notably, phenazines and naphthoquinones are not only released by *P. aeruginosa* and *M. tuberculosis* but are produced by a number of prokaryotes present at mucosal barriers and in the environment (Cella and Colonna, 2015).

1.1.2.1.3 Dietary AhR agonists

Many AhR ligands also come from naturally-occurring dietary compounds, including indole-3-carbinol (I3C) derivatives and flavonoids (Nguyen and Bradfield, 2008). I3C, present in many cruciferous vegetables such as broccoli, cabbage, cauliflower and Brussels sprouts, can form numerous di- and trimeric condensation derivatives in the acidic environment of the stomach which can act as AhR agonists; these include ICZ, 3,3'-diindolylmethane (DIM) and 2-(indol-3-ylmethyl)-3,3'-diindolylmethane (Bjeldanes *et al.*, 1991). Natural flavonoids are a class of phenolic compounds ubiquitously found in fruits and vegetables (Ross and Kasum, 2002). Flavonoids such as chrysin, galangin, baicalein, genistein, daidzein and apigenin activate the AhR (Zhang, Qin and Safe, 2003). Many of these phytochemicals have beneficial health effects such as anti-inflammation and anticancer; however, how these effects are related to AhR activation needs further investigation (Nguyen and Bradfield, 2008; Busbee *et al.*, 2013).

1.1.2.1.4 Environmental AhR agonists

The best-characterized AhR ligands are synthetic toxicants and environmental pollutants, namely polycyclic aromatic hydrocarbons (PAHs) and halogenated aromatic hydrocarbons (HAHs) (Bohonowych and Denison, 2007). PAHs are a class of aromatic compounds containing two or more fused benzene rings (Wang *et al.*, 2016). PAHs are formed

during incomplete combustion of organic matter and can be of both natural (*e.g*. forest fires and volcanic eruptions) and anthropogenic (*e.g*. burning of fossil fuels, wood, garbage, and tobacco) origins (Stejskalova, Dvorak and Pavek, 2011). Many PAHs are procarcinogens, including benzo[*a*]pyrene (B[*a*]P), benzo[*a*]anthracene (B[*a*]A), and benzo[*b*]fluoranthene (B[*b*]F) (Fan, 2014), and induce the expression of xenobiotic-metabolizing enzymes (XMEs), particularly cytochrome P450 (CYP) enzymes through the AhR pathway (Burczynski and Penning, 2000). XMEs can metabolically activate these PAHs to become genotoxic carcinogens capable of forming DNA adducts (Shimada and Fujii-Kuriyama, 2004; Alexandrov, Rojas and Rolando, 2006).

HAHs are chemicals that consist of benzene rings with one or more halogens (*i.e.* chlorine, fluorine, bromine and iodine) attached (Sparling, 2016). HAHs encompass many environmental pollutants such as polychlorinated dibenzo-p-dioxins (PCDDs; dioxins), polychlorinated dibenzofurans (PCDFs) and polychlorinated biphenyls (PCBs; Kopf and Walker, 2010). PCDDs and PCDFs are usually formed as by-products of industrial processes, such as bleaching of wood pulp and chlorination of phenols, whereas PCBs are used in industrial applications such as capacitor and transformer oils, hydraulic fluids, lubricating oils, and as plasticizers, due to their non-flammability, chemical stability, high boiling point and electrical insulating properties (Stejskalova, Dvorak and Pavek, 2011). Similar to PAHs, many HAHs are genotoxic (Fu *et al.*, 1999; Shaikh *et al.*, 2008). Dioxins are a class of chlorinated aryl hydrocarbons composed of two benzene rings connected through two oxygen atoms (Sparling, 2016). Dioxin is also commonly used as the synonym for one specific dioxin, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). TCDD is a metabolically-inert chemical that is a potent and the best-characterized AhR ligand (Simones, Shepherd and Moser, 2010).

Dioxins are environmentally and biologically persistent organic toxicants (Poland and Knutson, 1982). Importantly, studies have confirmed that the toxicity of dioxins is dependent on their interaction with the AhR (Fernandez-Salguero *et al.*, 1996; Bunger *et al.*, 2008). TCDD, being the most toxic of the 22 isomers of dioxins (Ayres *et al.*, 1985), is used as a reference to evaluate the toxicity of persistent organic pollutants that have related structures and elicit their toxicity by activating the AhR pathway (Simones, Shepherd and Moser, 2010).

1.1.2.1.5AhR antagonists

In addition to agonists, there are also antagonists that can inhibit AhR activation. Most AhR ligands are partial agonists that exhibit lower intrinsic activity than that of full agonists. However, partial agonists can still bind to AhR and may impair the effect of full agonists on AhR activation, thus behaving as functional AhR antagonists (Stejskalova, Dvorak and Pavek, 2011). Besides, ligand- and species-selective properties of some AhR antagonists have been observed. For example, 3'-methoxy-4'-nitroflavone (MNF) and 6,2',4'-trimethoxyflavone (TMF) inhibit TCDD- but not beta-naphthoflavone- (BNF, a PAH-like chemical) induced AhR activation (Zhao *et al.*, 2010). Moreover, although MNF behaves as an antagonist in rat and mouse cells, it is a full agonist in guinea pig cells, which may be the result of inter-species differences in the AhR ligand-binding domain (Henry and Gasiewicz, 2008; Zhao *et al.*, 2010). CH-223191 (1-methyl-N-[2-methyl-4-[2-(2-methylphenyl)diazenyl]phenyl]-1H-pyrazole-5-carboxamide) is a synthetic AhR antagonist that preferentially inhibits the ability of TCDD and related HAHs to bind to and activate AhR. However, CH-223191 does not antagonize the effects of other AhR agonists including BNF, certain PAHs (*e.g.* dibenz(a,h)anthracene, benzo(k)fluoranthene and B[*a*]A), certain synthetic flavonoids and indirubin (Zhao *et al.*,

2010). Thus, significant differences exist in the binding of different groups of chemicals to the AhR (Zhao *et al.*, 2010).

1.1.2.2 Canonical AhR signaling

The primary role of the canonical AhR signaling pathway was originally believed to be the sensing and metabolizing of xenobiotic chemicals (Kawajiri and Fujii-Kuriyama, 2017). As illustrated in **Figure 1.2**, in the absence of ligands, the AhR rests in a cytosolic complex consisting of an hsp90 dimer, an AhR interacting protein (AIP; also known as AHR-associated protein 9 [ARA9] and hepatitis B virus X-activating protein 2 [XAP2]) and the co-chaperone p23 (Nukaya *et al.*, 2010; Esser and Rannug, 2015). Among these cytosolic AhR binding partners, the hsp90 dimer stabilizes unliganded AhR in the cytoplasm, sequestering AhR intrinsic DNA binding ability and maintaining the proper conformation of the AhR ligand-binding domain (Pongratz, Mason and Poellinger, 1992). AIP retains the AhR in the cytoplasm and protects it from ubiquitin-mediated degradation (Monostory *et al.*, 2009). p23 stabilizes the AhR-hsp90 interaction, prevents AhR degradation and modulates AhR ligand responsiveness (Kazlauskas, Poellinger and Pongratz, 1999; Monostory *et al.*, 2009; Pappas *et al.*, 2018).

Upon ligand binding, the conformation of the AhR changes, resulting in exposure of the NLS and subsequent translocation into the nucleus (**Figure 1.2**) (Esser and Rannug, 2015). There, the AhR exchanges its chaperones for ARNT and binds to the XRE core sequence (5'-GCGTG-3') near the promoters of target genes, thereby inducing gene transcription (Lo and Matthews, 2012). Many genes that are targeted by AhR encode for phase I and II enzymes and proteins regulating the inflammatory response, such as the cytochrome P450 family 1 subfamily A member 1 (*CYP1A1*), tumor necrosis factor-alpha (TNFα) and interleukin 1 beta

(IL-1β) (Esser and Rannug, 2015). One of the other target genes induced by canonical AhR signaling is the AhR repressor (*AhRR*), which suppresses AhR activity by binding to ARNT, and therefore forms a negative feedback loop to dampen AhR activation (Larigot *et al.*, 2018). Ligand-bound AhR also mediates the induction of a mono-ADP-ribosyltransferase, TCDD-inducible poly ADP-ribose polymerase (TiPARP), which negatively regulates AhR activity by interfering with AHR/ARNT-XRE interaction and promoting AhR degradation (MacPherson *et al.*, 2013). After activation, AhR is exported from the nucleus to the cytoplasm and degraded through a proteasome-dependent mechanism (Monostory *et al.*, 2009). Activation of the canonical AhR signaling pathway is most well-known for mediating the toxic effects of dioxins, such as hepatotoxicity and immunosuppression (Bunger *et al.*, 2003; Stockinger, 2009).

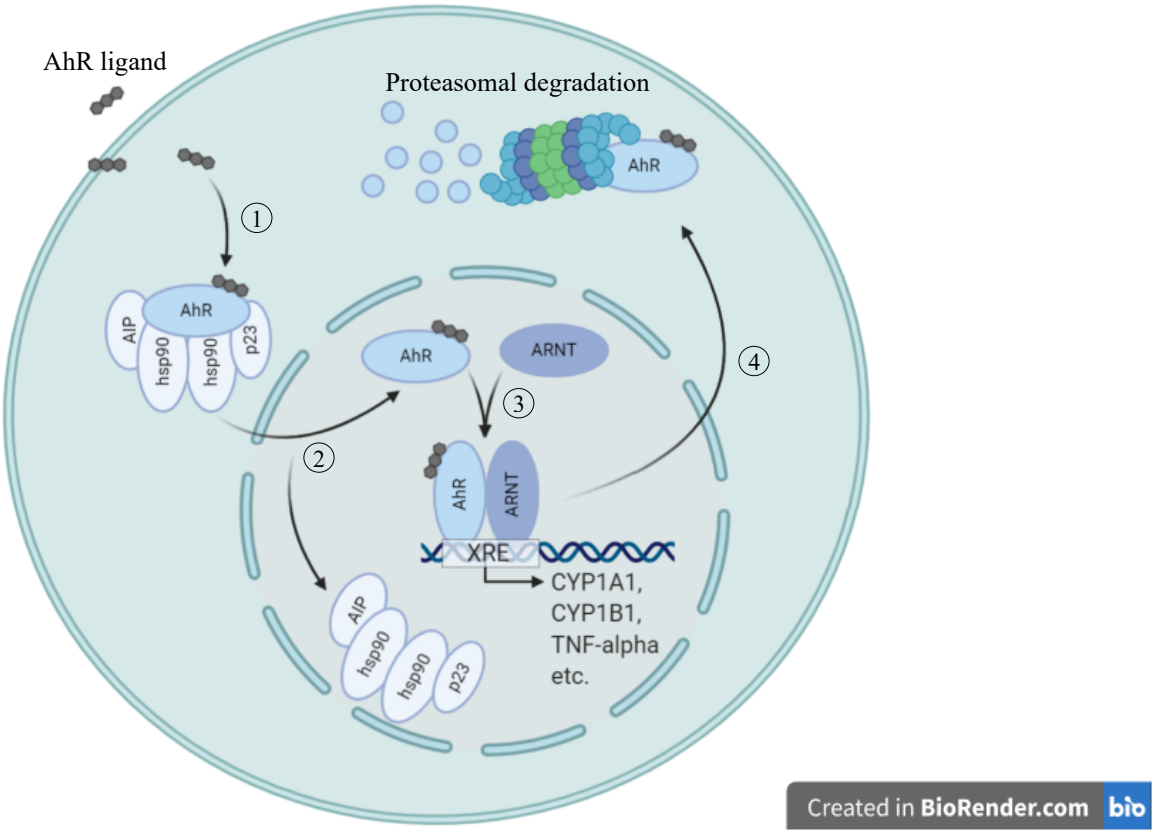

Figure 1.2. Diagrammatic representation of the canonical AhR signaling pathway. Inactive AhR resides in the cytosol in a protein complex consisting of an hsp90 homodimer, AIP and p23. AhR ligands are usually lipophilic and diffuse through the plasma membrane to bind the AhR (1). Upon ligand binding, AhR translocates into the nucleus and dissociates from its chaperone complex (2). Then, the AhR dimerizes with ARNT and binds to an XRE on the DNA to induce target gene transcription, resulting in cell-specific transcriptome changes (3). Finally, AhR is exported from the nucleus into the cytoplasm and degraded via the proteasomal degradation pathway (4). AhR, aryl hydrocarbon receptor; AIP, AhR interacting protein; hsp90, heat shock protein 90; ARNT, AhR nuclear translocator; XRE, xenobiotic responsive element; CYP1A1, cytochrome P450 family 1 subfamily A member 1; CYP1B1, cytochrome P450 family 1 subfamily B member 1; TNF-alpha, tumor necrosis factor-alpha.

1.1.2.3 Non-canonical AhR signaling

Not all AhR-mediated effects can be explained by the canonical signaling pathway (Wright et al., 2017). Indeed, a number of non-canonical AhR signaling pathways have been identified, including ligand-independent signaling, interactions with non-classical DNA-binding partners, regulation of non-coding RNAs (ncRNAs) and triggering non-transcriptional events in the cytoplasm; these will be further discussed in the following sections. The identification of non-canonical AhR signaling pathways is important for deepening our understanding of the mechanism of action of the AhR in the regulation of both toxic responses as well as essential physiological processes (Wright et al., 2017).

1.1.2.3.1 Ligand-independent AhR signaling

The AhR can regulate extracellular matrix (ECM) formation, cell cycle progression, and gene expression independent of its activation by either exogenous or endogenous ligands (Chang et al., 2007). For example, the rate of cell proliferation is significantly higher in AhR-expressing cells than in AhR-null cells (Chang et al., 2007). Chang et al. (2007) found that in AhR-null mouse embryonic fibroblasts (MEFs), the expression of growth-promoting genes (*e.g.* cyclin and cyclin-dependent kinase genes) were significantly decreased whereas that of the growth-arresting genes (*e.g.* ECM-related genes, transforming growth factor β1 (TGF-β1), and cyclin-dependent kinase inhibitor genes) were increased (Chang et al., 2007). It was also suggested that the AhR down-regulates *TGF-β1* mRNA in AhR-positive cells by regulating the RNA-binding protein tristetraprolin (TTP), which is responsible for mRNA destabilization (Chang et al., 2007).

1.1.2.3.2 Crosstalk between AhR and other transcription factors

Upon activation, AhR can also crosstalk with multiple transcription factor pathways to produce downstream effects not involved in the canonical AhR-XRE signaling. For example, the AhR can interact directly with estrogen receptor alpha (ERα), COUP transcription factor I (COUP-TFI; critical for normal embryogenesis) and estrogen-related receptor alpha1 (ERRα1; important in the regulation of cell differentiation and genes involved in energy production) (Bonnelye *et al.*, 1997; Villena *et al.*, 2007), in a ligand-specific manner (Klinge, Kaur and Swanson, 2000). For instance, β-napthoflavone (β-NF)-occupied AhR binds more strongly to ERα, COUP-TFI and ERRα1 compared with unoccupied AhR as well as AhR occupied by the partial antagonist α-naphthoflavone (α-NF) (Klinge, Kaur and Swanson, 2000).

The AhR also has extensive crosstalk with the Wnt/β-catenin pathway. The Wnt/β-catenin pathway regulates the transcription of genes involved in cell proliferation, differentiation, adhesion and migration (Yang *et al.*, 2016). When both the Wnt/β-catenin and the AhR pathway are activated, β-catenin can bind to the AhR-ARNT complex in the nucleus and act as a co-activator to enhance the induction of *CYP1A1* (Schulthess *et al.*, 2015). On the other hand, activated AhR functions as a substrate recognition subunit of the E3 ubiquitin ligase cullin 4B to mediate the degradation of sex steroid receptors (Ohtake *et al.*, 2007) and β-catenin in the nucleus (Kawajiri *et al.*, 2009).

Furthermore, AhR can interact with the nuclear factor-κB (NF-κB) subunits RelA and RelB. The NF-κB family of transcription factors play essential roles in the regulation of various cellular processes such as adaptive and innate immunity, cell differentiation, proliferation and apoptosis, and has been recognized as crucial players in many diseases

including cancer (Vogel *et al.*, 2007; Vogel and Matsumura, 2009). The RelA subunit is involved in the canonical NF-κB signaling while the RelB is involved in the alternative NF-κB pathway whose exact role has remained elusive (Vogel *et al.*, 2007). However, nuclear AhR can associate with RelB and bind to the RelB/AhR-response elements in the promoter of genes such as interleukin 8 (IL-8) (Vogel and Matsumura, 2009), suggesting a role in the regulation of inflammation. AhR has also been shown to physically interact with RelA in breast cancer cells and bind to NF-κB elements on DNA to induce c-*myc* gene expression (Kim *et al.*, 2000).

1.1.2.3.3 Regulation of ncRNA by AhR

The AhR also regulates many ncRNAs to affect cellular processes. microRNAs (miRNAs) are short ncRNAs that play important roles in the regulation of gene expression (Fabian, Sonenberg and Filipowicz, 2010), and usually function by binding to the three prime untranslated region (3'-UTR) of the target mRNA to destabilize the mRNA or interfere with translation efficiency (Fabian, Sonenberg and Filipowicz, 2010). In the absence of exogenous ligands, *Ahr* ablation reduced the basal expression of miRNAs such as miR-137, miR-196a, miR-133b, miR-96, miR-205 and miR-29a (Rogers *et al.*, 2017). There was also an approximately 4-fold increase of miR-96 after 4 weeks of chronic exposure to cigarette smoke in the lungs of AhR knock-out ($Ahr^{-/-}$) mice, but not that of the AhR heterozygous ($Ahr^{+/-}$) mice; the mechanism through which AhR regulated miR-96 is activation-independent (Rogers *et al.*, 2017). This suggests that AhR can regulate the levels of certain miRNAs in response to inhaled pollutants and may play a role in the development of diseases induced by these substances. Moreover, many of the miRNAs that can be regulated by the AhR are potential tumor suppressors or have cytoprotective effects. For instance, miR-137 suppressed tumor

growth and metastasis in clear cell renal cell carcinoma (Wang *et al.*, 2018), and miR-133b inhibited cell proliferation, migration and invasion of esophageal squamous cell carcinoma (Zeng *et al.*, 2019). Therefore, the regulation of these miRNAs by the AhR in relation to cancer progression is worth further investigation. Moreover, AhR-dependent regulation of miR-196a expression attenuated lung fibroblast apoptosis, suggesting that the ablation of AhR may render the cells more susceptible to environmental insults (Hecht *et al.*, 2014).

1.1.2.3.4 Functions of cytoplasmic AhR

After activation, the AhR also elicits non-genomic functions in the cytoplasm. Following exposure to TCDD, there was a rapid increase in intracellular calcium in multiple cell types which triggered subsequent signaling cascades involved in mitogenesis and inflammation (Puga, Nebert and Carrier, 1992; Tannheimer *et al.*, 1997; Matsumura, 2009). In addition, the proto-oncogene tyrosine-protein kinase c-Src is a binding partner of the cytoplasmic AhR complex (Enan and Matsumura, 1996; Ghotbaddini *et al.*, 2017). After a ligand is bound to AhR, c-Src will be activated and released from the AhR complex (Enan and Matsumura, 1996; Ghotbaddini *et al.*, 2017). As a consequence, activation of c-Src via the AhR pathway can modulate cell morphology, migration, integrin recycling and inflammation (Ghotbaddini *et al.*, 2017).

AhR can also shuttle between the cytoplasm and the nucleus in the absence of exogenous ligands, and this nuclear-cytoplasmic shuttling is influenced by cell density, with higher cell density being associated with increased cytoplasmic localization of AhR (Ikuta, Kobayashi and Kawajiri, 2004). This suggests that the AhR responds to cell-cell contact signals and may have implications for contact inhibition, adhesion, migration and tumor metastasis. Furthermore, TCDD triggers release from contact inhibition in the WB-F344 rat

liver epithelial stem-like cell line and the Madin-Darby canine kidney epithelial cell line via AhR-dependent, but ARNT-independent, induction of the transcription factor JunD and subsequent upregulation of the proto-oncogene cyclin A (Weiss *et al.*, 2008). The AhR also associates with caveolin-1 (a protein involved in directional cell migration) at the cell membrane microdomains to modulate caveolin-1 distribution and mobilization in mouse dermal fibroblasts (Takahashi, Kawamura and Uemura, 2013).

1.1.3 Regulation of AhR expression

The AhR is an evolutionarily-conserved protein that is expressed in all tissues and is especially abundant in the first-line defense organs such as the skin, liver, gut and lungs (Harper, Riddick and Okey, 2006). Changes in AhR protein levels may lead to altered functional outcomes and therefore have important implications in health and disease. Therefore, it is important to understand the mechanisms that regulate AhR expression. First, expression of the AhR can be regulated by transcription factors. In multiple species, including human, mouse and rat, there are regulatory motifs at the 5'-flanking regions of the *Ahr* gene which are binding sites for numerous transcription factors; these transcription factors include hepatocyte nuclear factor (HNF), distal-less homeobox 3 (DLX3), brain-specific homeobox/POU domain protein 3 (BRN3), signal transducer and activator of transcription 6 (STAT6), T-cell factor/lymphoid enhancer factor (TCF/Lef), LIM homeobox 3 (LHX3) and nuclear receptors (NR) (Harper, Riddick and Okey, 2006). There are also multiple GC islands and specificity protein-1 (Sp1) binding sites within both mouse and human *AhR* genes (Harper, Riddick and Okey, 2006). Evidence supports that Sp1 binds to the *Ahr* promoter region to increase *Ahr* expression in mouse aorta endothelial cells (Tang *et al.*, 2010).

The level of AhR can also be regulated by ncRNAs. For example, miR-203 bound to the 3'-UTR of *AhR* mRNA and reduced the levels of both *AhR* mRNA and protein (Li *et al.*, 2014). miR-124 (Zhao *et al.*, 2016) and miR-375 (Bleck *et al.*, 2013) inhibited AhR protein expression and promoted *AhR* mRNA degradation, respectively. Many other pathways that crosstalk with the AhR also influence AhR expression. For example, *AhR* is a target gene of the Wnt/β-catenin signaling pathway and activation of this pathway increased AhR expression (Schneider, Branam and Peterson, 2014). The NF-κB subunit RelB also plays an important role in the regulation of AhR expression. Here, knock-out or knock-down of RelB significantly decreased *AhR* mRNA and protein levels (Iu *et al.*, 2017).

In addition, single nucleotide polymorphisms (SNPs) and epigenetic modifications of the human *AHR* gene may also influence AhR expression. Cauchi *et al.* (2001) identified the presence of three SNPs of *AHR* in a French male population: 2417A/G (allele frequency: 0.005), 157G/A (allele frequency: 0.25) and 1721G/A (allele frequency: 0.086) (Cauchi *et al.*, 2001). The 1721G/A and 2417A/G SNPs are located in exon 10 and will lead to Arg554Lys and Met786Val substitutions, respectively, whereas the 157G/A SNP is localized in the 5'-untranslated region (Cauchi *et al.*, 2001). This study also examined the association of these polymorphisms with CYP1A1 inducibility and lung cancer risk (Cauchi *et al.*, 2001). Neither 1721G/A nor 157G/A showed significant associations with CYP1A1 inducibility and lung cancer, while the 2417A/G allelic variant was so rare that the number of subjects included in this study was not sufficient to analyze the phenotypic associations (Cauchi *et al.*, 2001). Nonetheless, in a subsequent study in a population of Caucasians, the AhR^{554}Lys/Lys homozygotes have significantly reduced *AHR* and *CYP1B1* mRNA expression in white blood cells compared with that of the AhR^{554}Arg/Arg homozygotes (Helmig *et al.*, 2011).

Besides genetic mechanisms, several epigenetic pathways modulate AhR expression. For example, long-term estrogen exposure of MCF-7 human breast cancer cells increased AhR expression and activity due to decreased trimethylation at histone 3, lysine 27 in the *AhR* proximal promoter region (Englert *et al.*, 2012). On the other hand, in MCF-7 cells cultured without 17β-estradiol (E_2) supplementation, AhR expression was slowly lost (Englert *et al.*, 2012). AhR expression can also be regulated by DNA methylation. Mulero-Navarro *et al.* found that acute lymphoblastic leukemia REH cells showed hypermethylation of the CpG islands in the promoter of the *AhR* gene, which resulted in reduced Sp1 binding and decreased AhR expression (Mulero-Navarro *et al.*, 2006)

1.1.4 Dysregulation of AhR expression in human disease

Changes in AhR expression have been observed in many chronic inflammatory diseases as well as cancers. The AhR is overexpressed in many types of neoplastic diseases, including actinic keratosis, Bowen disease, cutaneous squamous cell carcinoma (Pan *et al.*, 2018), lung adenocarcinoma, small cell lung carcinomas (Lin *et al.*, 2003) and gastric cancer (Peng *et al.*, 2009). In several chronic inflammatory diseases, there is reduced AhR expression. For example, in patients with multiple sclerosis, a disabling chronic immune-mediated demyelinating disease of the central nervous system (CNS), there was less circulating AhR than that of the healthy controls (Rothhammer *et al.*, 2016). Ulcerative colitis (UC) and Crohn's disease (CD) are two types of inflammatory bowel disease (IBD) (Qiu and Zhou, 2013). The level of AhR was found to be significantly lower in the inflamed mucosa of CD patients compared with that of the controls (Monteleone *et al.*, 2011). Although there was no significant difference in AhR expression between UC patients and the controls overall, there was a markedly reduced AhR level in some UC samples (Monteleone *et al.*, 2011). Another

disease with an inflammatory etiology is chronic obstructive pulmonary disease (COPD), an umbrella term referring to diseases such as emphysema and chronic bronchitis and a leading cause of morbidity and mortality around the world (Mannino, 2005). Cigarette smoke (CS) is the primary cause of COPD (Samet, 2013) and can contribute to COPD pathogenesis by promoting inflammation, oxidative stress, endoplasmic reticulum (ER) stress and lung structural cell death, *etc*. (Kelsen, 2016; Pezzuto *et al.*, 2019). Our laboratory has previously found that the lung fibroblasts derived from COPD patients expressed significantly lower AhR than both never-smokers and smokers; however, a causal relationship between reduced AhR level and COPD pathogenesis was unclear (Sarill *et al.*, 2015).

1.1.5 Physiological functions of AhR

Besides its role in the regulation of xenobiotic metabolism, the AhR also has other important endogenous physiological functions that are not well understood. These include observations that the AhR is necessary for proper liver and vascular development, immune system function, as well as cell growth and differentiation (Fernandez-Salguero *et al.*, 1995, 1997; Shimba *et al.*, 2002). Importantly, the AhR promotes cell proliferation and survival, potentially through exogenous ligand-independent mechanisms (Yin *et al.*, 2016). Previous studies and unpublished data from our laboratory also suggest that the AhR can suppress CS-induced apoptosis, inflammation, oxidative stress, ER stress and mitochondrial dysfunction in pulmonary cells (Baglole *et al.*, 2008a; De Souza *et al.*, 2011; Sarill *et al.*, 2015). Although many chemicals present in the cigarette smoke extract (CSE) such as B[*a*]P are classic AhR agonists, some of the cytoprotective effects of AhR against CSE were independent of XRE binding (Baglole *et al.*, 2008a; De Souza *et al.*, 2011; Sarill *et al.*, 2015). The mechanism through which the AhR produced beneficial effects is still not known and is likely not a

consequence of the canonical AhR signaling. Based on recent evidence suggesting that AhR can non-canonically interact with many other pathways, it is possible that the AhR produces its cytoprotective effects by regulating important pro-survival pathways, such as the AKT serine/threonine kinase (Akt) pathway.

1.2 The Akt pathway

The Akt pathway is well-known for its role in promoting cell survival, proliferation, protein synthesis, *etc*. (Szymonowicz *et al.*, 2018). The canonical pathway that leads to Akt activation begins with growth factors binding to and activating cell surface receptors such as receptor tyrosine kinases (RTKs) (Carnero and Paramio, 2014). Once activated, these receptors stimulate the activation of class IA phosphoinositide 3-kinases (PI3Ks), which are heterodimers consisting of a p110 catalytic subunit and a p85 regulatory subunit(Carnero and Paramio, 2014). Activated PI3Ks then phosphorylate phosphatidylinositol to produce phosphatidylinositol 3,4,5-triphosphate (PIP3) (Sheridan and Downward, 2013). This will result in the colocalization of inactive Akt and the enzymes that phosphorylate it to the cell membrane, leading to subsequent Akt activation (Carnero and Paramio, 2014). Akt has two main activating phosphorylation sites, namely the threonine 308 residue (T308) which is phosphorylated by phosphoinositide-dependent kinase-1 (PDK1) and the S473 residue which is phosphorylated by mammalian target of rapamycin complex 2 (mTORC2), and the phosphorylation of both results in the full activation of Akt (Sheridan and Downward, 2013). There are also proteins that negatively regulate the activation of the Akt pathway. For example, the phosphatase and tensin homolog (PTEN) dephosphorylates the phospholipid PIP3, and PH domain leucine-rich repeat protein phosphatase (PHLPP) directly dephosphorylates and inhibits Akt (Gao, Furnari and Newton, 2005; Sheridan and Downward, 2013). Reactive

oxygen species (ROS) also modulate the PI3K/Akt pathway by negatively regulating the activity of phosphatases such as PTEN (Chetram *et al.*, 2013; Uranga, Katz and Salvador, 2013; Koundouros and Poulogiannis, 2018).

1.3 Crosstalk between the AhR and Akt pathways

Many of the downstream effects of Akt overlap with the physiological functions of the AhR, particularly promoting cell proliferation and attenuating apoptosis. Previous studies have found that the AhR can enhance tumor cell growth and suppress apoptosis through the Akt pathway. For example, Wu *et al.* (2007) found that the AhR reduced serum starvation-, hydrogen peroxide-, and ultraviolet (UV) irradiation-induced apoptosis in a mouse hepatoma cell line due to increased Akt activation (Wu *et al.*, 2007). Other studies have found that AhR ligands such as TCDD and benzyl butyl phthalate (BBP) activate Akt in an AhR-dependent manner to promote cell proliferation, survival, migration and invasion in multiple non-small cell lung cancer and hepatocellular carcinoma cell lines (Tsai *et al.*, 2014; Ye *et al.*, 2018). It is suggested that ligand-activated AhR, while still in the cytosol, may translocate to the cell membrane and promote the activation of GPCRs or the nonreceptor tyrosine kinase Src to stimulate downstream signaling pathways, including Akt (Tsai *et al.*, 2014; Ye *et al.*, 2018). Moreover, Bölck *et al.* (2014) found that B[*a*]P increased Akt phosphorylation in human keratinocytes after 5 min of treatment through unknown mechanisms (Bölck *et al.*, 2014). Contrary to these previous findings, a recent *in vivo* study conducted by Moreno-Marín *et al.* found that there was more Akt activity in $Ahr^{-/-}$ mouse liver tissue, accompanied by higher PI3K activity, increased downstream target phosphorylation and enhanced cell proliferation compared with that in the AhR wild-type ($Ahr^{+/+}$) mice; the mechanism was poorly-described

(Moreno-Marín *et al.*, 2018). The sum of these studies also suggests that the regulation of Akt by AhR may be cell type specific.

It was also found that the AhR regulates many downstream targets of Akt, although whether the AhR produces these effects by directly regulating Akt is not known. For example, glycogen synthase 3 beta (GSK-3β) is a common target of Akt that is inhibited by Akt through phosphorylation at the S9 residue (Carnero and Paramio, 2014). Studies utilizing human orbital fibroblasts and A549 human lung adenocarcinoma cells found that knock-down of AhR decreased the activity of GSK-3β, which promoted myofibroblast formation and enhanced epithelial-mesenchymal transition, respectively (Woeller *et al.*, 2016; Li *et al.*, 2017). Interestingly, GSK-3β normally inhibits cell cycle progression, increases intrinsic apoptosis and promotes inflammation, which seems to be the opposite of AhR (Sun *et al.*, 2009; Carnero and Paramio, 2014). One possible explanation for this is that under certain conditions, the interactions between the AhR and GSK-3β pathways mainly control cell differentiation and have little effect on regulating cell proliferation, apoptosis and inflammation. Akt is also known to phosphorylate and inhibit the forkhead box O family of transcription factors (FOXOs) (De Souza *et al.*, 2011). Although FOXOs can induce the transcription of pro-apoptotic proteins such as the BCL-2-like protein 11 (BIM) (De Souza *et al.*, 2011), FOXOs are also important in the induction of antioxidants-notably superoxide dismutase (SOD) (Park and Bae, 2016). Our previous studies found that there was less manganese SOD (MnSOD) and copper-zinc SOD (CuSOD) in $Ahr^{-/-}$ cells compared to $Ahr^{+/+}$ cells (De Souza *et al.*, 2011). Whether this was caused by altered FOXO or Akt activities needs to be determined. To summarize, the AhR has complex interactions with the Akt pathway and its downstream targets, and the mechanisms through which the AhR regulates these may be species-, cell type-

and condition-specific. As our laboratory has previously identified important physiological roles of AhR in lung fibroblast (Baglole *et al.*, 2008a; De Souza *et al.*, 2011; Sarill *et al.*, 2015), it is worthwhile to investigate how the AhR interacts with the Akt pathway in lung fibroblasts.

CHAPTER 2: Hypothesis and aims

Hypothesis:

AhR enhances the activation of the Akt pathway in mouse lung fibroblasts to promote cell survival.

Aims:

1. Determine if the AhR regulates Akt activity at the basal level, in the presence of select growth factors, or the presence of AhR ligands in mouse lung fibroblasts (MLF).
2. Determine through which mechanism the AhR regulates the Akt pathway and its functional implications.

CHAPTER 3: Methods

3.1 Chemicals and reagents

B[*a*]P was purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich). Recombinant mouse platelet-derived growth factor-BB (PDGF-BB) was purchased from STEMCELL Technologies (Cambridge, MA) and dissolved in double-distilled water. LY294002 was purchased from Cell Signaling Technology (Danvers, MA) and dissolved in DMSO. Glutathione reduced ethyl ester (GSH-MEE) was purchased from Sigma-Aldrich and dissolved in double-distilled water. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) solution (#H0887) purchased from Sigma-Aldrich (St. Louis, MO). Urea and NaCl were purchased from (Thermo Fisher Scientific, MA).

3.2 Cell culture

Primary MLFs were derived from $Ahr^{+/+}$ and $Ahr^{-/-}$ C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) as previously described (Baglole *et al.*, 2005). Fibroblasts were cultured in Gibco® minimum essential medium (MEM) (Thermo Fisher Scientific: #11095080) supplemented with 10% fetal bovine serum (FBS) (Wisent Bioproducts: #080150), 1X Gibco® GlutaMAX™ (Thermo Fisher Scientific: #35050061), 1X antibiotic-antimycotic solution (Wisent Bioproducts: #450115) and 0.05 mg/ml gentamycin sulfate solution (complete medium) (Wisent Bioproducts: #450135), and maintained at 37°C in humidified air with 5% CO_2. Cells used in each experiment were within two passage differences and no fibroblasts exceeding passage ten were used. All cells were grown to

approximately 80-90% confluence and starved in serum-free medium for 18 h before conducting the experiments unless otherwise indicated. AhR protein expression in all MLFs used in the experiments has been verified at least once using Western blot.

3.3 Preparation of 2% CSE

CSE was generated from filtered research-grade cigarettes (3R4F) obtained from the Kentucky Tobacco Research Council (Lexington, KT) as previously described (Carp and Janoff, 1978; Baglole *et al.*, 2006). Briefly, smoke from one cigarette was bubbled through 15 ml of serum-free medium and the optical density (OD) of the CSE was measured using a SmartSpec™ Plus spectrophotometer (Bio-Rad Laboratories, Hercules, CA). An OD of 0.65 at 320 nm was considered to represent 100% CSE. Then, the CSE was passed through a Thermo Scientific™ Titan3™ syringe filter with a pore size of 0.45 μm (Thermo Fisher Scientific: #44525-NP) and diluted to 2% CSE using serum-free medium.

3.4 Cellular protein extraction and quantification

Upon sample collection, media was removed, and the fibroblasts were washed with 1X phosphate-buffered saline (PBS). Total cellular protein was extracted using Pierce™ radioimmunoprecipitation (RIPA) buffer (Thermo Fisher Scientific) supplemented with 1X cOmplete™ Mini protease inhibitor cocktail (Roche) and 1X PhosSTOP™ phosphatase inhibitor cocktail (Roche). Protein quantification was performed using a Thermo Scientific™ Pierce™ bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific) according to

manufacturer's instructions and the absorbance was measured with an iMark™ microplate reader (Bio-Rad Laboratories, Hercules, CA).

3.5 Western blot

Up to twenty micrograms of total cellular protein was mixed with Laemmli SDS sample buffer (Alfa Aesar) and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electro-blotted onto Immun-Blot® PVDF membranes (Bio-Rad Laboratories, Hercules, CA) as described (Mahmood and Yang, 2012). Membranes were blocked with 5% Oxoid^{TM} skim milk powder (Thermo Fisher Scientific) in 1X PBS and 0.1% Tween-20 (PBS-T) (Thermo Fisher Scientific) for one hour at room temperature. After that, membranes were incubated with a primary antibody **(Table 3.1)** on a rocking platform for either one hour at room temperature or overnight at 4 °C. All antibodies were diluted in 5% non-fat milk in PBS-T. After incubation with the primary antibody, the membranes were incubated with the corresponding secondary antibody for one hour at room temperature on a rocking platform. The secondary antibodies used in the experiments were horseradish peroxidase-conjugated anti-rabbit IgG (Cell Signaling Technology #7074; 1:10000) and anti-mouse IgG (Cell Signaling Technology #7076; 1:10000). Detection of protein levels was performed using enhanced chemiluminescence (ECL) and the signals were captured using a ChemiDoc™ MP Imaging System (Bio-Rad Laboratories, Hercules, CA). Densitometric analysis was performed using Image Lab™ Software (Bio-Rad Laboratories, Hercules, CA) and target phospho-protein levels were normalized to the corresponding total protein levels. In each experiment, the data are presented as the fold-changes relative to the wild-type control groups unless otherwise indicated.

Table 3.1. Primary antibodies used for western blot.

Target protein	Specificity	Dilution	Product information
p-Akt	p-Akt (S473)	1:2000	Cell Signaling Technology #4060
Akt	Total Akt	1:1000	Cell Signaling Technology #2920
p-GSK-3β	p-GSK-3β (S9)	1:1000	Cell Signaling Technology #5558
GSK-3β	Total GSK-3β	1:1000	Cell Signaling Technology #12456
p-FOXO3a	p-FOXO3a (S253)	1:1000	Abcam #ab154786
FOXO3a	Total FOXO3a	1:1000	Cell Signaling Technology #2497
p-Akt substrates	p-Akt substrate motif (RXXS*/T*)	1:1000	Cell Signaling Technology #9614
AhR	AhR	1:2000	Enzo Life Sciences #BML-SA210
PTEN	PTEN	1:1000	Cell Signaling Technology #9559
p85α	p85α	1:500	Cell Signaling Technology #13666
Tubulin	Tubulin	1:10000	Sigma-Aldrich #T7816

3.6 Transfection

Ahr$^{+/+}$ MLF were grown to 60-80% confluency and transfected with 100 nM of small interfering RNA (siRNA) against p85α (PI 3-kinase p85α siRNA (m): Santa Cruz Biotechnology #sc-36218) or non-targeting control siRNA (Control siRNA-D: Santa Cruz Biotechnology #sc-44232) using Lipofectamine® RNAiMAX (Thermo Fisher Scientific) and siRNA transfection MEM (Santa Cruz Biotechnology) according to manufacturer's instructions. Cells were incubated in the transfection medium containing the siRNA-lipid complexes for one hour, then, serum-containing medium was added. Twenty-four hours later, the cells were serum-starved for 18 h prior to analysis.

3.7 MTT assay

Equivalent numbers of fibroblasts were seeded in triplicates in 96-well cell culture plates. Cells were allowed to settle overnight then serum-starved for 18 h prior to conducting the experiments. For the viability experiment, *Ahr*$^{+/+}$ and *Ahr*$^{-/-}$ MLFs were either treated with

50 μM LY294002 alone or an equivalent volume of DMSO for 24 h, 0.25% or 0.5% CSE alone for 22 h, or pre-treated with 50 μM LY294002 for 2 h then co-treated with 0.25% or 0.5% CSE for 22 h. After incubation with the treatments, 10 μL of 5 mg/ml MTT (in 1X PBS) was added to each well and the plates were incubated for 4 h at 37 °C. Following this, the plates were centrifuged, and the media were removed. The precipitate in each well was then dissolved by adding 200 μL of DMSO and the absorbance was read with a BioRad microplate reader at 510 nm.

3.8 Quantitative mass spectrometry (MS) for phosphoproteomics

Ahr$^{+/+}$ and *Ahr*$^{-/-}$ MLFs were cultured in T175 cell culture flasks and serum-starved for 18 h before sample collection. Cells were rinsed and harvested in 20 mM HEPES supplemented with 150 mM NaCl. The cell-containing solution was then centrifuged to remove the HEPES saline. The cells were resuspended in 8M urea (pH 8) dissolved in 20 mM HEPES supplemented with protease and phosphatase inhibitors. The samples were sonicated briefly on ice to assist membrane breakdown. Cell debris was cleared by centrifugation. The final samples contained pooled protein extracts from two different *Ahr*$^{+/+}$ and *Ahr*$^{-/-}$ mouse subjects, respectively. Equal amount of protein from the samples was sent to Institut de recherches cliniques de Montréal (IRCM, Montréal) for mass spectrometry analysis. Briefly, approximately 290 μg of protein from each sample was digested by trypsin and the peptides were labeled with tandem mass tag (TMT). The phosphopeptides were enriched using titanium dioxide (TiO_2) and a liquid chromatography with tandem mass spectrometry (LC-MS-MS) was performed. Peptide and protein identification were carried out using Mascot (Matrix Science) by searching against a *Mus musculus* reference proteome database (UniProt, the

Universal Protein resource, http://www.uniprot.org). The results were then analyzed by Proteome Discoverer 2.4 (Thermo Scientific™). Identified proteins with an abundance ratio adjusted p-value (KO/WT) less than 0.05 and a Mascot score greater than 20 were selected for further analysis. Peptide sequences of each identified protein were also manually inspected. Information used for functional annotation of each protein was obtained from the Uniprot database. To perform a protein-protein interaction network analysis of the identified upregulated/downregulated proteins, the Search Tool for Retrieval of Interacting Genes (STRING, https://string-db.org) database was employed. Active interaction sources used for constructing the network include text mining, experiments, databases and co-expression. The species was limited to "*Mus musculus*" and a minimum required interaction score was set to 0.4.

3.9 Statistical analysis

Statistical analysis was performed using Prism 6 (v. 6.02; GraphPad Software, San Diego, CA). Statistical significance between two groups was analyzed by two-tailed parametric t-tests assuming unequal variance. Statistical differences among the means of more than two groups were determined using one-way analysis of variance (ANOVA) followed by a Tukey's multiple comparisons test. The interrelationships between more than two independent variables were assessed using two-way ANOVA followed by a Holm-Šídák test unless otherwise indicated. In all cases, values with $P<0.05$ were considered significantly different.

CHAPTER 4: Results

4.1 FBS and PDGF-BB significantly induced Akt and GSK-3β phosphorylation independent of the AhR

To determine the extent to which the AhR controls Akt phosphorylation in response to growth stimuli, we treated *Ahr*$^{+/+}$ and *Ahr*$^{-/-}$ MLFs with FBS and PDGF-BB, a growth factor that is well-known to induce Akt activation in fibroblasts (Franke *et al.*, 1995). The phosphorylation of GSK-3β, one of the key downstream targets of Akt (Manning and Toker, 2017), was also evaluated. To first determine if the AhR regulates the Akt pathway in MLFs growing in the complete medium (*i.e.* contains 10% FBS), cells were either serum-starved or cultured in the complete medium; serum-starved cells were used as negative controls for Akt activation. After 0.5, 3 and 6 h of changing either the serum-free or the complete medium, cells were collected and the phosphorylation of Akt and GSK-3β was determined by western blot. In both *Ahr*$^{+/+}$ and *Ahr*$^{-/-}$ MLFs, supplying the cells with 10% FBS significantly induced Akt phosphorylation after 30 minutes, which then gradually decreased over the 3- and 6-hour time frame (**Figure 4.1A**). However, the phosphorylation of Akt induced by FBS did not differ significantly between the *Ahr*$^{+/+}$ and *Ahr*$^{-/-}$ cells at the 30-minute timepoint (**Figure 4.1A**). There was also a slight increase in Akt phosphorylation over time in serum-starved cells (**Figure 4.1A**). FBS also increased GSK-3β phosphorylation, but the increase did not reach statistical significance (**Figure 4.1B**). Moreover, there was no significant difference in GSK-3β phosphorylation between *Ahr*$^{+/+}$ and *Ahr*$^{-/-}$ MLFs (**Figure 4.1B**).

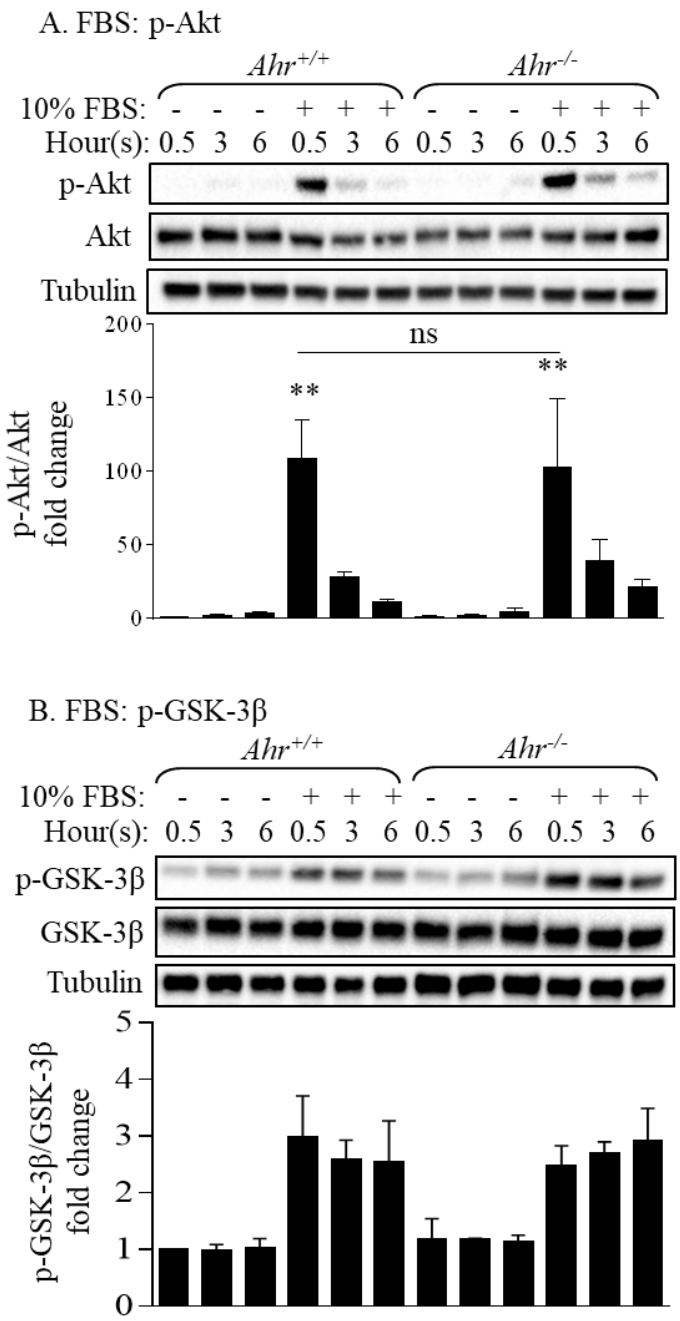

Figure 4.1. Effects of AhR expression on Akt and GSK-3β phosphorylation in MLFs exposed to 10% FBS. *Ahr*$^{+/+}$ and *Ahr*$^{-/-}$ MLFs were either serum-starved for 18 h or maintained in 10% FBS. Cellular protein was extracted after 0.5, 3 and 6 h of changing either the serum-free or 10% FBS containing medium for western blot analysis of **(A)** Akt and **(B)** GSK-3β phosphorylation. Depicted blots are representative of three independent experiments. Densitometric values of the phosphorylated proteins were normalized to the values of their corresponding total proteins and were converted to fold changes relative to that of the control *Ahr*$^{+/+}$ cells. Results are expressed as mean ± SEM (** $p<0.01$; ns, not statistically significant).

Next, we used PDGF-BB as another Akt pathway stimulator to determine if there was a difference in Akt and GSK-3β phosphorylation between $Ahr^{+/+}$ and $Ahr^{-/-}$ cells. In both $Ahr^{+/+}$ and $Ahr^{-/-}$ MLFs, PDGF-BB induced rapid and significant Akt phosphorylation within 5 minutes that persisted for at least 30 minutes (**Figure 4.2A** and **4.2B**). There was also a significant increase in GSK-3β phosphorylation caused by PDGF-BB (**Figure 4.2C**). Although PDGF-BB significantly induced Akt and GSK-3β phosphorylation in MLFs, the level of induction did not differ between $Ahr^{+/+}$ and $Ahr^{-/-}$ cells. Thus, the AhR does not control Akt or GSK-3β phosphorylation induced by growth factors in MLFs.

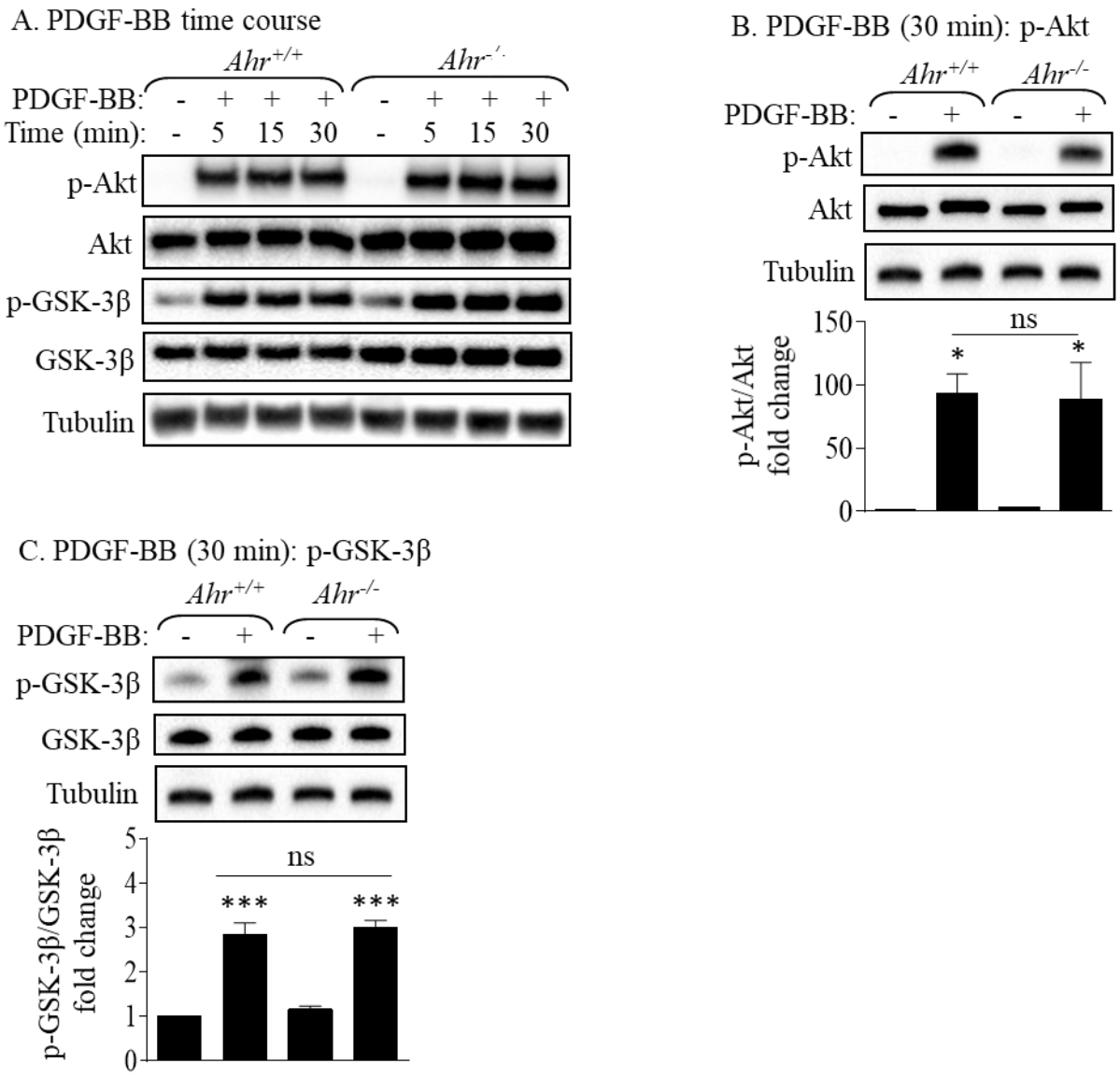

Figure 4.2. Effects of AhR expression on Akt and GSK-3β phosphorylation in MLFs exposed to PDGF-BB. Serum-starved *Ahr*$^{+/+}$ and *Ahr*$^{-/-}$ MLFs were either treated with 20 ng/ml PDGF-BB or the vehicle control (H_2O) and cellular protein was collected for western blot analysis. **(A)** Representative western blot of Akt and GSK-3β phosphorylation time course after PDGF-BB stimulation. **(B, C)** *Ahr*$^{+/+}$ and *Ahr*$^{-/-}$ MLFs were treated with PDGF-BB for 30 min and **(B)** Akt and **(C)** GSK-3β phosphorylation was determined by western blot. Depicted blots are representative of three independent experiments. Densitometric values of the phosphorylated proteins were normalized to the values of their corresponding total proteins and were converted to fold changes relative to that of the control *Ahr*$^{+/+}$ cells. Results are expressed as mean ± SEM (* $p<0.05$; *** $p<0.001$; ns, not statistically significant).

4.2 CSE did not cause Akt and GSK-3β phosphorylation

CS is a complex mixture of toxicants that contains numerous AhR ligands (Baglole *et al.*, 2008b). Our laboratory previously found that AhR protects lung fibroblasts against 2% CSE-induced cell death (De Souza *et al.*, 2011; Hecht *et al.*, 2014). Since Akt is part of a pro-survival pathway that has been shown to protect the cells from CSE-induced apoptosis (Park *et al.*, 2008, 2013; Manning and Toker, 2017), we next evaluated whether the AhR activates Akt in response to cigarette smoke. Akt phosphorylation is usually an immediate early event (0–2 h) after exposure to environmental stimuli that precedes other cellular responses (Park *et al.*, 2008). Therefore, we determined Akt phosphorylation in MLFs exposed to CSE at early time points. In response to 2% CSE for 5 minutes, there was a slight but non-significant increase in Akt phosphorylation in the $Ahr^{+/+}$ cells but not in the $Ahr^{-/-}$ cells (**Figure 4.3A**). There was also no change in the amount of GSK-3β phosphorylation in MLFs exposed to CSE (**Figure 4.3B**). We also evaluated whether more prolonged exposure to CSE altered the phosphorylation status of Akt in an AhR-dependent manner. However, exposure to 2% CSE for up to 6 hours did not significantly change the phosphorylation of either Akt or GSK-3β in MLFs (**Figure 4.3C** and **4.3D**). Therefore, we conclude that exposure to CS does not impact activation of the Akt pathway in MLFs.

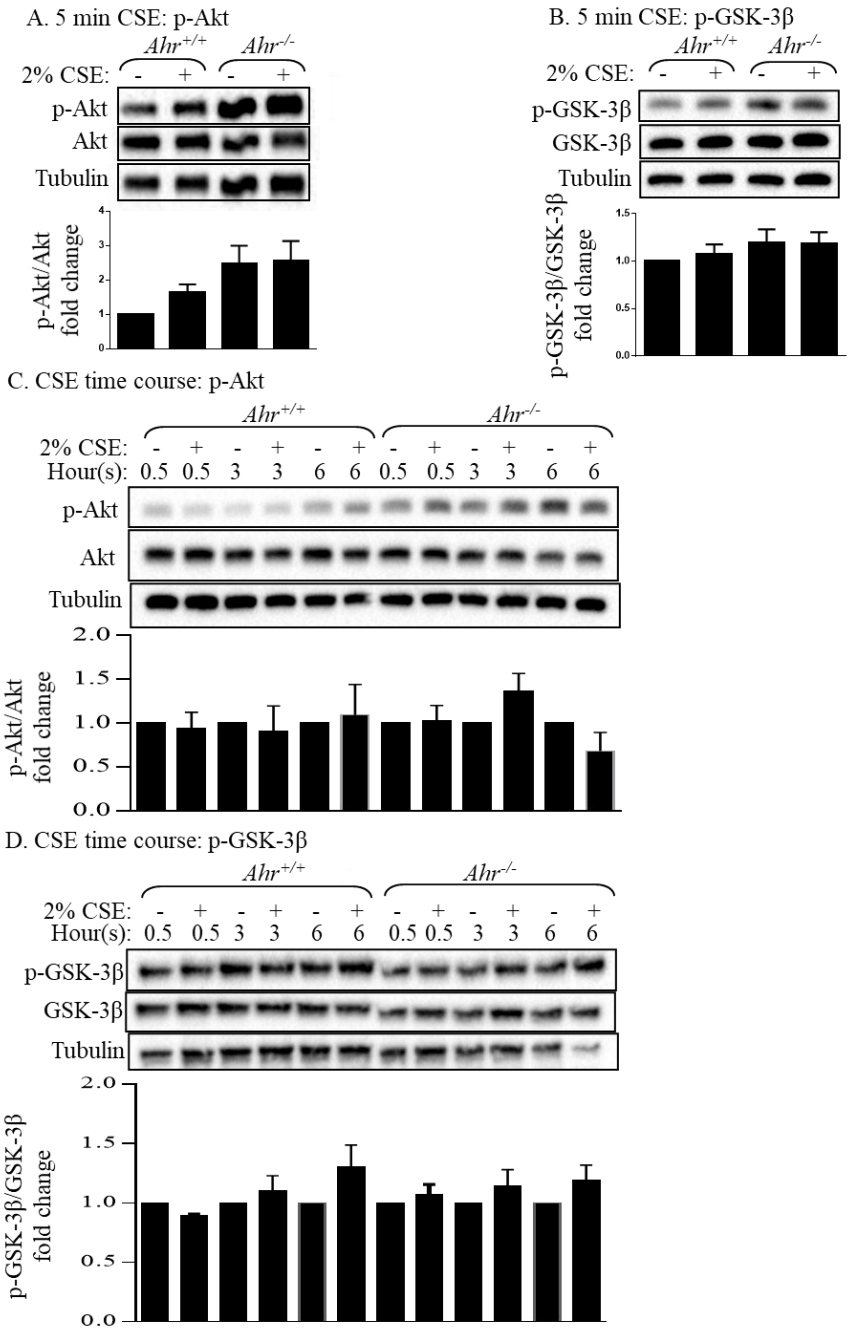

Figure 4.3. Effects of CSE on Akt and GSK-3β phosphorylation. (A, B) Serum-starved *Ahr*$^{+/+}$ and *Ahr*$^{-/-}$ MLFs were treated with or without 2% CSE for 5 min and cellular protein was collected for western blot analysis of **(A)** Akt and **(B)** GSK-3β phosphorylation. **(C, D)** *Ahr*$^{+/+}$ and *Ahr*$^{-/-}$ MLFs were treated with or without 2% CSE for 0.5, 3 and 6 h, and cellular protein was collected for western blot analysis of **(C)** Akt and **(D)** GSK-3β phosphorylation. Blots are representative of **(C, D)** three or **(A, B)** four independent experiments. Densitometric values of the phosphorylated proteins were normalized to the values of their corresponding total proteins and are converted to fold changes relative to that of **(A, B)** the control *Ahr*$^{+/+}$ cells or **(C, D)** untreated control groups at each time point. Results are expressed as mean ± SEM.

4.3 B[*a*]P and CH-223191 did not affect Akt phosphorylation

Next, we evaluated whether acute exposure to the AhR ligand B[*a*]P affected Akt and GSK-3β phosphorylation. For this study, we also included an AhR antagonist, CH-223191, in our experiments as B[*a*]P can affect cellular pathways not involving AhR activation (Bölck *et al.*, 2014). It was suggested that AhR can transiently translocate to the cell membrane after activation to stimulate pathways upstream of Akt (Tsai et al., 2014; Ye et al., 2018) and that AhR translocates into the nucleus as short as 5 to 15 min after ligand exposure (Tkachenko et al., 2016; Kim et al., 2019). Therefore, we first determined if B[*a*]P affects Akt phosphorylation in $Ahr^{+/+}$ and $Ahr^{-/-}$ MLFs after a 5-min exposure. We found that exposure to B[*a*]P (1 μM) did not affect Akt phosphorylation in $Ahr^{+/+}$ and $Ahr^{-/-}$ MLFs (**Figure 4.4A**). There was also no change in GSK-3β phosphorylation in either $Ahr^{+/+}$ or $Ahr^{-/-}$ MLFs after B[*a*]P exposure (**Figure 4.4B**). While these data support that transient exposure to either CSE or B[*a*]P minimally affects Akt phosphorylation, we also performed experiments in $Ahr^{+/+}$ cells with the AhR antagonist CH-223191. In these experiments, we pretreated cells with CH-223191 (10 μM) followed by co-treatment with B[*a*]P (1μM) for 5 min. However, CH-223191 pre-treatment did not affect Akt phosphorylation significantly (**Figure 4.4C**). Therefore, we conclude that AhR activation by classic ligands and AhR inhibition by CH-223191 have little direct impact on the Akt pathway.

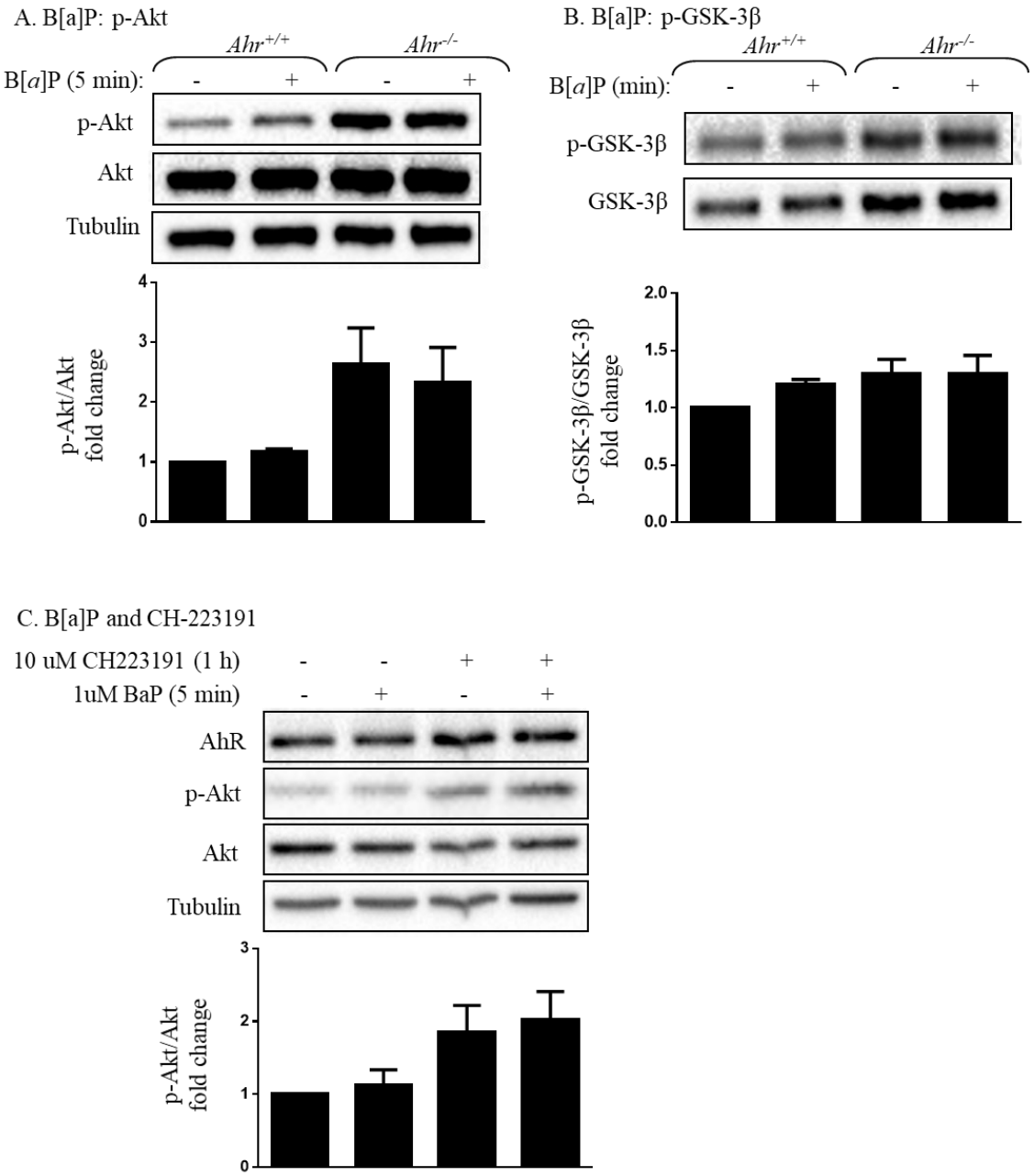

Figure 4.4. Effects of AhR activation on Akt and GSK-β phosphorylation. (A, B) Serum-starved *Ahr*$^{+/+}$ and *Ahr*$^{-/-}$ MLFs were either treated with 1 μM B[*a*]P or the vehicle control (DMSO) for 5 min and cellular protein was collected for western blot analysis of **(A)** Akt and **(B)** GSK-3β phosphorylation. **(C)** Serum-starved *Ahr*$^{+/+}$ and *Ahr*$^{-/-}$ MLFs were treated with 10 μM CH-223191 or the vehicle control (DMSO) for 1 hour followed by 5 min co-treatment with 1μM B[*a*]P or the vehicle control (DMSO) and cellular protein was collected for western blot analysis of Akt phosphorylation. Blots are representative of **(A, B)** three or **(C)** five independent experiments. Densitometric values of the phosphorylated proteins were normalized to the values of their corresponding total proteins and were converted to fold changes relative to that of the control *Ahr*$^{+/+}$ cells. Results are expressed as mean ± SEM.

4.4 AhR regulates basal phosphorylation of Akt

During the course of previous experiments, it became evident that *Ahr*$^{-/-}$ MLFs had noticeably higher basal Akt phosphorylation than that of the *Ahr*$^{+/+}$ cells (**Figures 4.1-4.4**). To further explore the possibility that the AhR regulates basal Akt activity in MLFs, Akt phosphorylation in serum-starved *Ahr*$^{+/+}$ and *Ahr*$^{-/-}$ MLFs was assessed by western blot. In the absence of exogenous stimuli, there was significantly higher Akt phosphorylation in the *Ahr*$^{-/-}$ MLFs than that in the *Ahr*$^{+/+}$ MLFs (**Figure 4.5A** and **4.5B**). However, the phosphorylation of GSK-3β at the basal level did not differ significantly between *Ahr*$^{+/+}$ and *Ahr*$^{-/-}$ MLFs (**Figure 4.5A** and **4.5C**). Collectively, these data suggest that the AhR controls basal activation of the Akt pathway without impacting GSK-3β, one of Akt downstream targets.

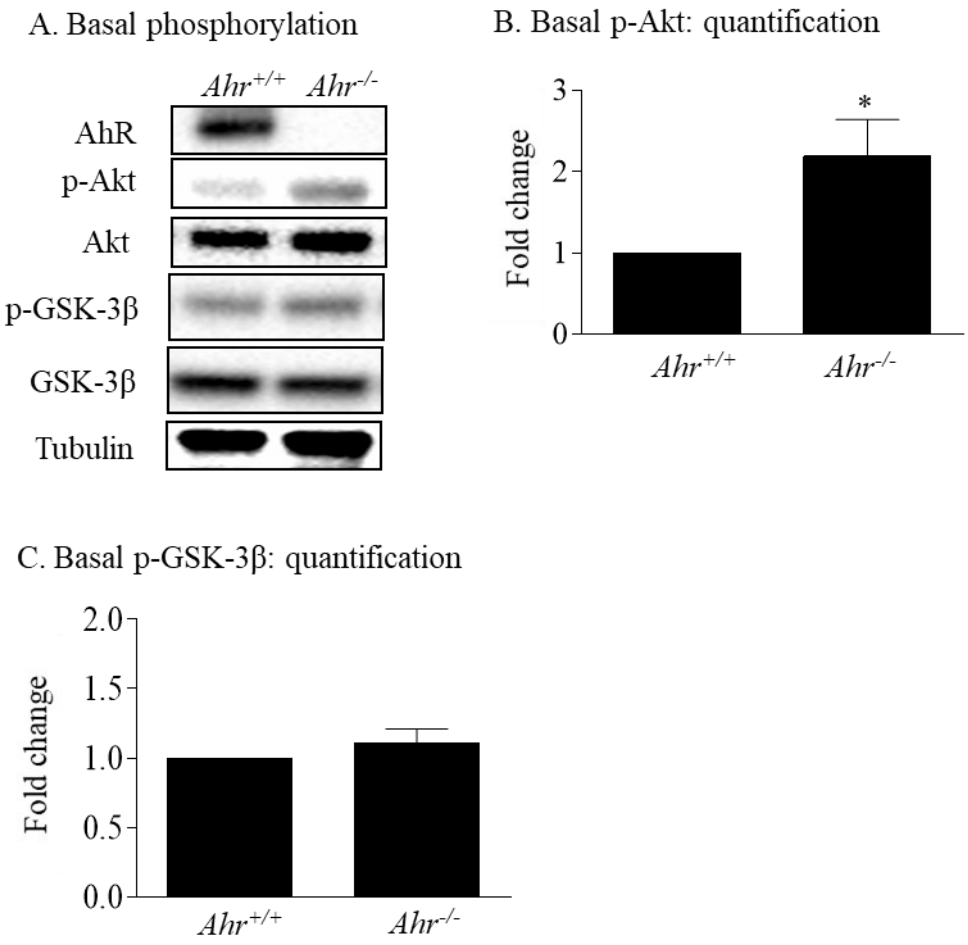

Figure 4.5. Basal Akt and GSK-3β phosphorylation in *Ahr*$^{+/+}$ and *Ahr*$^{-/-}$ MLFs. *Ahr*$^{+/+}$ and *Ahr*$^{-/-}$ MLFs were serum-starved for 18 h and cellular protein was collected for western blot analysis of **(A)** Akt and GSK-3β phosphorylation. **(B, C)** Densitometric analysis of basal **(B)** Akt and **(C)** GSK-3β phosphorylation in *Ahr*$^{+/+}$ and *Ahr*$^{-/-}$ MLFs. Depicted blots are representative of six independent experiments. Densitometric values of the phosphorylated proteins were normalized to that of their corresponding total proteins and are expressed as fold changes relative to that of the *Ahr*$^{+/+}$ group. Results are expressed as mean ± SEM (* $p<0.05$).

4.5 PI3K is required for basal phosphorylation of Akt and GSK-3β in MLFs

The mechanism through which the AhR controls the basal phosphorylation of Akt is not known but may be due to upstream mechanisms that are known to regulate Akt activation, including control over PI3K and/or oxidative stress. To first investigate if AhR regulates Akt phosphorylation by a mechanism that is dependent on PI3K, we utilized the PI3K inhibitor LY294002. In cells that were exposed to LY294002, there was a significant reduction in the basal phosphorylation status of Akt in both $Ahr^{+/+}$ and $Ahr^{-/-}$ MLFs (**Figure 4.6A**). Here, LY294002 completely abrogated the heightened p-Akt activity in $Ahr^{-/-}$ MLFs. Note that LY294002 had no effect on total Akt levels. While there was also a significant reduction in basal GSK-3β phosphorylation, LY294002 did not abrogate the phosphorylation of GSK-3β (**Figure 4.6B**).

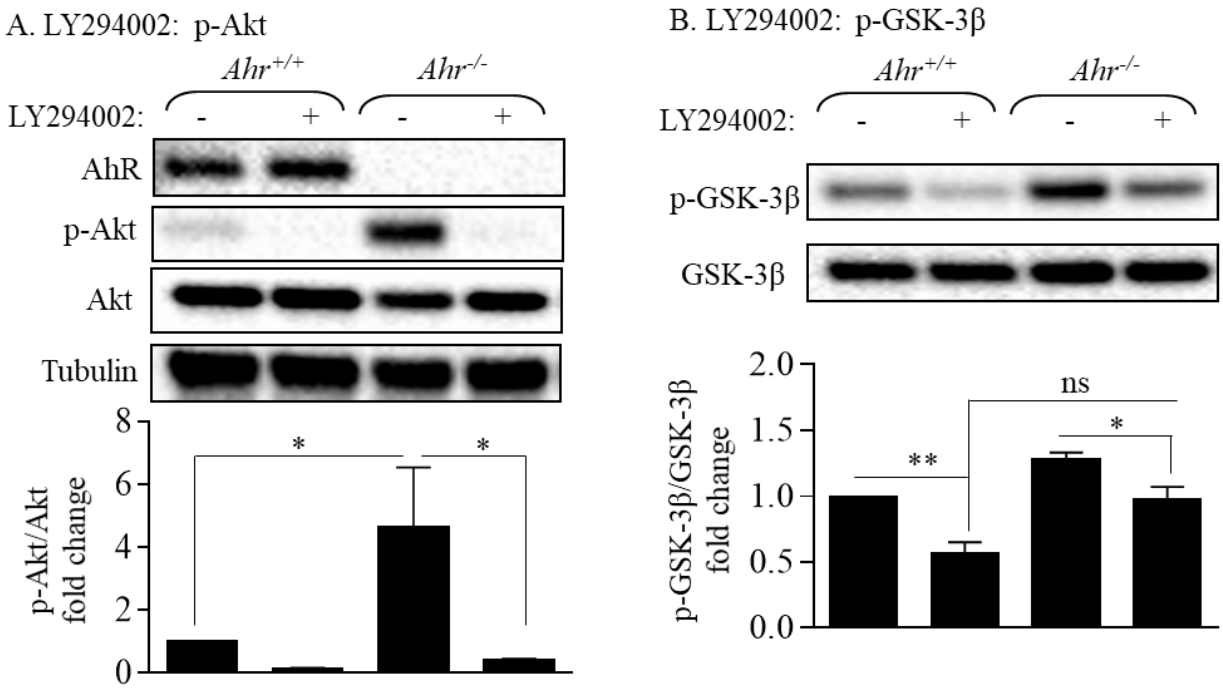

Figure 4.6. Effects of LY294002 on basal phosphorylation of Akt and GSK-3β. *Ahr*$^{+/+}$ and *Ahr*$^{-/-}$ MLFs were treated with either 50 μM LY294002 or the vehicle control (DMSO) for 2 h and cellular protein was collected for western blot analysis of **(A)** Akt and **(B)** GSK-3β phosphorylation. Depicted blots are representative of three independent experiments. Densitometric values of the phosphorylated proteins were normalized to that of their corresponding total proteins and are expressed as fold changes relative to that of the control *Ahr*$^{+/+}$ cells. Results are expressed as mean ± SEM. Post-hoc analysis was performed using the Newman-Keuls test (* $p<0.05$; ** $p< 0.01$; ns, not statistically significant).

4.6 Increased basal Akt phosphorylation in *Ahr*$^{-/-}$ MLFs was not due to altered PTEN expression or heightened oxidative stress

Next, we determined if *Ahr* ablation altered the expressions of the key upstream regulators of Akt activity. To assess this, we first examined the protein level of PTEN in *Ahr*$^{+/+}$ and *Ahr*$^{-/-}$ MLFs, as PTEN is the major negative regulator of the PI3K/Akt pathway (Georgescu, 2010). We observed similar PTEN protein expression in *Ahr*$^{+/+}$ and *Ahr*$^{-/-}$ MLFs (**Figure 4.7A**). PTEN activity is sensitive to oxidation, such that increased ROS can inhibit PTEN and promote the activation of the PI3K/Akt pathway (Leslie *et al.*, 2003). We and others have previously shown that the AhR controls the oxidative stress response, leading to the speculation that higher ROS signaling may be responsible for the increase in Akt phosphorylation in *Ahr*$^{-/-}$ MLFs at baseline (Sarill *et al.*, 2015). To test this, we pretreated the cells with GSH-MEE to boost intracellular glutathione levels, then determined Akt phosphorylation in *Ahr*$^{+/+}$ and *Ahr*$^{-/-}$ cells (Baglole *et al.*, 2006). While 1 h GSH-MEE (2 mM) treatment reduced Akt phosphorylation in *Ahr*$^{+/+}$ MLFs, there was no effect on *Ahr*$^{-/-}$ MLFs (**Figure 4.7B**). Therefore, we conclude that the AhR-dependent regulation of Akt phosphorylation involves PI3K but is likely independent of altered PTEN expression or oxidative stress.

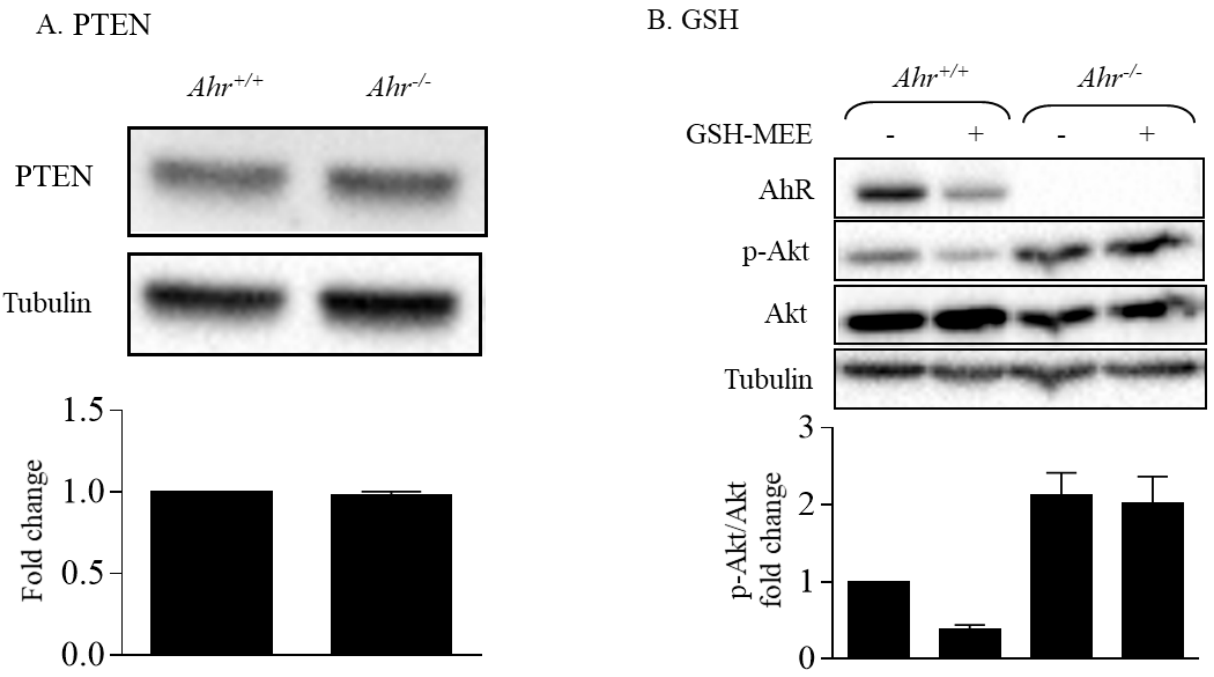

Figure 4.7. Involvement of PTEN and oxidative stress in AhR regulation of basal Akt phosphorylation. (A) *Ahr*$^{+/+}$ *a*nd *Ahr*$^{-/-}$ MLFs were serum-starved for 18 h and cellular protein was collected for western blot analysis of PTEN expression. **(B)** Serum-starved *Ahr*$^{+/+}$ and *Ahr*$^{-/-}$ MLF were treated with 2mM GSH-MEE or the vehicle control (H_2O) for 1 h and cellular protein was collected for western blot analysis of Akt phosphorylation. Depicted blots are representative of three to four independent experiments. Densitometric values of proteins were normalized to that of tubulin or the corresponding total protein and are expressed as fold changes relative to that of the *Ahr*$^{+/+}$ groups. Results are expressed as mean ± SEM.

4.7 *Ahr*$^{-/-}$ MLFs have reduced p85α-PI3K expression

p85α is an important and highly-expressed PI3K regulatory subunit that may modulate PI3K pathway activation (Luo and Cantley, 2005; Cheung *et al.*, 2015). We observed a significant reduction of p85α protein level in the *Ahr*$^{-/-}$ MLFs compared with that in the *Ahr*$^{+/+}$ MLFs (**Figure 4.8**). p85α is a tumor suppressor that has been reported to be downregulated in many cancers (Vallejo-Díaz *et al.*, 2019). It was shown that under baseline conditions, p85 can dimerize with the PI3K catalytic subunit to stabilize and inhibit its catalytic activity (Yu *et al.*, 1998). Previous studies also found that knocking down p85α may promote PI3K pathway activation (Taniguchi *et al.*, 2010). We hence proposed that increased Akt phosphorylation in *Ahr*$^{-/-}$ MLFs was associated with reduced p85α expression. However, knocking down p85α using siRNA in *Ahr*$^{+/+}$ MLFs did not affect Akt phosphorylation (**Figure 4.9**). We therefore conclude that decreased p85α expression does not induce increased Akt phosphorylation in serum-starved MLFs.

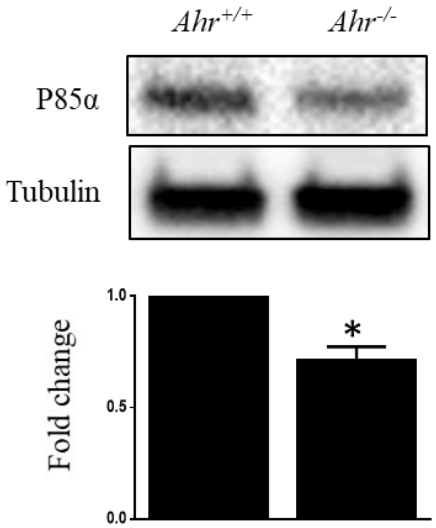

Figure 4.8. Effects of AhR expression on p85α-PI3K expression. *Ahr*$^{+/+}$ and *Ahr*$^{-/-}$ MLFs were serum-starved for 18 h and cellular protein was collected for western blot analysis of p85α-PI3K. Depicted blots are representative of four independent experiments. Densitometric values of p85α was normalized to that of tubulin and are expressed as fold changes relative to that of the *Ahr*$^{+/+}$ group. Results are expressed as mean ± SEM (* $p<0.05$).

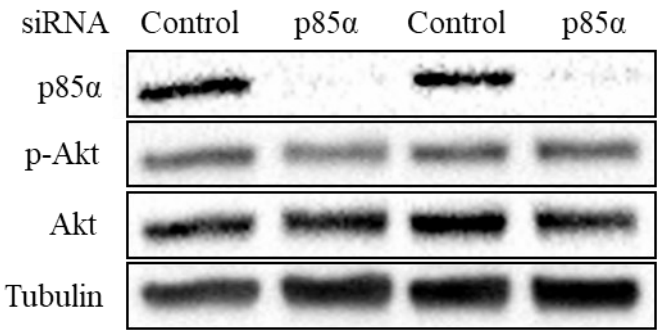

Figure 4.9. Effects of p85α knockdown on Akt phosphorylation. *Ahr*$^{+/+}$ MLFs were transfected with non-targeting control siRNA or siRNA against p85α. Transfected cells were serum-starved for 18 h and cellular protein was collected for western blot analysis of Akt phosphorylation.

4.8 *Ahr*$^{-/-}$ MLFs did not have increased Akt substrate phosphorylation

To determine the downstream effects of increased Akt phosphorylation in *Ahr*$^{-/-}$ MLFs, we assessed the levels of p-FOXO3a (S253), another well-known Akt target, in addition to GSK-3β (Brunet *et al.*, 1999; Nho, 2014). Phosphorylation of FOXO3a (S253) by Akt is crucial in regulating FOXO3a cytoplasmic/nuclear translocation (Brunet *et al.*, 1999; Nho, 2014). We first verified if Akt activation and inhibition in response to PDGE and LY294002, respectively, affect FOXO3a phosphorylation in MLFs. However, neither of these treatments altered FOXO3a (S253) phosphorylation (**Figure 4.10A** and **4.10B**). Basal FOXO3a phosphorylation was also not different between *Ahr*$^{+/+}$ and *Ahr*$^{-/-}$ cells (**Figure 4.10C**). Therefore, we conclude that Akt did not regulate FOXO3a (S253) phosphorylation in MLFs.

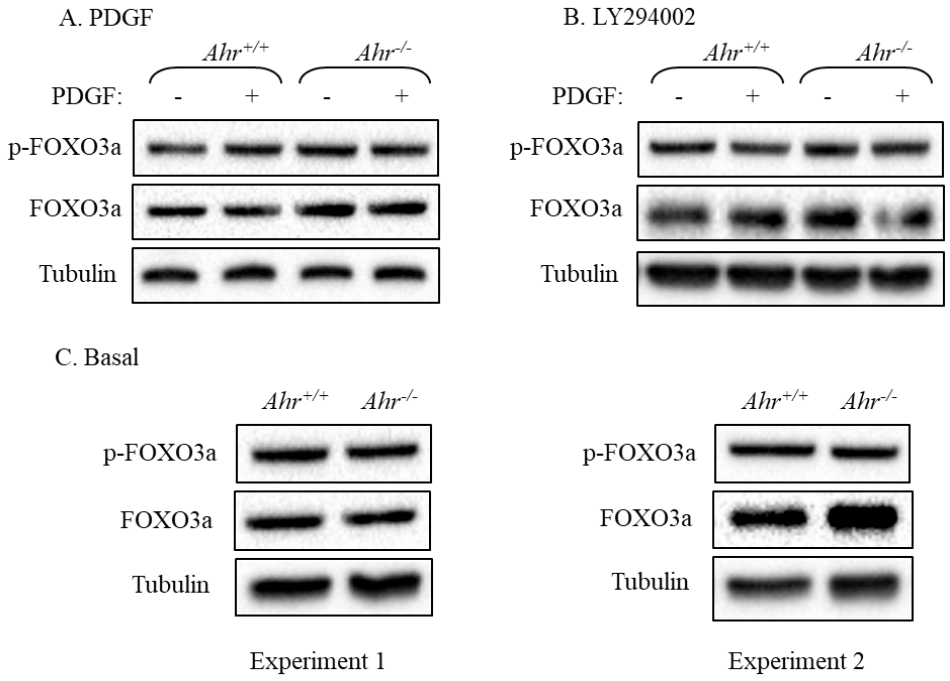

Figure 4.10. FOXO3a phosphorylation in *Ahr*$^{+/+}$ and *Ahr*$^{-/-}$ MLF at the basal level and in response to known PI3K/Akt activating/inhibiting stimuli. Serum-starved *Ahr*$^{+/+}$ and *Ahr*$^{-/-}$ MLFs were either **(A)** treated with 20 ng/ml PDGF-BB for 30 min, **(B)** treated with 50 μM LY294002 for 2 h or **(C)** untreated, and cellular protein was collected for western blot analysis of FOXO3a (S253) phosphorylation.

Akt phosphorylates its substrates at the serine/threonine residues preceded by arginine at positions -5 and -3 (Alessi, Caudwell, *et al.*, 1996). Therefore, to better understand if increased basal Akt phosphorylation in $Ahr^{-/-}$ MLF had downstream consequences, we utilized an antibody that recognizes the phosphorylation of Akt substrates at the minimum conserved motif Arg-Xaa-Xaa-Ser/Thr (Xaa: any amino acid) in quiescent $Ahr^{+/+}$ and $Ahr^{-/-}$ MLFs. This antibody produced a strong signal on the western blot near the 45 kDa region in both $Ahr^{+/+}$ and $Ahr^{-/-}$ MLFs (**Figure 4.11**). Thirty minutes of PDGF-BB treatment increased the intensity of this band (**Figure 4.11A**) while LY294002 treatment eliminated this band in both $Ahr^{+/+}$ and $Ahr^{-/-}$ cells (**Figure 4.11B**). This suggests that the targets detected by this antibody reflected Akt activation/inhibition. Exposure to 2% CSE did not affect Akt substrate phosphorylation (**Figure 4.11C**), which corresponds with the results for Akt phosphorylation. However, $Ahr^{-/-}$ MLFs did not show increased basal Akt substrate phosphorylation than that of the $Ahr^{+/+}$ MLFs (**Figure 4.11D**). Therefore, we concluded that elevated Akt phosphorylation in $Ahr^{-/-}$ MLFs did not result in enhanced Akt substrate phosphorylation as evaluated using this antibody.

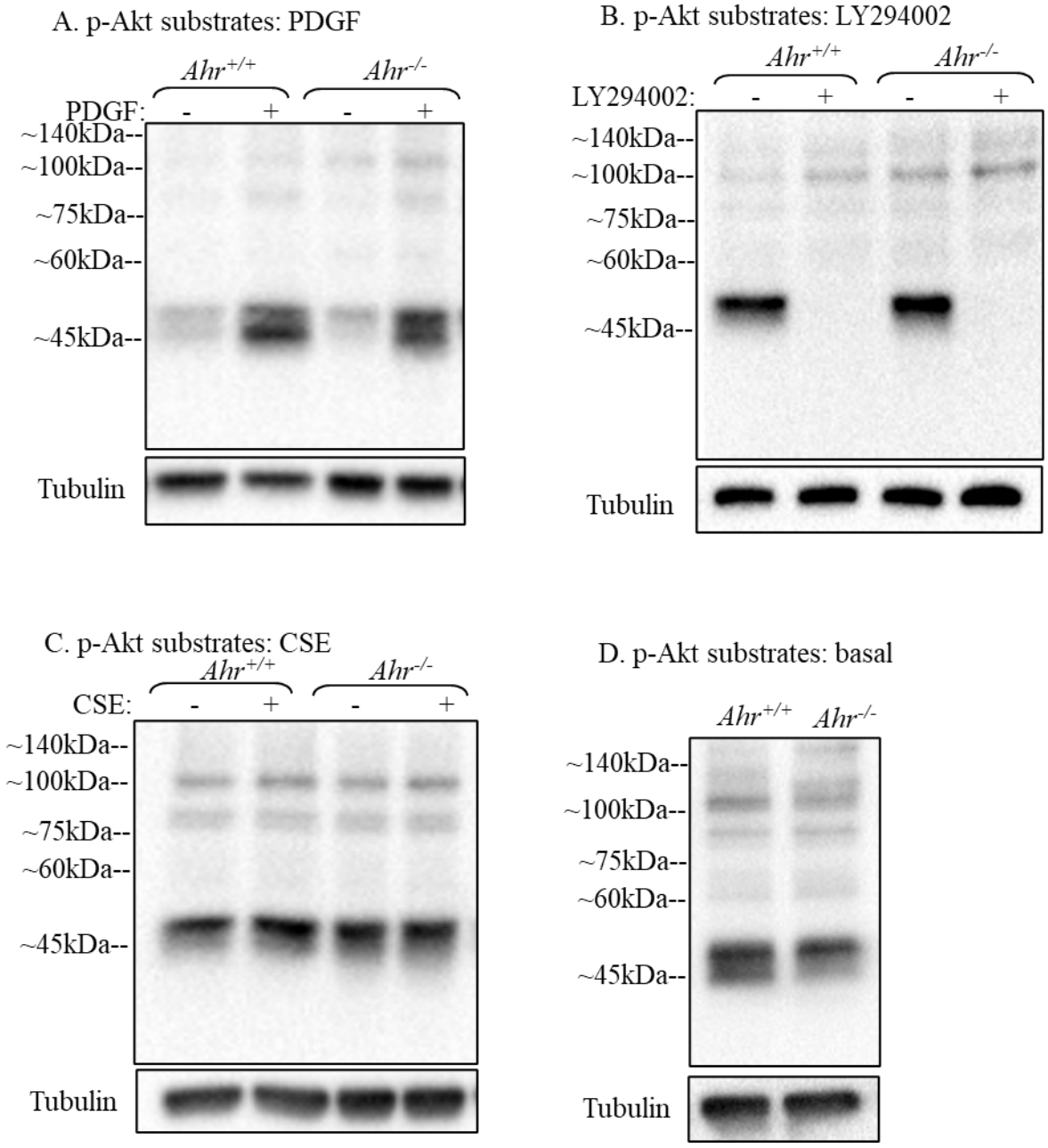

Figure 4.11. Akt substrate phosphorylation in *Ahr*$^{+/+}$ and *Ahr*$^{-/-}$ MLFs at the basal level and in response to different treatments. Serum-starved *Ahr*$^{+/+}$ *a*nd *Ahr*$^{-/-}$ MLF were either **(A)** treated with 20 ng/ml PDGF-BB for 30 min, **(B)** treated with 50 μM LY294002 for 2 h, **(C)** treated with 2% CSE for 5 min or **(D)** untreated, and cellular protein was collected for western blot analysis of proteins phosphorylated at the Akt substrate phosphorylation motif RXXS*/T*. Depicted blots are representative of three independent experiments.

4.9 *Ahr* ablation may affect the phosphoproteomics in MLFs

To determine how the phosphoproteomics changes in the $Ahr^{-/-}$ MLFs compared to that in the $Ahr^{+/+}$ MLFs, and if any Akt substrates are differentially phosphorylated based on AhR expression, a MS-based quantitative proteomics analysis was conducted on serum-starved $Ahr^{+/+}$ and $Ahr^{-/-}$ MLFs. A total of 29 proteins were found to be significantly up- or down-regulated ($p < 0.05$) in $Ahr^{-/-}$ cells compared with $Ahr^{+/+}$ cells (**Figure 4.12**); among these, 19 proteins have a Mascot score greater than 20 (**Table 4.1**). These 19 proteins are involved in a diverse range of biological processes, including proliferation, apoptosis, differentiation, metabolism, autophagy, ubiquitination, phosphorylation and dephosphorylation, cytoskeleton remodeling, ECM organization, intracellular cargo transport, regulation of transcription and translation, protein folding and modulation of various signal transduction pathways (**Table 4.1**). In particular, many of the proteins with differential phosphorylation between $Ahr^{+/+}$ and $Ahr^{-/-}$ MLFs play important roles in the regulation of cell morphology, adhesion and migration, such as fibrillin, fibronectin and various actin-binding proteins **(Table 4.1)**. It was also found that the level of phosphorylated Bcl2-associated agonist of cell death (BAD) at S155, a phosphorylation site that contributes to cell survival (Virdee, Parone and Tolkovsky, 2000), was almost halved in the $Ahr^{-/-}$ cells compared with $Ahr^{+/+}$ MLFs (fold change $Ahr^{-/-}$ / $Ahr^{+/+}$ = 0.587) **(Table 4.1)**. However, no well-known Akt substrates were identified in the data set. To predict potential functional interactions between the differentially phosphorylated proteins and Akt, the STRING database was used. The result indicates possible interactions between BAD, Fn1, Stub1, Notch2 and phospholipase A2 (PLA2) with Akt (**Figure 4.13**).

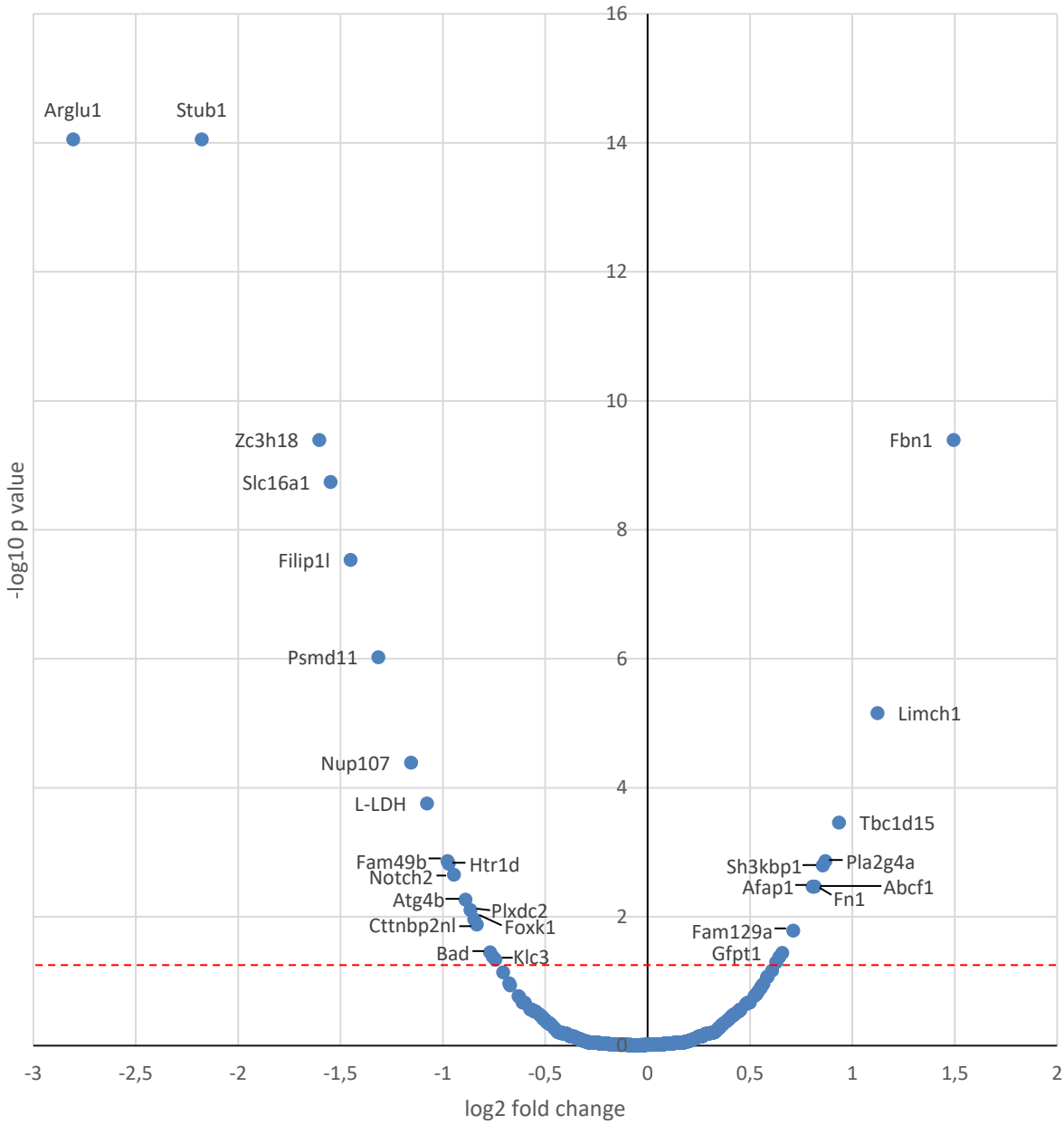

Figure 4.12. A Volcano plot showing the relative levels and the corresponding p values of proteins in the *Ahr*$^{+/+}$ and *Ahr*$^{-/-}$ samples identified by MS. Fold change indicates the intensity ratio of the reporter ions between the *Ahr*$^{-/-}$ and *Ahr*$^{+/+}$ samples. The red horizontal dashed line represents p = 0.05. The plot was generated using Microsoft Excel.

Table 4.1. List of differentially phosphorylated proteins between *Ahr*$^{+/+}$ (WT) and *Ahr*$^{-/-}$ (KO) MLFs identified by MS (Mascot score > 20, $p < 0.05$). Protein information was obtained from UniProt.

Protein	Gene	Phosphorylated site(s)	Ratio: KO/WT	Functions
Fibrillin-1	*Fbn1*	[S2704]	2.817	Metal ion binding; ECM structural constituent; hormone activity regulation; a cell adhesion mediator
LIM and calponin homology domains-containing protein 1	*Limch1*	[S560]	2.178	Metal ion binding; actin binding; actin stress fibers assembly; focal adhesion stabilization; inhibition of cell migration
TBC1 domain family member 15	*Tbc1d15*	[S32; S201; S203; S205; S616]	1.912	Regulation of intracellular protein trafficking; acting as a GTPase activating protein for Rab family proteins
Phospholipase A2	*Pla2g4a*	[S429]	1.825	Ca^{2+}-dependent phospholipase and lysophospholipase activities; membrane lipid remodeling and biosynthesis of lipid inflammatory mediators
SH3 domain-containing kinase-binding protein 1	*Sh3kbp1*	[S274; S631; S633]	1.81	Regulation of diverse signal transduction pathways; apoptosis; endocytosis; ubiquitin protein ligase binding; cell migration and cytoskeletal organization
Actin filament-associated protein 1	*Afap1*	[S669]	1.76	Regulation of cytoskeleton organization; an adapter molecule linking other proteins to the actin cytoskeleton
ATP-binding cassette sub-family F member 1	*Abcf1*	[S194]	1.753	mRNA translation initiation mediator; ATP binding and ATPase activity; RNA binding; ribosome binding
Fibronectin	*Fn1*	[S2294]	1.748	Regulation of cell shape, adhesion and migration; ECM organization; regulation of focal adhesion signaling
Protein Niban 1	*Fam129a*	[S581; S601; S755]	1.638	Regulation of phosphorylation of proteins involved in translation regulation; ER stress response
Glutamine--fructose-6-phosphate aminotransferase [isomerizing] 1	*Gfpt1*	[S259; T261]	1.578	Regulation of glucosamine biosynthesis and nucleotide sugar metabolism; circadian regulation of gene expression
Kinesin light chain 3	*Klc3*	[S173]	0.592	Microtubule-associated force-producing protein; organelle and intracellular cargo transport
Bcl2-associated agonist of cell death	*Bad*	[S155]	0.587	Positive regulation of mitochondrial membrane potential, release of cytochrome c, and cell death
CTTNBP2 N-terminal-like protein	*Cttnbp2nl*	[T490; S567]	0.561	Regulation of lamellipodial actin dynamics; protein phosphatase 2A binding; protein dephosphorylation; negative regulation of transporter activity
Forkhead box protein K1	*Foxk1*	[S431]	0.556	mTORC1-mediated metabolic reprogramming; transcriptional regulator; forkhead DNA binding; negative regulation of autophagy and cell growth; regulation of glucose metabolism
Plexin domain-containing protein 2	*Plxdc2*	[S456]	0.549	Transmembrane protein; Regulation of tumor angiogenesis
Neurogenic locus notch homolog protein 2	*Notch2*	[S2082]	0.519	Transmembrane receptor for membrane-bound ligands; transcriptional regulator; regulation of cell differentiation, proliferation and apoptotic programs
CYFIP-related Rac1 interactor B	*Fam49b*		0.508	Regulation of mitochondrial function; attenuation of actin filament polymerization, phagocytosis and cell migration; protection against infection
Filamin A-interacting protein 1-like	*Filip1l*	[S789; T992]	0.366	Antiangiogenic; negative regulation of proliferation and migration; positive regulation of apoptosis
STIP1 homology and U box-containing protein 1	*Stub1*	[S20]	0.221	Quality control for misfolded or incompletely synthesized proteins; regulation of the activity of several chaperone complexes and protein ubiquitination

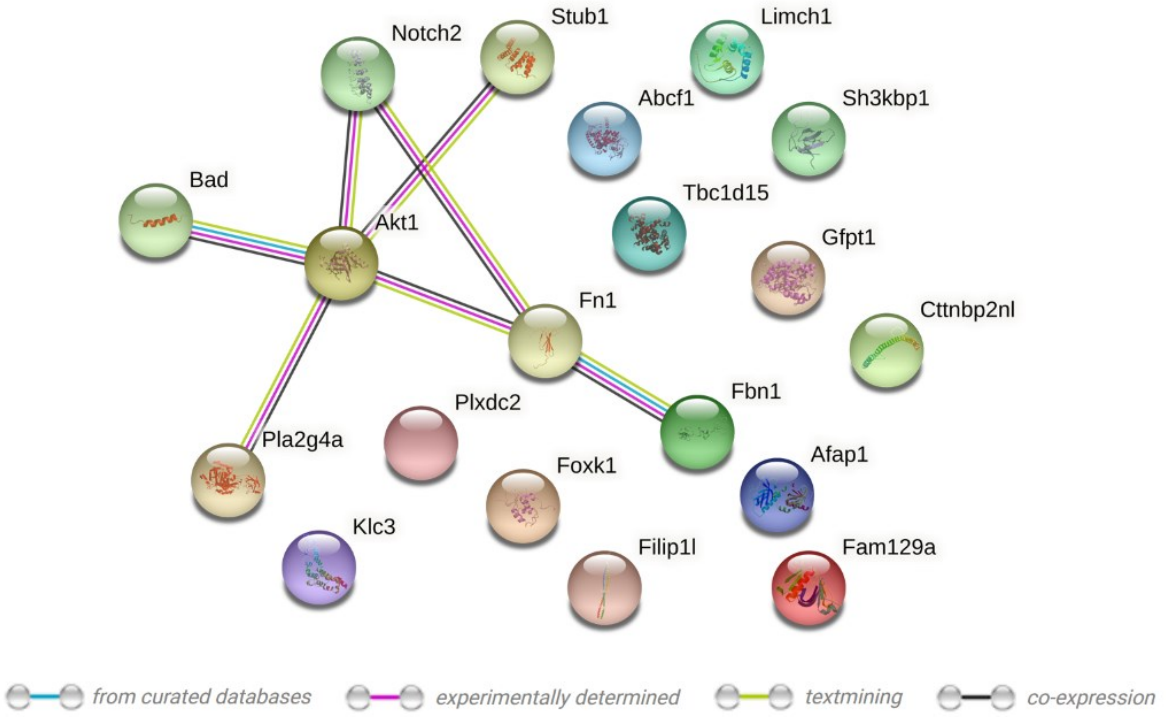

Figure 4.13. A graph showing potential interactions among listed phosphoproteins in Table 4.1 and Akt. The colored nodes correspond to proteins and the edges represent the possibility of both functional and physical protein associations. Line color indicates the type of interaction evidence. Interaction scores: Akt1-Bad: 0.974; Akt1-Fn1: 0.902; Akt1-Stub1: 0.788; Akt1-Notch2: 0.644; Akt1-Pla2g4a: 0.504; Fn1-Fbn1: 0.986; Fn1-Notch2: 0.413. Note: Fam49b from **Table 4.1** was excluded from this analysis as no phosphorylated site was detected. The plot was generated using STRING.

4.10 LY294002 significantly decreased cell viability in *Ahr*$^{-/-}$ MLFs but not in *Ahr*$^{+/+}$ MLFs after 24 h

Finally, we suspected that the increased Akt phosphorylation in *Ahr*$^{-/-}$ MLFs could be a result of a compensatory mechanism for cell survival. Therefore, an MTT assay was performed to assess differences in cell viability between serum-starved *Ahr*$^{+/+}$ and *Ahr*$^{-/-}$ MLFs following LY294002 and CS exposure. *Ahr*$^{-/-}$ MLFs treated with 50 μM LY294002 for 24 h showed significantly reduced cell viability, whereas that of *Ahr*$^{+/+}$ MLF was not significantly affected (**Figure 4.14**). We also assessed the effect of low dose CSE and LY294002 co-treatment on MLF viability to determine if LY294002 can reduce the concentration threshold of CSE-induced cell death. In our previously-published results, CSE concentrations equal to or below 0.5% did not induce MLF cell death (De Souza *et al.*, 2011); therefore, we treated the cells with 0.25% and 0.5% CSE in this experiment. We found that 0.25% and 0.5% CSE alone did not affect MLF viability (**Figure 4.14**). LY294002-CSE co-treatment significantly reduced *AhR*$^{-/-}$ MLF cell viability to the same extend as LY294002 alone treatment (**Figure 4.14**).

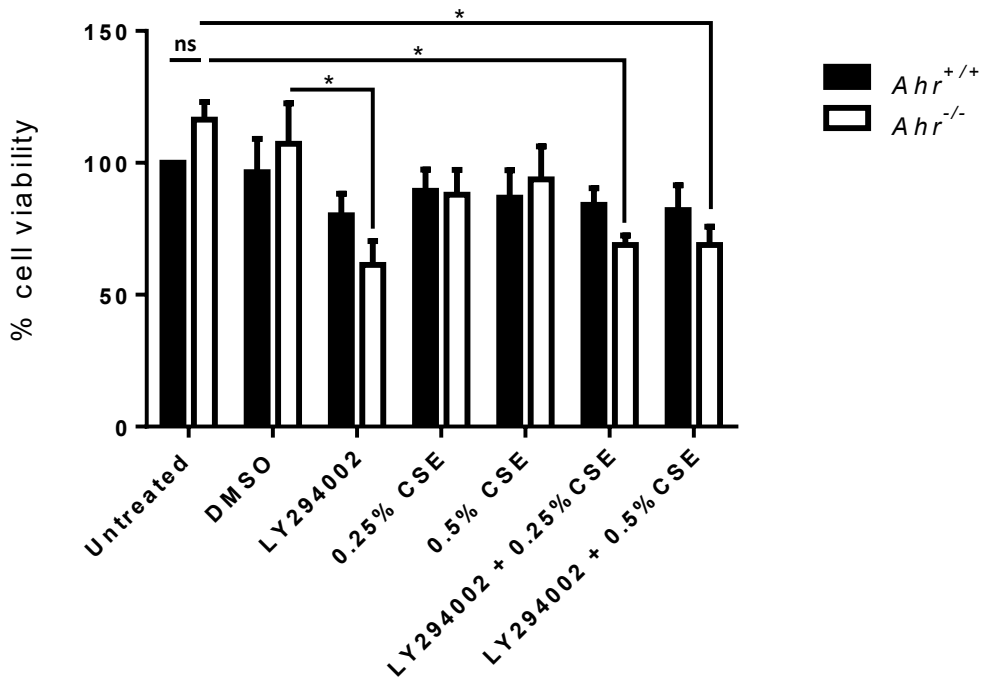

Figure 4.14. Differences in viability between *Ahr*$^{+/+}$ and *AhR*$^{-/-}$ MLFs exposed to LY294002 and CSE. *Ahr*$^{+/+}$ and *AhR*$^{-/-}$ MLFs were either treated with 50 μM LY294002 alone or an equivalent volume of DMSO for 24 h, 0.25% or 0.5% CSE alone for 22 h, or pre-treated with 50 μM LY294002 for 2 h then co-treated with 0.25% or 0.5% CSE for 22 h, and viability was assessed by colorimetric MTT assay. Results are expressed as percent viability relative to the untreated *AhR*$^{+/+}$ cells and are presented as mean ± SEM (n = 5 independent experiments). Post-hoc analysis was performed using the Tukey's multiple comparisons test (* $p<0.05$; ns, not statistically significant).

In conclusion, our current study has demonstrated three novel findings:

1) *Ahr*$^{-/-}$ MLFs cultured *in vitro* had increased phospho-Akt (S473) during serum-starvation compared with their wild-type counterparts.
2) Using an MS-based approach, we were able to identify proteins that were differentially phosphorylated due to *Ahr* ablation, which could potentially influence the regulation of cell shape, adhesion, migration, signaling transduction and survival.
3) The reason for increased Akt phosphorylation in *Ahr*$^{-/-}$ MLFs could be a compensatory mechanism for cell survival during serum starvation, as inhibiting PI3K/Akt activity in the *Ahr*$^{-/-}$ MLFs led to significant loss of cell viability.

CHAPTER 5: Discussion

The AhR is a ligand-activated transcription factor that was first identified as the binding protein for TCDD in 1976 (Poland, Glover and Kende, 1976). Since then, the role of AhR in mediating dioxin toxicity has been extensively investigated, while little was known about its endogenous functions. In the past few years, advances in molecular biology and technological innovations have led to the discovery of several non-canonical interactions between the AhR and other signaling proteins that may shed light on the physiological functions of AhR. For instance, it was found that AhR can act as a strong modulator of the Wnt/β-catenin pathway, NF-κB pathway and pathways associated with epidermal growth factor receptor (EGFR) signaling (*i.e.* tyrosine kinases, extracellular signal-regulated kinase, Src and Akt) (Madhukar *et al.*, 1988; Tian *et al.*, 1999; Wu *et al.*, 2007; Procházková *et al.*, 2011). Furthermore, an increasing number of studies have revealed the molecular and physiological significance of AhR in processes involved in cell cycle regulation, tissue differentiation, organ development and immune system function amongst others (Gasiewicz and Henry, 2011). Although the mechanisms have yet to be elucidated, these findings greatly broadened our view of the role of AhR beyond that of a xenobiotic response mediator.

Among the demonstrated physiological functions of AhR, its role in protecting pulmonary cells against environmental insults, especially against CS, has important implications in human health and diseases. Exposure to tobacco smoke is a critical risk factor for developing chronic respiratory diseases, including lung cancer and COPD, as well as increasing the risk of death from pneumonia, by causing inflammation, redox disturbance and cell death (Centers for Disease Control and Prevention, 2010). Recently, our laboratory showed that AhR has various cytoprotective effects in pulmonary cells against CSE and that *Ahr*$^{-/-}$ MLFs exhibited dramatically

reduced cell viability in response to CSE compared to the *Ahr*$^{+/+}$ MLFs (De Souza *et al.*, 2011). Therefore, we postulated that AhR expression may have significant impacts on the activation of central survival pathways within the MLFs.

One of the most fundamental pro-survival pathways within all cells of higher eukaryotes that govern cell growth and proliferation is the Akt pathway (Szymonowicz *et al.*, 2018). In the present study, we investigated whether AhR regulates the Akt pathway in MLFs exposed to various conditions. We expected that the AhR would promote Akt activation. To test this, we first treated the *Ahr*$^{+/+}$ and *Ahr*$^{-/-}$ MLFs with known Akt activating stimuli (*i.e.* serum and PDGF) and measured Akt phosphorylation at the S473 residue as an indicator of Akt activation (Chu *et al.*, 2018). It was found that these treatments did not result in differential phosphorylation of Akt between the *Ahr*$^{+/+}$ and *Ahr*$^{-/-}$ MLFs (**Figures 4.1** and **4.2**). We also observed no impact of AhR agonists or antagonists (*i.e.* CSE, B[*a*]P and CH-223191) on Akt phosphorylation (**Figures 4.3, 4.4** and **4.5**). In contrast, previous studies conducted by Park *et al.* (2008, 2013) demonstrated induction of Akt phosphorylation in cells treated with CSE (Park *et al.*, 2008, 2013). The discrepancy between their results and ours may be because that the CSE concentration used by Park *et al.* was 20% whereas that used in our experiments was 2% (Park *et al.*, 2008, 2013). Although it has been reported that components in the cigarette smoke, such as nicotine, B[*a*]P and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), could induce rapid Akt activation (West *et al.*, 2003; Bölck *et al.*, 2014), the concentrations of these components in our 2% CSE preparation may be too low to induce detectable Akt phosphorylation. However, when we treated the MLFs with 1 μM B[*a*]P for 5 min, the phosphorylation of Akt was still unchanged (**Figure 4.4**), which differed from the results from a previous study showing that 1 μM B[*a*]P significantly induced Akt phosphorylation in human immortalized (HaCaT) keratinocytes after 5 min of treatment (Bölck *et al.*, 2014). One

possible explanation that could account for this discrepancy is that the Akt phosphorylation site evaluated by Bölck *et al.* (2014) was T308, whereas that evaluated in our study was S473 (Bölck *et al.*, 2014). Although it is known that the phosphorylation of Akt1 at both S473 and T308 is crucial and obligatory for its maximal activation, downstream of PI3K (Alessi, Andjelkovic, *et al.*, 1996), and that the phosphorylation of both sites is usually tightly coupled and can both reflect Akt activity under most conditions (Kumar *et al.*, 2007), it may be possible that these two sites were differently phosphorylated in different types of cells under different experimental conditions.

Although there was no difference between the phosphorylation status of Akt in $Ahr^{+/+}$ and $Ahr^{-/-}$ MLFs in response to growth factors or AhR ligands, to our surprise, Akt phosphorylation was significantly upregulated in serum starved, untreated $Ahr^{-/-}$ cells than in the $Ahr^{+/+}$ cells (**Figure 4.5**), suggesting that the AhR controls basal Akt phosphorylation. This result is inconsistent with previous studies that compared cancer cells with their AhR-deficient counterparts and found that AhR promoted Akt phosphorylation and activation to enhance cancer cell survival and proliferation in response to apoptotic stimuli (Wu *et al.*, 2007; Tsai *et al.*, 2014). On the other hand, and in support of our result, one previous study found that knocking-out AhR increased Akt phosphorylation in mouse livers (Moreno-Marín *et al.*, 2018). The inconsistency between the results for these different studies may suggest that the regulation of Akt activity by the AhR is cell-type specific and/or be reflective of differences between primary and cancer cells. Specifically, the AhR-deficient cancer cells used by Wu *et al.* were a low-AhR expressing variant derived from the parental murine (Hepa1c1c7) hepatoma cells (Miller, Israel and Whitlock, 1983), which expresses approximately 10% of the wild-type AhR level, meaning that they are not AhR knock-out cells (Wu *et al.*, 2007). Other genetic and epigenetic changes may be present in those AhR deficient derivatives of Hepa1c1c7 that can partially account for the impairment of Akt activation. Moreover,

the types of stimuli used by Wu *et al.* were different than those used in our experiments (Wu *et al.*, 2007). In the study conducted by Wu *et al.*, the cells were treated with apoptotic stimuli known to induce mitochondrial-mediated cell death, such as UV irradiation (Wu *et al.*, 2007). It is possible that AhR promotes the activation of Akt to mitigate cell death under certain apoptosis-inducing conditions which were not investigated in our study. Moreover, as we only determined protein phosphorylation but did not directly measure Akt activity in the experiments, an Akt kinase assay should be performed in $Ahr^{+/+}$ and $Ahr^{-/-}$ cells in the future to validate the results.

We sought to identify the mechanisms through which AhR could regulate basal Akt activity. We proposed that heightened Akt phosphorylation in the $Ahr^{-/-}$ cells at the basal level resulted from other biological changes induced by AhR knock-out instead of a direct protein-protein interaction between the Akt and AhR. Specifically, we postulated that the expression of key upstream regulators of Akt, such as PTEN or PI3K, are transcriptionally controlled by the AhR. We verified that the basal activation of Akt was dependent on the PI3K as the phosphorylation of Akt in both $Ahr^{+/+}$ and $Ahr^{-/-}$ cells was depleted by a PI3K inhibitor, LY294002 (**Figure 4.6**). This inhibitor inhibits PI3K by acting on the ATP binding site of the enzyme (Franke *et al.*, 1995). Notably, though, LY294002 may non-specifically interact with other proteins. For example, it also binds to and may perturb the functions of casein kinase 2 (CK2), GSK-3β and mammalian target of rapamycin (mTOR) (Gharbi *et al.*, 2007), which can be a limitation in the interpretation of data obtained from this study. We first examined the level of PTEN, a main negative regulator of the PI3K/Akt pathway, and found it was not regulated by AhR (**Figure 4.7A**). However, because PTEN activity is also regulated by phosphorylation (Chen *et al.*, 2016), absolute protein levels may not be reflective of its activation state. Therefore, it may be worthwhile to also determine if PTEN phosphorylation is altered in AhR deficient cells in the future.

As the activity of the PTEN and PI3K/Akt pathway can be regulated by oxidative stress (Chen *et al.*, 2016), we then determined if administering the antioxidant, GSH-MEE, would decrease Akt phosphorylation in *Ahr*$^{+/+}$ and *Ahr*$^{-/-}$ MLFs. GSH is a tripeptide (L-γ-glutamyl-L-cysteinylglycine) naturally synthesized by eukaryotic cells to protect against oxidative stress (Lushchak, 2012). GSH contains a cysteine residue with an active thiol group, which can act as an antioxidant to reduce reactive oxygen and nitrogen species and be oxidized to glutathione disulfide (GSSG) in the process (Lushchak, 2012). GSH can be synthesized via a two-step process catalyzed by the enzymes γ-glutamyl-L-cysteine ligase (γGLCL) and glutathione synthetase (GLS) (Lushchak, 2012). GSH can also be re-generated via reducing oxidized GSSG by glutathione reductase (GR) (Lushchak, 2012). GSH-MEE is a lipophilic molecule that can be hydrolyzed by intracellular esterases to release GSH, thereby replenishes the intracellular GSH reservoir and mediates the elimination of oxidants (Tsan, White and Rosano, 1989; Chung *et al.*, 2016). However, we found that this antioxidant only decreased Akt phosphorylation in the *Ahr*$^{+/+}$ cells but not that in the *Ahr*$^{-/-}$ cells (**Figure 4.7B**). This may suggest that ROS signaling plays a role in basal Akt activation in the *Ahr*$^{+/+}$ cells whereas that in the *Ahr*$^{-/-}$ cells was not. This has also raised the suspicion that the Akt pathway in the *Ahr*$^{-/-}$ cells had reduced sensitivity to fluctuations in the ROS level, but more experiments are needed to determine this. Finally, it is also possible that GSH-MEE treatment did not successfully alleviate oxidative stress in the *Ahr*$^{-/-}$ cells, which could mean that the speed of consumption of GSH in the *Ahr*$^{-/-}$ cells exceeded that of the conversion of GSH-MEE to GSH. In future experiments, intracellular GSH and ROS levels in the antioxidant-treated *Ahr*$^{+/+}$ and *Ahr*$^{-/-}$ cells should be evaluated to ensure the replenishment of the GSH pool and the reduction of oxidative stress to similar levels.

Another key regulator of the PI3K pathway is the PI3K regulatory subunit, p85α (Carnero and Paramio, 2014). We found that the p85α level was significantly decreased in the *Ahr*$^{-/-}$ cells compared with that in the *Ahr*$^{+/+}$ cells (**Figure 4.8**). One possible reason accounting for the higher p85α level in AhR expressing MLFs is that *PIK3R1* (the gene name of p85α) is an AhR target gene (Sartor *et al.*, 2009; Rouillard *et al.*, 2016). We thus proposed that decreased p85α level could lead to increased basal Akt phosphorylation. However, when we knocked down p85α in wild-type MLFs, basal Akt phosphorylation remained unchanged (**Figure 4.9**). This suggests that downregulation of p85α alone is not a cause of elevated Akt phosphorylation. Therefore, more research is needed to understand the mechanisms through which AhR regulates Akt phosphorylation. However, the consequences of p85α level reduction in *Ahr*$^{-/-}$ MLFs are worth further investigation, as p85α has emerged as a potential therapeutic target for many diseases, including cancer (Mauvais-Jarvis *et al.*, 2002; Toste *et al.*, 2015). Besides its importance in cancer, p85α also plays a role in insulin resistance and it was found that reducing the expression level of p85α significantly improved insulin sensitivity and decreased the incidence of diabetes development in mouse models with insulin resistance (Mauvais-Jarvis *et al.*, 2002).

In addition to examining the upstream regulators of the PI3K/Akt pathway that could account for the elevation of Akt phosphorylation in *Ahr*$^{-/-}$ MLFs, we also assessed the phosphorylation of Akt downstream targets. Our goal was to determine whether increased Akt phosphorylation in *Ahr*$^{-/-}$ cells resulted in any functional consequences in MLFs, as many previous studies have linked increased Akt activity to lung cancer and fibrosis (Calvo, Bolós and Grande, 2009; Nho *et al.*, 2011; Dakhlallah *et al.*, 2019). Because *Ahr*$^{-/-}$ cells exhibited higher Akt phosphorylation than that of the *Ahr*$^{+/+}$ cells, we predicted that the *Ahr*$^{-/-}$ MLFs also had elevated phosphorylation of Akt substrates. The specific downstream targets of Akt we selected to examine

in this study were GSK-3β (S9) and FOXO3a (S253), which are well-established substrates of Akt (Manning and Toker, 2017). We found that FOXO3a (S253) phosphorylation was not regulated by Akt in MLFs, as its level did not change in response to Akt activation and inhibition stimuli (**Figure 4.10**). On the other hand, GSK-3β phosphorylation in MLFs correctly reflected Akt activity in response to growth factor stimuli and PI3K inhibition (**Figures 4.1, 4.2** and **4.6**); however, despite that serum-starved *Ahr*$^{-/-}$ cells showed increased basal Akt phosphorylation than that of the *Ahr*$^{+/+}$ cells, they did not show increased GSK-3β phosphorylation than the *Ahr*$^{+/+}$ cells (**Figure 4.5**). These results may suggest that increased Akt phosphorylation at S473 in the *Ahr*$^{-/-}$ cells have not resulted in increased Akt kinase activity. However, it is in contrast to the study that Moreno-Marin *et al.* conducted, which showed that *Ahr* ablation increased the phosphorylation of both Akt and GSK-3β in mouse livers (Moreno-Marín *et al.*, 2018). Nonetheless, Moreno-Marin *et al.* conducted their experiments using homogenized mouse liver tissue while we utilized isolated MLFs (Moreno-Marín *et al.*, 2018). This disagreement could be caused by the fact that increased Akt phosphorylation in *Ahr*$^{-/-}$ MLFs was uncoupled with increased Akt activity and the phosphorylation efficiency of GSK-3β by Akt may be different in different types of cells and tissues. Many post-translational modifications of Akt besides phosphorylation at S473 and T308, such as acetylation, ubiquitylation, methylation, hydroxylation, glycosylation and SUMOylation, have also been identified and may serve to fine-tune Akt activity, cellular localization and substrate specificity, therefore potentially affect the ability of Akt to phosphorylate GSK-3β (Manning and Toker, 2017). There are also other proteins that could regulate GSK-3β to maintain its phosphorylation in the *Ahr*$^{+/+}$ MLFs in addition to Akt. For example, GSK-3β (S9) can be phosphorylated by cAMP-dependent protein kinase (PKA) in the absence of a functional PI3K/Akt pathway (Fang *et al.*, 2000). Protein kinase C (PKC) and p70 S6 kinase are also able to mediate

PI3K/Akt-independent phosphorylation of GSK-3β (S9) (Beurel, Grieco and Jope, 2015). In our study, LY294002 did not completely block GSK-3β phosphorylation, supporting that other mechanisms regulate basal GSK-3β phosphorylation besides PI3K/Akt in MLFs (**Figure 4.6**).

We have also utilized an antibody that recognizes proteins that are phosphorylated at the Akt phosphorylation motif (Arg-Xaa-Xaa-Ser/Thr) to determine the phosphorylation of Akt substrates in general. We found that the levels of phosphorylation of the targets identified by this antibody correctly reflected PI3K/Akt activity in response to the activation and inhibition signals (**Figure 4.11A** and **4.11B**). However, we did not observe increased phosphorylation of Akt substrates detected by this antibody in the *Ahr*$^{-/-}$ MLFs compared with that in the *Ahr*$^{+/+}$ MLFs, which may be another evidence supporting that increased basal Akt S473 phosphorylation in the *Ahr*$^{-/-}$ MLFs was not correlated with increased Akt activity against its targets (**Figure 4.11**). We were not able to identify the individual proteins that this phospho-Akt substrate antibody recognized and therefore could not determine their corresponding total protein levels. The number of proteins that were visualized by this antibody may also be limited, such that only the most abundant phosphoproteins could be revealed. Importantly, besides Akt, other members of the Arg-directed kinases or AGC-family kinases, including PKA, cGMP-dependent protein kinase (PKG) and PKC, all share a substrate specificity characterized by Arg at position -3 relative to the Ser or Thr phosphorylation site (Pearson and Kemp, 1991; Montminy, 1997). Therefore, this antibody may have also recognized non-Akt-specific substrates. Furthermore, in addition to Akt activity, factors such as the accessibility of the phosphorylation sites on the substrates and their subcellular compartmentalization may also be important to regulate their phosphorylation by Akt (Manning and Toker, 2017).

It is also possible that elevated Akt phosphorylation in the *Ahr*$^{-/-}$ cells induced additional proteomics changes. To further investigate this, MS analysis of the phosphorylated proteins in *Ahr*$^{+/+}$ and *Ahr*$^{-/-}$ cells was performed to identify if the phosphorylation of any known downstream targets of Akt are altered due to *Ahr* ablation. Using MS, we identified several proteins that showed differential phosphorylation between the *Ahr*$^{+/+}$ and the *Ahr*$^{-/-}$ cells (**Figure 4.12** and **Table 4.1**). It has been shown that AhR plays a role in the regulation of cell morphology, focal adhesion and migration (Dietrich and Kaina, 2010; Tomkiewicz *et al.*, 2013). Here, we found that many proteins regulating these processes were differentially phosphorylated between *Ahr*$^{+/+}$ and *Ahr*$^{-/-}$ cells (**Table 4.1**). According to the results, the protein with the most upregulated phosphorylation due to *Ahr* ablation was fibrillin-1 (fold change *Ahr*$^{-/-}$/*Ahr*$^{+/+}$ = 2.817) (**Table 4.1**). Fibrillin-1 is a major structural component of microfibrils (Yoshiba *et al.*, 2015); however, it is not only a structural protein but also regulates the storage, release and activation of extracellular TGF-β1, an important cytokine modulating the transdifferentiation of resident fibroblasts into myofibroblasts (Yoshiba *et al.*, 2015). Previous studies showed that fibrillin-1 degradation and downregulation are likely to be implicated in the differentiation of myofibroblasts (Yoshiba *et al.*, 2015); however, little is known about how phosphorylation of fibrillin-1 will affect its function. An ECM protein that is closely associated with fibrillin and has been shown to be required for proper fibrillin assembly, fibronectin (Sabatier *et al.*, 2009), was also found to be more highly phosphorylated in the *Ahr*$^{-/-}$ than *Ahr*$^{+/+}$ cells (fold change *Ahr*$^{-/-}$/*Ahr*$^{+/+}$ = 1.748) **(Table 4.1)**. Fibronectin is a glycoprotein in the ECM that binds to integrins and many ECM proteins and regulates various biological processes including cell-to-cell adhesion and fibroblast migration (Proctor, 1987). It is known that fibronectin can be phosphorylated in the C-terminal cross-linked region, and the phosphorylation state is important for the structural stability of this domain (Kulke *et al.*, 2019). Phosphorylated

fibronectin may enhance cell attachment (Yalak *et al.*, 2019). Studies have also suggested a regulatory role of fibronectin on Akt activity through regulating the focal adhesion kinase (FAK)-PI3K/Akt pathway (Han, Khuri and Roman, 2006; Yousif, 2014). The activity of the PI3K/Akt activity is regulated by many factors (Carnero and Paramio, 2014). Our results suggest that AhR did not regulate growth factor-induced Akt activation, and increased Akt phosphorylation in *Ahr*$^{-/-}$ MLFs was not due to altered PTEN expression or oxidative stress (**Figure 5.1**). How AhR regulates Akt phosphorylation in MLFs remained unknown. Nevertheless, the finding that AhR knockout likely resulted in abnormal phosphorylation of fibrillin and fibronectin gave rise to the speculation that increased Akt phosphorylation in *Ahr*$^{-/-}$ MLFs was associated with dysregulation of the ECM (**Figure 5.1**).

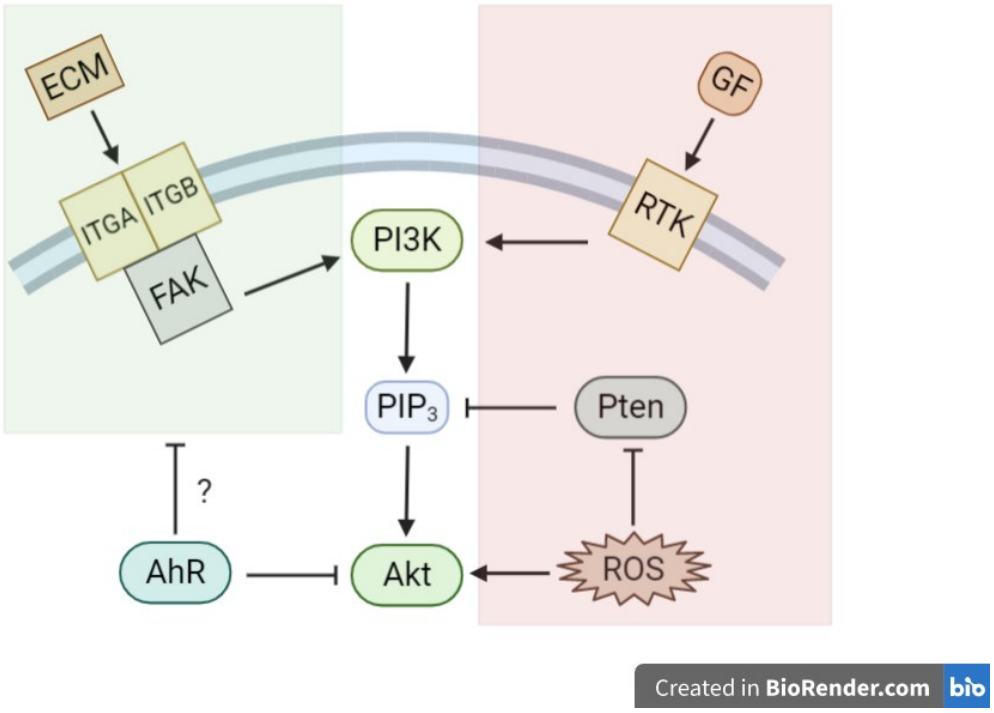

Figure 5.1. A diagram showing the Akt activating pathways that AhR may regulate. In this study, we show that the presence of AhR reduced basal Akt phosphorylation; however, the mechanism is still unclear. PI3K/Akt signaling pathway is activated by many types of cellular stimuli. Besides the canonical growth factors-mediated RTK/PI3K/Akt pathway, the interactions between integrin and ECM can also transduce signals into the cell by activating the FAK/PI3K/Akt pathway (Carnero and Paramio, 2014; Wu *et al.*, 2016). We suspected that AhR affected Akt phosphorylation through the regulation of these pathways. The red shaded area corresponds to the pathways that we found not likely to be involved in AhR-dependent downregulation of Akt phosphorylation. The green shaded area represents the potential pathway by which AhR could modulate Akt phosphorylation and further investigation is warranted. ECM: extracellular matrix; GF: growth factors; ITGA: integrin subunit alpha; ITGB: integrin subunit beta; FAK: focal adhesion kinase; RTK: receptor tyrosine kinase; PI3K: phosphoinositide 3-kinase; AhR: aryl hydrocarbon receptor; PIP_3: phosphatidylinositol 3,4,5-triphosphate; Akt: AKT serine/threonine kinase; Pten: phosphatase and tensin homolog; ROS: reactive oxygen species.

Protein-protein interaction analysis of the proteins with differential phosphorylation in *Ahr*$^{-/-}$ MLFs allowed us to identify their potential interactions with the Akt (**Figure 4.13**). Some of these proteins may play a role in the regulation of Akt phosphorylation, such as PLA2, Stub1 and Notch2. Indeed, numerous studies investigating the interactions between Akt and these proteins can be found in the literature. Cytosolic PLA2 is a calcium-dependent enzyme that cleaves fatty acids from phospholipids by hydrolyzing the SN-2 acyl bond, producing various second messengers (Leslie, 1997). Lipid second messengers produced by cytosolic PLA2 can activate cell surface receptors to affect the Akt pathway (Feuerherm, Dennis and Johansen, 2019). Activation of PLA2 and production of the lysophosphatidylcholine was required for radiation-induced activation of Akt in vascular endothelial cells (Yazlovitskaya *et al.*, 2008). Stub1, a U-box containing E3 ubiquitin ligase, can act as an Akt suppressor or an activator depending on the context (Tawo *et al.*, 2017; Cheng *et al.*, 2018). Depletion of Stub1 in a human embryonic kidney cell line activated Akt signaling (Tawo *et al.*, 2017), whereas overexpression of Stub1 in prostatic cancer cells promoted cell proliferation through activation of the Akt pathway (Cheng *et al.*, 2018). Notch2, a transmembrane receptor that plays an important role in development and the determination of cell fate, negatively regulates cell invasion and metastasis by inhibiting the PI3K-Akt signaling pathway in gastric cancer and nasopharyngeal carcinoma (Guo *et al.*, 2012; Zou *et al.*, 2019). How the phosphorylation of PLA2, Stub1 and Notch2 affects their functions and their interactions with the Akt in MLFs are worth further investigation.

One protein found to be differentially phosphorylated between and the *Ahr*$^{+/+}$ and *Ahr*$^{-/-}$ cells was BAD (**Table 4.1**), with *Ahr*$^{+/+}$ cells having intrinsically higher BAD (S155) phosphorylation than that of the *Ahr*$^{-/-}$ cells (fold change *Ahr*$^{-/-}$/*Ahr*$^{+/+}$ = 0.587) (**Table 4.1**). The S155 residue of BAD is primarily phosphorylated by PKA but has also been shown to be weakly

phosphorylated by Akt (Datta *et al.*, 2000). BAD is a pro-apoptotic BH3-only protein (Adachi and Imai, 2002) and previous studies have reported that phosphorylation of BAD at S155 within the BH3 domain inhibits its death-promoting activity (Tan *et al.*, 2000). Therefore, decreased BAD phosphorylation in $Ahr^{-/-}$ cells could have conferred a survival disadvantage compared to the $Ahr^{+/+}$ cells, which may lead to increased susceptibility of the $Ahr^{-/-}$ cells to cell death.

Compared to antibody-based assays, MS offers greater specificity, reproducibility and sensitivity (Jayasena *et al.*, 2016). MS has been a technique of choice for large-scale quantitative phosphoproteomics analysis and screening for novel protein phosphorylation sites (Nita-Lazar, Saito-Benz and White, 2008; Mayya and Han, 2009). However, MS results can be largely affected by sample quality, sample preparation, calibration strategies and the use of different databases and search algorisms (Nita-Lazar, Saito-Benz and White, 2008; Mayya and Han, 2009). There are also limitations existed in MS-based protein quantification and data interpretation, including incomplete proteolytic digestion of certain proteins, inefficient mass tag labeling and difficulties in ascertaining fold change in multisite phosphorylation (Nita-Lazar, Saito-Benz and White, 2008; Mayya and Han, 2009). In our experiment, phosphopeptide enrichment was performed to increase the chance of detecting low abundance phosphoproteins; however, this process may cause sample loss and sequence coverage to be low, giving rise to challenges in protein identification. Possibly due to this, we were not able to identify any Akt-specific peptide sequences in this experiment. Different protein extraction buffers can also affect sample quality and MS results. Because detergents and phosphate-containing buffers may interfere with the phosphopeptide enrichment procedure and MS, 8M urea in HEPES buffer was used for the solubilization and denaturation of proteins in our experiment (Chen *et al.*, 2007; Sun *et al.*, 2014). However, in aqueous solution, urea can gradually decompose to isocyanic acid and cause carbamylation at the N-termini and at

the side chain amino groups of lysine and arginine residues (Chen *et al.*, 2007; Sun *et al.*, 2014). Protein/peptide carbamylation can block sites of trypsin digestion and isotopic/isobaric labeling and affect protein identification and quantification by changing peptide charge states, retention time and masses (Chen *et al.*, 2007; Sun *et al.*, 2014). The process of urea decomposition is dramatically accelerated by elevated temperature (37°C) (Chen *et al.*, 2007; Sun *et al.*, 2014). Therefore, in our experiments, fresh urea solution was prepared at the time of protein extraction and was kept at low temperature. MS-based quantitative assays also usually exhibit high degrees of variability between replicates due to the dynamic range of protein expression (Jayasena *et al.*, 2016). In the future, replicates for MS-based phosphoproteomics analysis of $Ahr^{+/+}$ and $Ahr^{-/-}$ cells can be conducted to validate our results. Nevertheless, despite the potential difficulties of MS-based phosphoproteomics analysis, this technique offers important advantages over the other analytical methods, particularly the ability to unambiguously identify and quantify phosphoproteins (Jayasena *et al.*, 2016). MS allowed us to detect protein phosphorylation sites that had not been documented previously. Since phosphorylation often alters the structural conformation of a protein (Ardito *et al.*, 2017), differential phosphorylation of proteins between the $Ahr^{+/+}$ and $Ahr^{-/-}$ cells strongly indicates changes in their functions and activation status.

Although we were unable to detect upregulated Akt substrate phosphorylation in $Ahr^{-/-}$ MLFs by western blot and MS, we suspect that $Ahr^{-/-}$ MLFs are dependent on increased Akt phosphorylation for cell survival. To test this, we inhibited the PI3K/Akt pathway using LY294002 in serum-starved $Ahr^{+/+}$and $Ahr^{-/-}$ MLFs for 24 h and performed MTT assays to determine cell viability. We found that while LY294002 did not significantly affect the viability of $Ahr^{+/+}$ MLFs, it significantly reduced that of $Ahr^{-/-}$ MLFs (**Figure 4.14**). Our results suggest that $Ahr^{-/-}$ MLFs may be vulnerable to PI3K/Akt inhibition and that increased Akt phosphorylation was needed by

Ahr$^{-/-}$ MLFs for survival during serum starvation. On the other hand, the viability of LY294002 and low concentration CSE co-treated MLFs did not differ from that of the MLFs treated with LY294002 alone, indicating that losing Akt activity does not affect cell viability in response to low concentrations of CSE (**Figure 4.14**). The fact that inhibiting Akt activity caused cell death in the *Ahr*$^{-/-}$ cells also gave rise to the possibility of a survival bias such that only serum-starved *Ahr*$^{-/-}$ cells with high Akt phosphorylation survived, and hence the higher Akt phosphorylation in the *Ahr*$^{-/-}$ compared to the *Ahr*$^{+/+}$ MLFs. Nevertheless, we did not observe statistically significant lower cell viability in LY294002 treated *Ahr*$^{-/-}$ MLFs than *Ahr*$^{+/+}$ MLFs (**Figure 4.14**). MTT is an assay that assesses cellular metabolic activity, which relies on the reduction of MTT to formazan (Riss *et al.*, 2004). Although the exact cellular mechanism of this reaction is not well understood, it likely involves reduced nicotinamide adenine dinucleotide (NADH) or similar reducing molecules that transfer electrons to MTT (Riss *et al.*, 2004). The amount of formazan signal generated will depend on the number of viable cells and their metabolic activity (Riss *et al.*, 2004). Other methods determining cell death not dependent upon metabolic activity can be used in the future to confirm the results we obtained from this experiment. LY294002 has been shown to induce apoptotic cell death (Jiang *et al.*, 2010). Therefore, the levels of apoptotic markers, such as caspase 3 and poly (ADP-ribose) polymerase (PARP), can be determined in LY294004 treated MLFs. The cysteine protease, caspase 3, is an executioner caspase involved in the apoptotic process, whose activation is marked by the cleavage of the interdomain linker and then subsequent removal of the N-terminal pro-domain (Han *et al.*, 1997). Once caspase 3 is activated, it catalyzes the specific cleavage of many key cellular proteins, one of them being the PARP (Boulares *et al.*, 1999). The cleavage of caspase 3 and PARP have been considered hallmarks of apoptosis (Kaufmann *et al.*, 1993; Chaitanya, Alexander and Babu, 2010).

In conclusion, in the present study, we observed that the presence of AhR negatively regulated the phosphorylation of Akt in untreated, serum-starved MLFs. However, AhR did not regulate Akt phosphorylation in MLFs in response to growth factors, CSE and AhR ligands. Analysis of cell viability suggests that elevated Akt activation in *Ahr*$^{-/-}$ MLFs could be a compensatory mechanism needed for cell survival during serum starvation. We also show that basal Akt activation in MLFs was dependent on PI3K, but the link between AhR and the PI3K/Akt pathway needs further investigation. We did not identify any well-known Akt substrates that were differentially phosphorylated between *Ahr*$^{+/+}$ and *Ahr*$^{-/-}$ MLFs; however, we found that AhR may regulate the phosphorylation of several proteins in MLFs, including those that can potentially interact with Akt and regulate its activity. Our findings have raised intriguing questions about how AhR may regulate protein phosphorylation in mammalian cells, which would be exciting avenues of research for future studies. Further molecular verification of the proteomic changes caused by *Ahr* ablation will lead to the identification of novel interactions between AhR and other cellular pathways and advance our understanding of the physiological functions of AhR.

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