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Effects of Prenatal Exposure to Bisphenol A on Fetal Lung Development

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A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Pharmacology and Toxicology
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Abstract

Prenatal exposure to bisphenol A (BPA), one of the most prevalent endocrine disrupting chemicals, is associated with lung dysfunction and diseases in later life. However, it is unknown if this association has a fetal origin. In this thesis, a series of *in vivo* and *in vitro* experiments were conducted to determine the effects of prenatal exposure to BPA on fetal lung development, and define the underlying molecular mechanisms. Environmentally relevant doses of BPA were administered to pregnant mice via diet from embryonic day (E) 7.5 to 18.5. Fetal lungs were analyzed at E18.5 for changes in structure and expression of key molecular markers of lung maturation. My main findings were: ***(a)*** BPA severely retards fetal lung maturation as evidenced by diminished alveolar airspace and thickened septa, hallmarks of lung immaturity; ***(b)*** this immaturity is characterized by aberrant alveolar epithelial type I cell differentiation; and ***(c)*** the effects of BPA are likely mediated through the glucocorticoid signaling pathway, because the expression of epithelial sodium channel γ (ENaCγ), a well-known glucocorticoid receptor (GR) target gene, is down-regulated in BPA-exposed fetal lungs. Moreover, maternal administration of dexamethasone rescues the lung immaturity phenotype. However, the precise molecular mechanisms by which BPA represses ENaCγ in lung cells were unknown. This important question was addressed using the A549 human lung epithelial cell line as an *in vitro* model system. I found that that ***(d)*** both dexamethasone and siRNA-mediated knockdown of GR expression blocked the inhibitory effects of BPA on ENaCγ expression, indicating that BPA suppresses ENaCγ via inhibition of GR activity. Given that BPA is known to function as a pro-inflammatory factor via the estrogen receptor β (ER β), and a mutual antagonism exists between the pro-inflammatory transcriptional factor NF-κB and GR, I then explored and provide evidence supporting the notion that ***(e)*** BPA acts on ER-β to activate the NF-κB signaling pathway, which in turn leads to diminished GR activity and consequent repression of ENaCγ expression in lung cells. Taken together, these findings demonstrate that prenatal exposure to BPA disrupts fetal lung maturation, and suggest a fetal origin for BPA-induced lung dysfunction and diseases.

Keywords

Endocrine-Disrupting Chemical, Bisphenol A, Fetal Exposure, Fetal Development, Lung Maturation, Reduced Lung Function, Lung Disease, Glucocorticoid Signaling Pathway, NF-κB signaling pathway, Estrogen Receptor-β signaling pathway

Co-Authorship Statement

Chapter 3:

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AH, GH, and KY designed the experiments. AH was responsible for animal care, blood and tissue collection and conducted the experiments. AH and KY analyzed and interpreted the data. AH, MC, and KY wrote the manuscript. All authors approved the final version of the manuscript.

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AH, GH, and KY designed the experiments. AH conducted the experiments. AH and KY analyzed and interpreted the data. AH and KY wrote the manuscript. All authors approved the final version of the manuscript.

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List of Abbreviations

11β-Hsd II	11β-hydroxysteroid dehydrogenase-II
AEC	Alveolar epithelial cell
AQP5	Aquaporin 5
AR	Androgen receptor
BPA	Bisphenol A
BPA-GA	Monoglucuronide conjugate of BPA
COPD	Chronic obstructive pulmonary disease
CRH	Corticotrophin-releasing hormone
DES	Diethylstilbestrol
DNA	Deoxyribonucleic acid
E	Embryonic day
EDC	Endocrine disrupting chemical
ENaC	Epithelial sodium channel
EPA	Environmental protection agency
ER	Estrogen receptor
Gapdh	Glyceraldehyde 3-phosphate dehydrogenase
GCs	Glucocorticoids
GI	Gastrointestinal
GPX	Glutathione peroxidase

GR	Glucocorticoid receptor
HSP	Heat shock protein
IκB-α	Nuclear factor of kappa light polypeptide gene enhancer in B-cell inhibitor-α
IP	Intraperitoneal
LOAEL	Lowest observed adverse effect level
LOQ	Limit of quantification
mRNA	Messenger ribonucleic acid
Mrp-2	Multi-drug resistance associated protein-2
N-CoR	Nuclear receptor co-repressor 1
NF-κB	Nuclear factor of kappa light polypeptide gene enhancer in B
NMDRC	Non-monotonic dose response curves
NOAEL	No observed adverse effect level
Oatp	Organic anion-transporting polypeptide
PAS	Periodic acid-Schiff
PCNA	Proliferating cell nuclear antigen
PD	Postnatal day
RDS	Respiratory distress syndrome
SC	Subcutaneous
siRNA	Small interfering ribonucleic acid

Snx5	Sorting nexin 5
SP	Surfactant Protein
SULT	Sulfotransferase
TR	Thyroid hormone receptor
UGT	Uridine diphosphate glucuronosyltransferase

1 INTRODUCTION

1.1 Bisphenol A

An endocrine disrupting chemical (EDC) is defined by the U.S. Environmental Protection Agency (EPA) as "an exogenous agent that interferes with the production, release, transport, metabolism, binding action, or elimination of natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes"[1]. Exposure to EDCs has been associated with a range of human diseases including cancer, diabetes, obesity, and decreased fertility[2–4]. Bisphenol A (BPA), one of the most prevalent EDCs in the environment, has gained world-wide attention due to growing evidence of its association with adverse health effects in humans and experimental animal models[4,5]. BPA is an industrial chemical used to manufacture polycarbonate plastics, epoxy resins lining the inside of food and beverage containers, thermal print papers and composites used in dental sealants[6–8]. Almost seven billion tonnes of BPA were produced in 2013[9], making it one of the highest volume chemicals produced worldwide. The pervasiveness of BPA is such that it is detected in the air, water and soil, making human exposure to BPA unavoidable[4,10]. Exposure to BPA occurs through multiple routes including inhalation, dermal absorption, and ingestion, which is the most common route of exposure[4,10]. Thus, it is not surprising that over 95% of Canadians have detectable levels of BPA in their urine[11].

BPA was first synthesized in 1891 by A.P. Dianin and was investigated in the 1930s for potential pharmaceutical use during the search for synthetic estrogens[8]. Although BPA has estrogenic activity, its pharmaceutical use was abandoned when Diethylstilbestrol (DES) was found to be a far more potent estrogen[8]. The commercial use of BPA developed with the plastics industry in the 1940s and 1950s, where it was used as a building block of polycarbonate plastics and epoxy resins[8]. Although BPA is a synthetic estrogen, its endocrine disrupting effects are not limited to the estrogen system, but extend to other endocrine systems including those mediated by glucocorticoids, androgens, thyroid hormones, and insulin, among others[8].

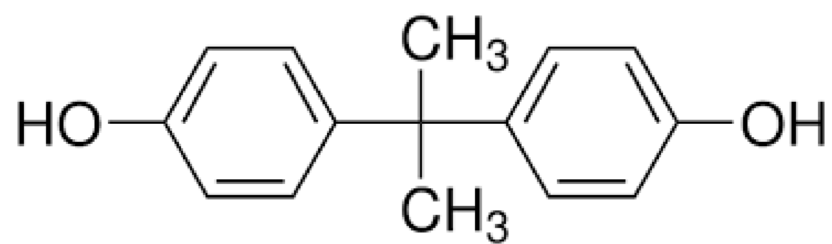

Figure 1.1: Structure of Bisphenol A[8].

1.1.1 Mechanisms of Action

ER: Since BPA was initially synthetized as a synthetic estrogen, initial studies on the mechanisms of BPA actions focused primarily on its effects on the estrogen receptor (ER). The ER is mainly located in the cell nucleus[12,13]. Once bound to estrogenic compounds, the ER undergoes conformational changes and binds to estrogen response elements in the promoter region of estrogen target genes, thereby activating gene transcription[12,13]. In mammals, the two major ER subtypes are ERα and ERβ[14]. Biochemical assays have demonstrated that BPA can bind to both ERα and ERβ, with a 10-fold higher affinity for ERβ[15]. Moreover, BPA acts as an agonist on ERβ, while demonstrating both agonist and antagonist effects on ERα[16]. In addition to acting via ERα and ERβ, BPA can also act on non-classical ERs such as binding to the membrane-bound G-protein coupled receptor 30, and the orphan nuclear receptor estrogen-related receptor γ [15,17]. BPA has been classified as a weak estrogen, binding to ERs with 1,000-10,000 fold lower affinity than estradiol[15,17–19]. This has led to the notion that BPA might be unable to create an impact in the midst of the already strong effects of endogenous estrogens. However, Rajapakse et al. demonstrate that the combination effect of estradiol and BPA is in fact additive[18]. The spare receptor hypothesis describes how typically, a maximal biological response can be achieved by low concentrations of a hormone, and well before receptor occupancy becomes saturated[20]. The presence of these “spare receptors” provides a mechanism for why low doses of an EDC such as BPA might exert a response, regardless of its low affinity for the receptor[15]. In addition, BPA has been shown to interact with ER in a non-classical manner that is distinct from other known groups of ER ligands[15,19], leading to the induction of a unique subset of ER-responsive genes and associated physiological responses[21]. Differences have also been noted in the recruitment of transcriptional co-regulators, indicating that BPA is not merely an estrogen mimic[8].

GR: Additional endocrine related effects of BPA include potential actions on the glucocorticoid receptor (GR)[8,22,23]. The GR belongs to the superfamily of nuclear receptors[24,25], acting as a steroid-hormone activated transcriptional factor, and is expressed by almost all cell types[25,26]. Glucocorticoids (GC), cortisol in humans and corticosterone in rodents, are the physiological ligands for GR[25,26]. GCs play a critical role in regulating

various physiological processes such as glucose homeostasis, cell differentiation, lung maturation, and inflammation[25,26]. In the absence of GCs, the inactive GR is sequestered in the cytoplasm bound within a large multiprotein complex that includes heat shock proteins (HSP) 90 and 70[26]. These proteins contribute to GR stability by ensuring proper protein folding and conformation, maintaining the receptor in a high affinity binding state and stabilizing the chaperone receptor complex[27]. When GCs bind to the GR, chaperones are released and the receptor dimerizes and translocates to the nucleus. In the nucleus, the GR binds to DNA promoter elements and can either activate or repress transcription of target genes[22]. Recent findings indicate that BPA can directly bind to the GR as both an agonist and antagonist, depending on the cell type[22,23,28]. However, the molecular mechanism of the direct effect of BPA on the GR signaling pathway remains unclear. In addition to the direct effects of BPA on GR, recent studies suggest that BPA may potentially alter the GR signaling pathway through indirect mechanisms.

NF-κB: Nuclear factor- κB (NF-κB) is a widely expressed inducible transcription factor that is of particular importance in the immune response, as it positively regulates the expression of many genes involved in inflammatory responses[29,30]. NF-κB is a heterodimer, typically consisting of p50, and a transcriptionally active p65 subunits[31]. It is latently present in the cytoplasm, under tight control by its associated inhibitory protein IκB-α, which binds to and sequesters it in the cytosol, preventing the nuclear translocation of NF-κB and its interaction with DNA to induce gene expression[29–31]. Interestingly, when different cell types were treated with BPA, a significant increase in both the expression and activity of NF-κB was detected[32–39], concomitant with a decrease in IκB-α expression[32]. Recent studies suggest that BPA increased NF-κB activity through binding to, and activating ERβ, suggesting that BPA may function as a pro-inflammatory inducer via ERβ[36,37].

Since the potential of synthetic GCs as immunosuppressive anti-inflammatory agents was discovered, increased focus has been directed to the mechanisms by which GCs act to suppress the NF-κB pathway[31,40–42]. Indeed, a mutual antagonism has been described between GR and NF-κB, whereby NF-κB represses GR and *vice versa*[43–46]. Since BPA has been shown to increase NF-κB activity, this provides a possible mechanism of action of

the repressive effects of BPA on the GR signaling pathway. Additional work is required to elucidate the molecular mechanisms of how BPA alters the GR signaling pathway, and whether NF-κB activation is involved in this process.

Other receptors: Other endocrine-related activities of BPA include its antagonistic actions on the androgen receptor (AR), another receptor that belongs to the superfamily of nuclear receptors. Results of several *in vitro* studies have suggested that BPA may have anti-androgenic effects at multiple steps of AR activation and function[47–50]. This is demonstrated through BPA inhibition of AR interaction with its coactivator, androgen binding of AR, AR nuclear translocation, and androgen-induced AR transcriptional activity[51]. Androgens are crucial for normal male sexual development and reproduction as they stimulate masculinization of the fetus and induce male imprinting of the developing brain[52]. As such, interference with androgen action during development can result in abnormalities of the male reproductive system. BPA also has been shown to disrupt the thyroid hormone pathway[8]. BPA binds to thyroid receptor (TR) and can act as an antagonist to inhibit transcriptional activity stimulated by thyroid hormone (triiodothyronine-T3)[53,54]. In addition, data from *in vitro* studies have demonstrated the ability of low levels of BPA to inhibit thyroid hormone receptor-mediated gene expression by enhancing recruitment of the nuclear receptor co-repressor (N-CoR) to the TR[54]. Collectively, these findings highlight the potential for BPA to disrupt several endocrine pathways and indicate its ability to commandeer multiple mechanisms and pathways upon exposure.

Epigenetic alterations: The epigenome is particularly susceptible to dysregulation during embryogenesis, as DNA methylation and chromatin patterning required for normal tissue development is programmed during early development. Accumulating evidence indicates that developmental exposure to BPA is thought to alter the epigenome[8,55]. Several *in vivo* studies have demonstrated changes in DNA methylation following early life exposure to BPA[55,56]. For example, when exposed to BPA prenatally and during lactation, viable yellow agouti (Avy) mice showed epigenetic alterations manifested as a shift in coat color towards yellow[55]. The shift in coat color is associated with decreased DNA methylation in 9 CpG sites examined in the promoter region of the *Avy* gene and in additional loci[55]. Effects of developmental exposure to BPA on the epigenome have also been reported in

organs such as the forebrain[56], uterus[57], and prostate[58], where BPA exposure resulted in changes in DNA methylation. Taken together, these studies provide compelling evidence that early developmental exposure to BPA can change the offspring's phenotype by altering the epigenome. Since numerous studies indicate that several chronic adult diseases and disorders result from epigenetic alterations early in development, early life exposure to BPA poses a risk to the development of chronic diseases later in life.

1.1.2 Toxicokinetics

The bioavailability of BPA, defined as the amount of parent compound reaching the systemic circulation, and biological activity of BPA are largely dependent on the route of exposure, which include oral ingestion, dermal contact, and inhalation[20]. While oral exposure to BPA is subject to first-pass metabolism in the gut and liver, other routes of exposure bypass these organs, leading to higher concentrations of active BPA in circulation[59]. Since BPA leaches into food from plastic packaging and resin linings of food and beverage containers, it has been generally assumed that the consumption of contaminated food and beverages represents the major route of human exposure. However, new sources of exposure continue to be discovered. For example, thermal receipts used for daily transactions have been shown to contain high levels of free BPA, raising the possibility that transdermal absorption may be a significant source of exposure[60]. Indeed, a recent study found rapid transfer of free BPA (due to holding a thermal receipt for two seconds) from the surface of thermal receipt paper to the hand, providing evidence that exposure to free BPA through contact with thermal paper could be an important factor in accounting for the high levels of bioactive serum BPA reported previously in human biomonitoring studies[61]. BPA is also present in cigarette filters, so smoking may be another previously unrecognized source of exposure[60]. Blood concentrations of bioactive BPA are significantly higher, indicative of higher bioavailability, in rodents following subcutaneous (SC) or intraperitoneal (IP) injections compared with oral exposure[59]. These findings demonstrate the importance of the route of exposure in determining BPA potency and explain the more detrimental effects of BPA administered via SC or IP routes even at much lower doses.

Since exposure to BPA is thought to occur primarily through ingestion of contaminated water or food[6,8], the toxicokinetics of orally administered BPA has been studied extensively in rodents, non-human primates, and humans. After oral administration, BPA is absorbed from the gastrointestinal (GI) tract where it undergoes pre-systemic Phase II metabolism by intestinal enterocytes or by hepatocytes after transport to the liver[62,63]. Phase II metabolism increases the polarity of lipophilic compounds by conjugation reactions through the attachment of an ionized group to the compound. The resultant metabolites are less active than the parent compound. The active form of BPA (unconjugated BPA) is rapidly metabolized to its inactive metabolite, BPA glucuronide (BPA-GA), by uridine diphosphate glucuronosyltransferase (UGT) enzymes that are found in the endoplasmic reticulum of enterocytes and hepatocytes[64]. These enzymes, most notably UGT2B15[64], catalyze the conversion of lipophilic compounds, such as BPA, to charged, water-soluble glucuronides[6,65]. Sulfonation of BPA via sulfotransferase (SULT1A1) enzymes accounts for other minor metabolites of BPA[6,65].

Previously, it has been suggested that orally ingested BPA is rapidly inactivated by UGT enzymes in the liver. However, this postulation was based on a single study that failed to detect unconjugated BPA in the plasma and urine of humans[66]. This study was characterized by several limitations, which include a small sample size (three woman and six men) as well as the use of a limit of quantification (LOQ; the smallest concentration of a substance that can be reliably measured by an analytical procedure) that was more than 10 times higher than those employed in other published studies using similar techniques[66]. In fact, recent studies using non-human primates[63] and rodents[67] demonstrate that first-pass metabolism of BPA after oral administration, although rapid, it is not complete. For example, the detection of free BPA in human urine samples in biomonitoring studies indicates internal exposure to active BPA[6,68]. These studies suggest that a portion of ingested BPA bypasses first-pass metabolism and/or that BPA metabolites, mainly BPA-GA, are de-conjugated in the body[20]. Indeed, several tissues express the enzyme β-glucuronidase, which has the ability to de-conjugate and re-activate conjugated BPA[10,69]. For instance, studies using rodents show that conjugated BPA is de-conjugated by β-glucuronidase in the lower intestine and colon[10]. Since β-glucuronidase is expressed in the

digestive tract of humans, especially those of infants, it is possible that conjugated BPA may be de-conjugated and activated during digestion[70].

The kinetics of BPA metabolism are remarkably similar in humans, non-human primates, and rodents, as demonstrated by detection of comparable levels of BPA in serum following oral administration[62]. Nonetheless, a key difference amongst these species is the route of BPA elimination[63]. In rodents, BPA-GA is subjected to enterohepatic recirculation and is primarily excreted in feces, with only small amounts excreted in urine[70]. Enterohepatic recirculation prolongs the elimination process, thereby increasing internal exposure to BPA-GA. In contrast, humans and non-human primates eliminate BPA from circulation more rapidly than rodents, with no evidence of enterohepatic recirculation, primarily excreting BPA-GA via urine within six hours[70]. This species difference in the route of elimination prompted some investigators to emphasize the importance of enterohepatic recirculation in rodents as a critical factor resulting in higher serum levels of BPA-GA relative to humans and non-human primates after a similar oral dose. However, Taylor et al. reported that the increase in concentration of free BPA in the serum of mice relative to that of primates/humans following oral exposure was not statistically significant[63]. Thus, although findings from rodent studies assessing the potential risks to humans should be interpreted with caution, they should not be discounted. The difference in the route of elimination of BPA between rodents (primarily via the feces) and primates (primarily via the urine) has also been incorrectly interpreted as supporting the prediction of a different level of clearance of BPA. However, Taylor et al. clearly demonstrate that in rhesus monkeys and mice, the rate of clearance of free BPA from serum during the 24 h after oral BPA administration is virtually identical[63]. These findings are consistent with other studies that also reported comparable pharmacokinetics of BPA between humans and rodents[20,63]. As a result, regulatory agencies have deemed the rodent model as a valid predictor to assess the potential effects of BPA in humans[20].

1.1.3 Dose-Response and Low-Dose Effects

The traditional toxicological concept that "the dose makes the poison" implies that the higher the dose of the drug/toxin, the greater the harm. It further suggests that effects that are not observed at high doses will similarly not be observed at low doses. This model

generates a “safe” dose by assessing different doses of a chemical until the lowest observed adverse effect level (LOAEL) and/or the no observed adverse effect level (NOAEL) are determined[20]. The discovery of EDCs challenges this model. One of the characteristics of EDCs, similar to naturally occurring hormones, is that they produce non-monotonic dose response curves (NMDRCs)[71]. Monotonic dose response curves (also known as the threshold model) can be both linear and non-linear. In the linear model, treatment with increasing doses of a drug has no effect until the “threshold” dose is reached, at which point an increase in response is observed[20]. In the non-linear model, a response occurs even at the lowest treatment dose, and therefore effects at high doses can be used to predict responses at low doses[20]. On the other hand, in a NMDRC, an increase in the dose does not necessarily correspond to an increase in response[20,71]. The dose response curve may be U-shaped with high responses at low and high levels of exposure, or as an inverted U, with high responses at intermediate levels of exposure[20]. Low-dose effects of BPA that cannot be predicted by the response seen at high doses of exposure, demonstrate that BPA produces a NMDRC[71,72]. Since the actions of BPA at low doses cannot be inferred by the presence or absence of a response of BPA at higher doses, the traditional approach of using high dose testing to assess chemical safety at low doses is particularly problematic for BPA.

In toxicology studies, a reference dose, defined as the maximal acceptable dose of a compound that is considered safe for human exposure, is typically calculated using the NOAEL[20]. However, in the case of BPA, the reference dose for human exposure was calculated using the LOAEL. This is because a NOAEL could not been determined, as adverse effects of BPA were detected even at the lowest dose administered[20]. This further supports the notion that exposure to BPA, even at very low doses, can have a wide range of adverse effects in humans. This is very alarming as the scientific literature shows that human exposure to BPA is within the range predicted to be biologically active in over 95% of individuals sampled[11]. Moreover, extensive evidence from animal studies indicate that some outcomes may not become apparent until long after BPA exposure has occurred, especially those effects resulting from exposure during development. These developmental effects are irreversible and can occur due to low-dose exposure during brief sensitive periods in development, even though BPA may not be detected during the time at which the damage or disease is expressed. Indeed, it is evident that the effects of BPA vary with

the dose and time of exposure. This is especially the case when exposure occurs during the prenatal and neonatal period, the most vulnerable window of exposure as it marks the critical period of organogenesis. As a result, exposure to BPA during perinatal development may have permanent adverse effects on the developing fetus.

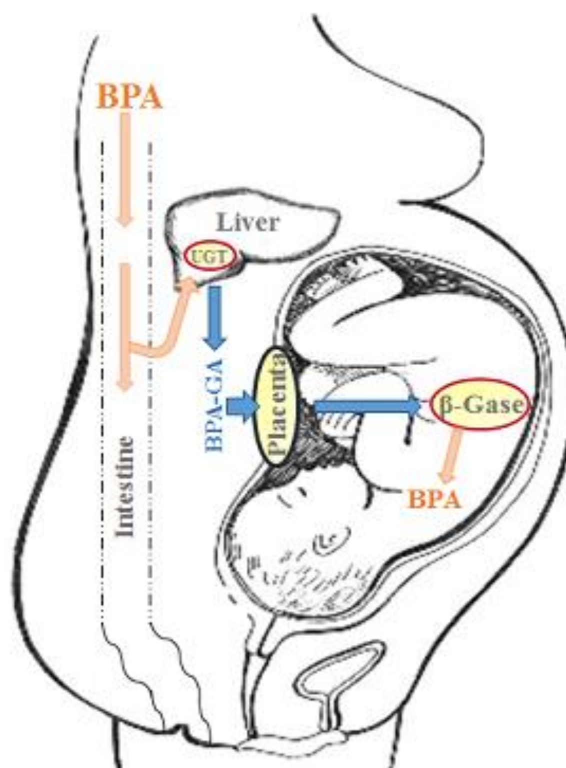

Figure 2.2: The predicted mechanism of adverse effects on the fetus induced by maternal BPA exposure during pregnancy. Following oral ingestion, BPA is conjugated in the liver and released into maternal blood. BPA-GA is transferred across the placenta, by the help of placental transporters (i.e. Oatp and Mrp transporter families) found in trophoblast cells, into the fetus. Certain fetal organs, such as the lung, liver, and heart, have high β-glucuronidase (β-Gase) activity, which de-conjugates BPA. De-conjugated BPA may remain in the fetus because of a deficiency in fetal UGT activities. Active BPA is capable of altering the development of target organs such as the lungs.

1.2 BPA during Pregnancy

While exposure to EDCs following puberty is an important factor in adverse health outcomes, the primary concern is fetal/neonatal exposure to EDCs since developing organisms are extremely vulnerable to perturbation by chemicals with hormone-like activity. Adverse effects from adult exposure to EDCs are usually reversible and are therefore termed "activational" effects[73]. On the other hand, exposure to EDCs during organ development, beginning in the prenatal period and continuing through puberty, may result in persistent "organizational" effects where the affected systems are altered even in the absence of subsequent exposure[73]. Furthermore, although exposure to BPA in adulthood requires relatively large doses to induce adverse response (in the milligrams per kilogram of body weight range), *in utero* exposure to much lower doses (in the nanograms per kilogram of body weight range) can induce alterations in organs of the fetuses that are observed later in life. As a result, adverse effects in the developing fetus may occur at concentrations that are far below levels that would be considered harmful in adults. Since BPA can readily cross the placenta and reach the developing fetus[69], investigations of the effects of prenatal exposure to low doses of BPA concluded that BPA can alter the development of several organs including the brain[74,75], heart[76], mammary glands[77–79], ovaries[80,81], uterus[82], and testes[83]. Such defects or malformations are not usually apparent at birth, but rather manifest as subtle functional changes (i.e. altered gene expression, increased cell differentiation, proliferation, and apoptosis) that increase the risk of diseases in adult life.

1.2.1 Developmental Origins of Adult Disease

The British epidemiologist David Barker hypothesized in 1990 that the interactions between the developing fetus and the environment determine the susceptibility of that individual to develop metabolic disease later in life[84,85]. Subsequent epidemiological investigations strongly supported the Barker hypothesis, finding that adverse events *in utero* (i.e. maternal hypoxia, undernutrition, infection, stress) can permanently alter physiological processes in adult life[86–88]. These clinical and animal studies sought to understand the role of the fetal environment in the long-term regulation or 'programming' of adult diseases[87,88]. Moreover, these studies attempted to elucidate the molecular

mechanisms involved in the etiology of these diseases in adult life. An understanding of the role of the perinatal and/or postnatal environment on long-term health can lead to the development of targeted therapeutic intervention strategies to prevent the onset of diseases in adulthood.

There are concrete examples of diseases that manifest in adulthood that originated from adverse events during fetal development[87,88]. For instance, pregnant women who were administered DES, a classified EDC, to reduce the risk of pregnancy complications were unharmed by the drug. However, their daughters who were exposed *in utero* had significantly increased rates of clear cell adenocarcinoma in their reproductive tissues[8,89]. They also suffered structural abnormalities in the development of their reproductive tract, infertility, and higher rates of pregnancy complications such as spontaneous abortion, ectopic pregnancies, and pre-term delivery. Alarmingly, the sons of women who took DES had higher rates of non-cancerous epididymal cysts than found in the general population, and this trend continued in the sons of DES daughters[90]. It is apparent that the adverse effects of EDCs on organ systems are critically dependent on the timing of exposure. Consequently, the vulnerability of the fetus and neonate to the adverse effects of BPA has become the focus of intensive investigations. Perinatal and/or postnatal exposure to BPA at doses below the established LOAEL have revealed effects on behavior, reproductive tissues, and altered glucose homeostasis in both humans and rodents[8,20].

1.2.2 Fetal Exposure to BPA

Relatively high levels of active BPA have been detected in umbilical cord blood, amniotic fluid, fetal blood, and blood of pregnant women[69,91–93], indicating that BPA readily crosses the maternal-fetal placental barrier and can act on the fetus during critical periods of development. Indeed, studies that examined the transfer of BPA across the placenta show that active BPA crosses the placenta bi-directionally, mainly via passive diffusion, due to BPA's high lipid solubility[69,91,92]. This finding was confirmed in rodent studies where pregnant rats were exposed to ^{14}C-BPA[69]. Following exposure, both active and conjugated BPA were detected in placentas and fetuses. Since the placenta expresses both UGT and SULT enzymes[94], it is possible that BPA is conjugated to the inactive form before reaching the fetus. However, in studies that explored this possibility, only negligible amounts of

BPA were conjugated by the placenta, suggesting that the fetus is exposed to the active form of BPA[94].

Due to alterations in the drug metabolizing and transporter systems that occur during pregnancy, the physiological state of a pregnant mother and fetus differs from that of non-pregnant individuals[69]. For example, the hepatic expression of the multi-drug resistance associated protein 2 (Mrp2), which plays an important role in excreting conjugates of lipophilic substances, is reduced during pregnancy in the rat liver[95,96]. This might account for the increased concentration of BPA in maternal blood during the entire gestational period[3,69], which potentially increases the risk of transferring conjugated BPA across the placenta[97]. In addition to the active form of BPA crossing the placenta through passive diffusion, conjugated BPA can actively cross the placenta with the help of organic anion-transporting polypeptide (Oatp) and Mrp transporter family members[69,96]. BPA-GA is carried from maternal blood vessels to trophoblast cells by the Oatp4a1 (influx) transporter, and then transported to the fetus via the Mrp1 (efflux) transporter[69,98,99]. Since the conjugated form of BPA is water soluble, it cannot cross the placenta once excreted in fetal urine, thus becomes trapped in the amniotic fluid with the potential to be swallowed and re-circulated in the fetus[97]. Although BPA-GA is an inactive metabolite, fetal tissues possess the ability to de-conjugate BPA-GA to active BPA via the enzyme β-glucuronidase[69,97]. The majority of de-conjugation occurs in the fetal liver, also a major site of BPA accumulation. In addition, although the presence of UGT isoforms has been observed in fetal liver, the levels are considerably below those observed in neonates and adults[70]. Due to the low expression of UGT enzymes in the fetus, fetal liver microsomes will have limited ability to detoxify BPA through glucuronidation[69,70]. Consequently, free BPA may be detected in fetal circulation as a result of the high de-conjugation ability of β-glucuronidase, in combination with the limited drug metabolizing system of the fetus that further exacerbates fetal exposure to BPA.

Pharmacokinetic disposition studies, which examine the tissue distribution of BPA, conclude that BPA is able to distribute to various organs. Interestingly, BPA accumulated in the lung most extensively, followed by kidneys, heart, liver, and brain, when expressed in BPA concentration terms (i.e. amount accumulated per gram organ weight)[100].

Accumulation of BPA in the lungs may have severe implications. Indeed, exposure to BPA is associated with lung dysfunction and is associated with various lung diseases in both animal models as well as in clinical investigations[101–112]. However, it remains elusive if this association has a fetal origin.

1.3 The Lungs

The mammalian respiratory system consists of the lungs, trachea, and vasculature[113]. The lungs are air-filled organs that are divided into lobes; the right lung encompasses three lobes, while the left has two lobes to accommodate the heart. The lungs fulfill multiple functions, of which the most critical is to facilitate gas exchange. Structurally, the lung consists of two intertwined and highly branched tree-like tubular systems, one conducting air and the other blood[113,114]. While this arrangement is highly efficient, breathing air is not without risks, such as exposure to airborne pathogens and dehydration[115]. In fact, respiratory disorders are among the most common reasons for doctor visits in industrialized countries, where there are higher concentrations of air-borne chemicals, pollutants, bacteria, and viruses[114].

1.3.1 The Structure and Physiological Function of the Lungs

Anatomically, the respiratory system is divided into the upper and lower respiratory tracts. Functionally, these structures make up the system's conducting portion, which consists of the nasal cavities, nasopharynx, larynx, trachea, bronchi, bronchioles, and terminal bronchioles; and a respiratory portion where gas exchange takes place, consisting of respiratory bronchioles, alveolar ducts, and alveoli[113,114]. The conducting portion serves two main functions: to provide a conduit through which air moves to and from the lungs and to condition the inspired air. To ensure an uninterrupted supply of air, a combination of cartilage, elastic and collagen fibers, and smooth muscle provides the conducting portion with rigid structural support and the necessary flexibility and extensibility[113].

Airflow into the lungs starts at the nose and mouth, and proceeds within the thoracic cavity via the trachea, which divides into left and right main primary bronchi. Each bronchus branches multiple times into progressively narrower and shorter bronchi which contain cartilage and mucous secreting glands in their walls. The bronchi branch further into

bronchioles, which are small (<2 mm diameter) thin-walled membranous airways that lack cartilage and glands[113]. Both bronchi and bronchioles are lined with a ciliated pseudostratified columnar epithelium, also known as the respiratory epithelium, where the cilia on the ciliated columnar cells beat in a coordinated fashion to impel an overlying layer of mucous toward the pharynx, where it is swallowed[113,114,116]. This system, known as the mucociliary blanket, plays an important role in cleansing the lungs of inhaled particles and microorganisms that deposit on the mucous lining of airways[115]. Goblet cells, brush cells and other cells in the respiratory epithelium also aid in this process. Table 1.1 summarizes each cell type and their respective functions.

Tubular branching continues further into the distal regions of the lung where the terminal bronchioles branch into respiratory bronchioles, then alveolar ducts until millions of individual lung units, known as alveoli, generate a very large surface area suitable for gas exchange. The alveoli have a continuous lining of simple squamous epithelium that is composed of connecting cells, known as alveolar epithelial cells (AECs). This epithelium is composed of two types of AECs. Type I cells are flattened with a large surface area to facilitate gas exchange, and cover about 95% of the alveolar surface while constituting only 40% of all epithelial cells[117,118]. Type II cells account for the remaining 60% of cells lining the alveoli, but represent only 5% of the alveolar surface, because of their cuboidal shape[117,118]. Type II cells produce and secrete pulmonary surfactant, composed of a complex of phospholipids (mainly phosphatidylcholine) and several surfactant associated proteins (SP): SP-A, SP-B, SP-C, and SP-D[119,120]. The hydrophobic SP-B and SP-C proteins enhance the spreading, absorption, and stability of surfactant phospholipids[118,119]. Whereas the SP-A and SP-D proteins are important factors in host defense and are less important in the processing of surfactant[118,121]. Surfactant is essential in reducing surface tension at the air-liquid interface of the alveoli and prevents alveolar collapse during exhalation, thereby optimizing lung compliance and facilitating the work of breathing[120]. Lack of pulmonary surfactant, whether caused by premature birth, lung injury, or mutations in genes critical to surfactant production or function, causes respiratory failure[119,120,122]. In addition to type I and type II cells, some resident alveolar macrophages are found within the alveolar space, where they act as a first line of defense against pathogens[115,123]. Finally, endothelial cells of the pulmonary capillaries occupy nearly 88% of the alveolar walls[124].

The close proximity of the capillary endothelial cells and the epithelial cells of the alveoli promotes diffusion of oxygen and carbon dioxide between the vast network of alveoli and the capillaries that envelop them.

Table 1.1. The different lung cell types and respective functions.

Cell Type	Location	Function
Clara cells	Trachea and Bronchi	- Secrete glycosaminoglycans: protects the bronchial epithelium
Basal cells	Trachea and Bronchi	- Attach the columnar epithelium with the basement membrane - Progenitor cells of the airway epithelium (following injury)
Ciliated cells	Trachea and Bronchi Bronchioles Bronchioalveolar duct junction	- Work in tandem with goblet cells to propel mucus away from the lungs
Neuroendocrine cells	Trachea and Bronchi Bronchioles	- Chemoreceptors for hypoxia detection - Regulate epithelial cell growth and regeneration
Dendritic cells	Trachea and Bronchi Bronchioles	- Innate and adaptive immunity
Goblet cells	Bronchioles	- Secrete mucus: maintains epithelial moisture and traps particulate material and pathogens from moving through the airways
Fibroblasts	Alveoli	- Airway inflammation and airway remodeling
Type II cells	Alveoli	- Secretion of pulmonary surfactant - Progenitor cells of the alveoli (following injury)
Type I cells	Alveoli	- Gas exchange
Macrophages	Alveoli	- Host defense - Innate immune response

1.4 Fetal Lung Development

Lung development during gestation is divided into two phases; lung growth (structural development) and lung maturation (functional development)[116,125]. Lung growth includes the progressive branching of the airways and the development of alveolar airspaces capable of gas exchange[123,125]. This phase is followed by lung maturation and the achievement of functionality, which is primarily a biochemical process largely regulated by hormones[125]. Survival at birth depends on (1) the architecture of the peripheral pulmonary acini and the maturation of the gas exchange region, (2) differentiation of type I cells and their close interface with endothelial cells of the pulmonary microvasculature system, (3) synthesis and secretion of surfactant lipids and proteins by type II cells, and (4) clearance of lung fluids to establish ventilation[116,125]. Successful lung development requires the completion of both structural and functional development phases. Incomplete development of lung structure and/or premature birth prior to the development of the surfactant system will lead to respiratory compromise and/or insufficiency in the newborn[124].

The embryonic lung is formed by the process of branching morphogenesis generating the conducting airways that lead to peripheral saccules from which alveoli are produced during the perinatal and postnatal periods of lung development[123]. In late gestation and after birth, septation and continued lung growth creates the extensive alveolar surfaces mediating efficient gas exchange. Five developmental stages have been delineated in fetal lung development, based on anatomic and histologic characteristics[116,117,123,125,126]. The early embryonic and pseudoglandular stages are characterized by the conducting airways; the later canalicular, saccular, and alveolar stages are characterized by reduction of mesenchyme and increased vascularization to form a thin air-blood barrier. The development of a highly branched vascular system occurs in concert with the development of the respiratory system. Birth does not signal the end of lung development, especially in humans, as after birth there is a continuing process of lung growth until childhood (approximately 8 years of age)[117,127].

1.4.1 The Embryonic Stage

The first stage of mouse fetal lung development is the embryonic stage, which is characterized by organogenesis. The respiratory system arises from the anterior foregut endoderm, a tissue that generates multiple organs including the esophagus, thyroid, and liver[116]. In the mouse, lung specification begins at embryonic day (E) 9.0 following the expression of the transcription factor Nkx.2.1 in endodermal cells on the ventral side of the anterior foregut[116,125,126]. By E9.5, evagination of these epithelial cells results in the formation of the trachea, which completes its separation from the esophagus, splitting the foregut into the esophagus and trachea with two lung buds, and initiating the embryonic stage of lung development (E9.5-E12.5). The lung buds are lined with an endodermally-derived epithelium, which further differentiates into both the respiratory epithelium that will line the airways and the specialized epithelium that will line the alveoli. The lungs are innervated by the ectoderm, while the mesoderm supplies the pulmonary blood vessels, smooth muscle, cartilage, and other connective tissue.

1.4.2 The Pseudoglandular Stage

The pseudoglandular stage (E12.5-E16.5 in the mouse) is characterized by branching morphogenesis, which is essential for forming both the structural airways as well as the terminal alveolar compartments in which gas exchange occurs[116,125,126]. At E12.5, the two primary lung buds undergo a highly regulated branching process and begin to extend into the surrounding mesenchyme. This process depends on multiple signaling pathways present in the mesenchyme, including the Wnt, bone morphogenetic protein (Bmp), and fibroblast growth factor (Fgf) pathways, which regulate lung specification, branching and patterning[126]. For that reason, the endodermal lung buds must be exposed to the mesenchyme in order to undergo branching morphogenesis. Additionally, the rate and extent of branching is directly proportional to the amount of mesenchyme present at the time. At the end of this stage, a tree-like network of airways with thousands of terminal branches are formed and following this point, further growth occurs by elongation and widening of existing airways only. Furthermore, towards the end of this stage proximal progenitor cells begin to give rise to clara, ciliated, and neuroendocrine cells. Distal

progenitor cells also begin to appear that will differentiate into the alveolar epithelial cells at the later stages of lung development.

1.4.3 The Canalicular Stage

In the murine model, between E16.5-E17.5, lung development enters the canalicular stage, where branching morphogenesis is marked by the narrowing of the terminal buds, while the respiratory tree continues to expand in diameter and length. During this stage, the gas exchanging portion of the lung is formed and vascularized. Vascularization occurs by vasculogenesis, the development of a vascular network from endothelial precursor cells of lung mesenchymal origin, and by angiogenesis, the sprouting of new vessels from existing vessels. The increased number of capillaries stimulates the terminal bronchioles to divide further into respiratory bronchioles and alveolar ducts. In the primitive alveoli, glycogen granules, which are later converted to glucose and used as a substrate for synthesis of surfactant phospholipids, become evident in the undifferentiated alveolar epithelial cells[126,128]. Towards the end of this stage, the undifferentiated alveolar epithelial cells begin to differentiate into the two main specialized cell types of the alveoli, the alveolar epithelial type I and type II cells. This is an important step as it indicates that at the end of the canalicular phase, surfactant production starts and the gas-exchange units of the lungs are formed[122]. Another crucial step at this stage is the decrease in interstitial tissue, a prerequisite for blood-gas exchange, and the growth of the capillary network in very close proximity to the distal surface of the alveolar cells.

1.4.4 The Saccular Stage

In the saccular stage, which extends from E17.5 to postnatal day (PD) 5 in mice, a substantial decrease in the interstitial tissue occurs, the alveolar walls thin, and the terminal buds begin to develop small sacs, called saccules, that are precursors of the alveoli. Saccule development results from apoptosis as well as ongoing differentiation of mesenchymal cells. Also during this stage, the AECs become recognizable as they differentiate into mature squamous type I and secretory rounded type II cells containing surfactant-storing lamellar bodies. At the same time the blood vessels, which have developed in parallel with the airways, become closely associated with the epithelium, enveloping the alveoli. This

encircling behavior of the blood vessels is critical to the development of the alveoli and for the subsequent gas exchange that occurs in the postnatal period. The establishment of a comprehensive lymphatic network in the lung tissue also occurs during this stage of development. Towards the end of this stage, the fetal lung becomes capable of supporting air exchange, even in prematurely born human neonates. A key factor in determining the ability of whether the newborn lung will successfully sustain gas exchange without collapse is the maturation of the processes of surfactant synthesis and secretion.

1.4.5 The Alveolar Stage

Following birth in mice, the saccules continue to mature in the alveolar stage (PD5 to PD30). While the saccules are lined with mature type I and type II alveolar cells, the shape of the saccules does not achieve the mature configuration until a few weeks after birth. In the final stages of lung development and maturation, the terminal respiratory sacs become functioning alveoli that are connected to alveolar ducts, lined with type I cells, which are in intimate contact to pulmonary capillaries, and contain surfactant-producing type II cells.

Table 1.2. The five different stages of lung development in the human and mouse. Since the stages of lung development are comparable between the human and mouse, the mouse has been extensively used as an embryological model when studying lung development and maturation. The use of gene knockout strains has especially made the mouse a remarkable tool that is essential in understanding the development of various respiratory disorders, congenital defects in human neonates, and how the disruption of hormonal (i.e. glucocorticoids) and morphogenetic programs early in development can lead to deficiencies that persist throughout life.

Stage	Human	Mouse	Features
Embryonic	Week 4-5	E9.5-E12.5	- Lung bud formation - Differentiation into trachea and bronchi
Pseudoglandular	Week 5-17	E12.5-E16.5	- Formation of major airways - Branching of bronchial tree
Canalicular	Week 16-25	E16.5-E17.5	- Primitive alveoli - Epithelial differentiation - Air blood barrier
Saccular	Week 24-Birth	E17.5-PND5	- Formation of alveolar saccules - AECs differentiate into mature type II and type I cells
Alveolar	Late fetal period to childhood (8 years)	PND5-PND30	- Expansion of gas exchange area

1.4.6 Functional Maturation of the Lungs

The cells in the lungs must undergo extensive cell differentiation to form cells with distinct structures that perform various functions in the lungs. Specific transcriptional factors, peptide growth factor receptor-mediated signaling pathways, extracellular matrix components, and integrin-signaling pathways interact to direct lung cell differentiation. Nkx2.1 is a hallmark transcription factor expressed in the lung epithelium[116,126]. Indeed, the earliest known step in lung development is the establishment of localized Nkx2.1 expression[129]. Lungs from Nkx2.1 knockout mice do not branch and exhibit decreased expression of many crucial lung genes, including the clara cell marker (Scgb1a1) and type II cell markers (Surfactant proteins A-B-C-D)[130]. During the pseudoglandular stage of lung development, the cells in the tips of the buds constitute a pool of highly proliferative multipotent progenitor cells. In the course of branching morphogenesis, the Nkx2.1-positive endoderm begins to develop distinct cell lineages along its proximal-distal axis, regulated by the Sox gene family of transcription factors. Sox2 expression marks the proximal endoderm progenitor lineage[131,132], whereas the combined expression of Sox9 and the transcriptional regulator Id2 marks the distal endoderm progenitor lineage[133]. Importantly, these two populations have distinct fates: the proximal progenitors give rise to airway neuroendocrine cells, clara (secretory) cells, ciliated cells, and goblet (mucosal) cells, whereas the distal progenitors give rise to type I and type II AECs. Both Sox2 and Sox9 are thought to be essential for the differentiation of proximal and distal progenitors into their various progeny as a lack of Sox2/Sox9 expression leads to the loss of mature clara, ciliated, and alveolar epithelial cells in the lungs.

Lung epithelial cells in the tips of the buds continue to divide and populate the distal tubules throughout the canalicular and saccular stages. Importantly, at the end of the canalicular stage, immature AECs begin to form in the alveoli. The current model of AEC differentiation indicates that type II cells themselves act as alveolar progenitors and give rise to type I cells during lung development[116,122,126,128,134]. Furthermore, investigations in animal models show that type II cells function as progenitor cells in the alveoli and differentiate into type I cells following lung injury[135–138].

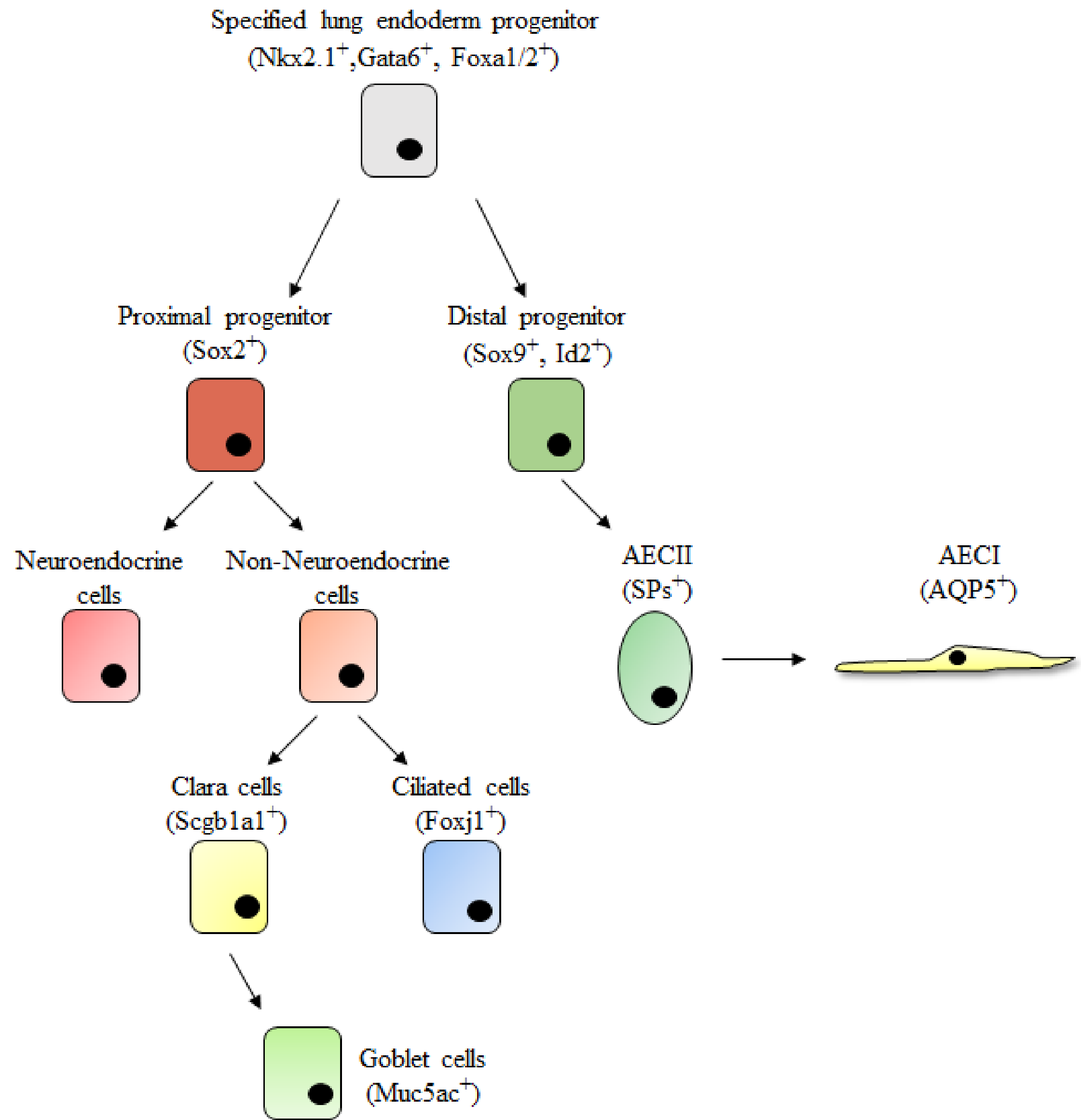

Figure 1.3: Epithelial cell lineages in the developing lung[116,126].

1.4.7 The Impact of Abnormal Lung Development

Normal airways develop sequentially by early epithelial tube branching and later septation of terminal air sacs in concert with the development of the pulmonary vasculature within lung mesenchyme and in close conjunction with epithelial differentiation[125]. Premature birth, intrauterine infection, genetic mutations, or hormonal insufficiency (i.e. glucocorticoids) can disrupt these developmental processes and result in abnormal lung structure, deficiency of gas exchange, and neonatal respiratory failure[122]. Clinical examples of such disruption of normal lung growth include neonatal respiratory distress syndrome (RDS), a common cause of morbidity and mortality associated with premature birth due to the insufficient production of pulmonary surfactant by immature type II cells[124]. More subtle non-lethal lung dysfunctions may emerge later in life such as asthmatic wheezing and predisposition to early onset of chronic obstructive pulmonary disease (COPD)[124]. Given that the correlation between impaired lung development and the risk of developing chronic lung disease is undisputable, it is important to delineate whether developmental exposure to environmental insults, such as BPA, contribute to lung dysfunction and diseases in adult life.

1.5 The Importance of Glucocorticoids during Lung Development and Maturation

Lung development is a complex maturational process that starts in the early stages of fetal life and continues after birth. At birth, the lungs must undergo an important change, as the fluid-filled alveoli adjust to respiratory gas exchange. Several endocrine factors, specifically GCs, are critical in the regulation of pulmonary development and the transition to extrauterine life[120,139]. Towards the end of gestation in mammals, a surge in GCs is essential for both structural and functional lung maturation[122,140,141]. Perhaps the most compelling clinical evidence for the importance of GC-induced lung maturation was the 1972 discovery by Liggins and Howie of the positive role of GCs on fetal lung maturation in lambs[139,142]. They demonstrated that administration of antenatal GCs reduced both morbidity and mortality in preterm infants at risk for respiratory distress syndrome. Since then, antenatal GCs are routinely administered to women who are at risk of preterm delivery to induce maturation of the fetal lungs[143,144].

GCs exert their effects by binding to and activating the GR[140]. Hollenberg et al. identified that the gene structure encoding GR in humans, by alternative splicing, generates the classic GRα prototype and a variant isoform termed GRβ[145]. GRα is capable of ligand binding and resides in the cytoplasm, while GRβ does not seem to bind GCs *in vivo* and is located in the nucleus[146,147]. The function of GRβ in fetal tissues has not been addressed, however, it has been shown that GRβ could repress the activity of GRα and therefore may have the ability to antagonize the function of GRα and decrease the activity of GCs[147,148]. Since mice are known to be deficient in the GRβ isoform, any mention of the GR will be in reference to the classic GRα isoform[149].

Although the GR is abundantly expressed in fetal lungs, even during early gestation, the number of receptors varies among cell types and with gestational age[150,151]. In human fetal lungs, GCs are capable of binding to the GR with high affinity as early as the second month of gestation[152,153]. During this time, the GR is highly expressed in the bronchial epithelium and terminal sac canaliculi in the lungs[153]. In addition, the enzyme 11-beta hydroxysteroid dehydrogenase type II (11β-HSD II), which inactivates GCs in cells[154], is expressed at very low levels, suggesting that GCs are not being metabolized to their inactive form[153,155]. This results in high local concentrations of GCs in the fetal lungs and implies a critical regulatory role for GCs in the developing lung. Indeed, maternal administration of GCs to pregnant primates, rabbits and rodents accelerates the transition through the pseudoglandular phase of lung development, in association with premature terminal differentiation and secretion of surfactant, and the initiation of structural changes required for gas exchange, most notable alveolar thinning[156–158]. These *in vivo* effects of GCs on distal lung maturation are reflected *in vitro* in both explants from developing lungs and in cultured cell models[159–161]. In lung explants from humans, GCs induce expression of SP-B and SP-C mRNA[159–161]. In cultured type II cells, GCs induce the expression of numerous markers of distal differentiation of the respiratory epithelium, including Nkx2.1, a transcription factor that is implicated in directly regulating SP-C expression, and the expression of other genes associated with a mature alveolar epithelium[162]. The effects of GCs on isolated alveolar epithelial cells thus appear to be consistent with many of the effects of GCs *in vivo*, supporting an epithelial cell autonomous model for the effects of

GCs on surfactant production in which ligand-activated GR in the developing epithelium drives distal differentiation.

The role of GCs in regulation of lung development was further investigated using corticotrophin-releasing hormone (CRH) knockout mice[163], as well as mice with mutations in the GR gene[164,165]. Although CRH-null mice have normal expression of GR in their fetal lungs, decreased levels of GC in both maternal and fetal circulation cause postnatal death of CRH-null offspring due to acute respiratory failure[163]. Maternal administration of GCs reverses the abnormal lung phenotype in CRH-null offspring, confirming the crucial role for GC in lung development[163]. Similarly, in a GR-null mouse model, where GR expression was eliminated, GR-null offspring suffered severely impaired lung maturation[164,165]. Biochemical analysis of fetal lungs from GR-null mice showed a marked reduction in type II and type I cells which indicates that receptor-mediated GC signaling is critical in facilitating terminal alveolar differentiation. Thus, GC signaling via the GR is essential for both structural and biochemical maturation of the fetal lungs. Indeed, in studies conducted in lambs, rabbits, and rodents, treatment with GCs resulted in maturation of lung epithelial cells and stimulated differentiation of type II cells[156,166].

A potential mechanism underlying this effect may be GC-mediated inhibition of DNA synthesis, as indicated by a lower rate of thymidine incorporation into DNA in the presence of GCs[167]. Inhibition of DNA synthesis restricts cell proliferation, which could stimulate cell differentiation. This effect is observed in CRH-null mice who show continued cell division combined with delayed maturation of type II cells in their lungs[163,168]. Another possible mechanism by which GCs regulate cell differentiation is via a GC-induced increase in aminopeptidase N, a factor that regulates cell growth and differentiation[169]. In addition to cell maturation, GCs regulate structural changes in the developing lung. The thinning of the alveolar wall during the later stages of intrauterine lung development is critical to effective gas exchange at birth[117,125]. Administration of GCs cause a decrease in interstitial tissues in the lungs with a consequent decrease in alveolar wall thickness, facilitating gas exchange after birth[170–172].

The late gestational increase in GCs in fetal circulation coincides with important pulmonary maturational events, such as surfactant synthesis and alveolar septal thinning[127,141]. Preterm birth can precede the late gestational increase in GCs, resulting in the failure of GC-induced lung maturation, low levels of surfactant, and subsequent development of neonatal RDS[122,124]. GC administration to pregnant women at risk of preterm delivery has been shown to result in lung maturation, as it stimulates the production and activity of surfactant proteins and phospholipids, components of surfactant[144,173]. Furthermore, GCs influence structural changes, growth factors, and lung liquid metabolism in the developing fetal lung that facilitate survival after preterm delivery.

1.6 Relationship between BPA and Lung Diseases

The notion that early disturbances during the development of organ systems can result in long-term consequences for the health of the individual is well-established. Numerous animal studies demonstrate that prenatal exposure to BPA affects fetal organ development and may account for adverse health outcomes observed later in life. The respiratory health effects of BPA have become the recent focus of attention. Adverse effects on lung function and associated lung diseases have been demonstrated by early life exposure to BPA in animal models as well as in clinical investigations. In humans, exposure to BPA during various periods of development is associated with impaired lung function[101] and the development of asthma[102,103] and wheeze[104] in children. Recently, high serum levels of BPA have been linked to chronic obstructive pulmonary disease (COPD)[105]. In addition, perinatal BPA exposure induces asthma in mice[106–108]. More importantly, recent studies suggest that exposure to BPA exclusively during the prenatal period is sufficient to disrupt lung function in later life. For example, prenatal, but not postnatal exposure to BPA is associated with diminished lung function and the development of a persistent wheeze in children[109]. These findings are supported by an animal study demonstrating that exposure to BPA during gestation, but not lactation, leads to asthma in mice[110]. In addition, BPA exposure during late gestation significantly increased secretory cell expression, as indicated by increased Clara cell secretory protein and MUC5AC mRNA and protein expression, in the proximal conducting airways in non-human primates[111,112]. This increase in airway mucins is a hallmark of a number of lung diseases that may result following

exposure to BPA. More importantly, if mucous cell abundance is increased following exposure to BPA, this could increase airway obstruction, making lung diseases, such as asthma, more severe. Collectively, these findings demonstrate that early life exposure to BPA is detrimental to lung function, and suggest that BPA may alter fetal lung development contributing to the development of lung diseases later in life. However, evidence in support of this hypothesis is lacking, and the underlying mechanisms are still unclear.

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2 HYPOTHESIS AND SPECIFIC AIMS

2.1 Focus of the Thesis

Increasingly, studies are linking exposure to synthetic estrogens, such as BPA, during sensitive periods of development to the increased incidence of infertility, genital tract abnormalities, obesity, and various cancers. Humans are inevitably exposed to BPA through contact with numerous consumer products, including plastic wares, inner-linings of canned food containers, and various paper products as well as contaminated water and food[1]. Importantly, BPA has been shown to accumulate in human body tissues, resulting in much higher exposure levels than previously thought[2]. Of particular concern is BPA exposure during pregnancy, a critical period during which the developing fetus is especially vulnerable to this EDC[3,4]. Indeed, high levels of bioactive BPA have been detected in amniotic fluid, umbilical cord blood, fetal and maternal blood of pregnant woman[5,6]. Alarmingly, recent epidemiological studies indicate that early life exposure to BPA is associated with impaired lung function[7] and the development of asthma[8,9] and wheeze[10] in children. Moreover, high serum levels of BPA are associated with COPD in humans[11]. Collectively, these findings demonstrate that early life exposure to BPA is detrimental to lung health, and suggest that BPA may alter fetal lung development, consequently leading to lung dysfunction and diseases in adult life. However, evidence in support of this hypothesis is lacking. ***The overall hypothesis of this thesis is that early life exposure to BPA impairs fetal lung development, providing a fetal origin for BPA-induced lung diseases.*** The focus of the thesis is to identify whether early life exposure to BPA disrupts fetal lung maturation, thus providing a fetal origin to BPA-induced lung diseases in adult life. The thesis will also examine the underlying molecular mechanisms through which BPA disrupts fetal lung maturation, providing a molecular pathway for the disruptive effects of BPA on fetal development.

2.2 Specific Aim 1

To determine whether prenatal exposure to environmentally relevant doses of BPA disrupts fetal lung maturation in the mouse.

BPA is one of the most prevalent EDCs in the environment, as a result human exposure to BPA is inevitable[12,13]. Of particular concern is exposure to BPA during pregnancy, a critical period during which organs undergo growth and differentiation to form functional organ systems[3]. Indeed, early life exposure to BPA has been shown to affect the development of various fetal organs including the brain[14,15], heart[16], mammary gland[17,18], ovary[19,20], and uterus[21]. Altered fetal organ development following early life exposure to BPA may provide a fetal origin for various BPA-induced diseases in adult life.

Proper lung development and maturation during fetal life is essential for neonatal survival[22,23]. Disruptions in this process result in malformation of the lung, and can result in neonatal death, or to more subtle non-lethal lung diseases in adult life, such as asthma and predisposition to early onset of COPD[24,25]. In Chapter 3, we sought to determine whether prenatal exposure to BPA impairs fetal lung maturation. Using the mouse as an experimental model we ***hypothesized that prenatal exposure to BPA may lead to impaired fetal lung maturation in the mouse.*** Environmentally relevant doses of BPA were administered to pregnant mice via maternal diet from embryonic day (E) 7.5 to E18.5, at which point fetal lungs were analyzed for changes in structure and expression of key molecular markers of lung maturation. Histological analysis of the fetal lungs demonstrated diminished alveolar airspace and thickened septa, hallmarks of lung immaturity. This immaturity is characterized by aberrant alveolar epithelial type I cell differentiation, as the expression of the type I cell marker, but not type II cell markers, is dramatically reduced. Collectively, these findings suggest that BPA disrupts fetal lung maturation, and suggest that BPA-induced lung dysfunction and diseases in adult life, as reported previously in clinical and animal studies, may have a fetal origin. Moreover, this was the first study to demonstrate that early life exposure to BPA affects the development of the fetal lungs, highlighting the potential impact of BPA exposure during pregnancy on the adverse health outcomes observed later in life.

2.3 Specific Aim 2

To determine whether maternal administration of synthetic glucocorticoids rescues the BPA-induced fetal lung immaturity.

During late gestation, a surge in glucocorticoid levels is essential for the proper development and maturation of the fetal lungs[26]. Synthetic glucocorticoids, such as betamethasone and dexamethasone, are commonly administered to pregnant women at risk of preterm delivery to promote fetal lung maturation in an attempt to decrease the incidence and severity of respiratory distress syndrome[26,27]. Furthermore, the lung immaturity characterized in glucocorticoid-signaling deficient (i.e., $CRH^{-/-}$) fetal mice[28–30], which is remarkably similar to lung immaturity phenotype observed in BPA exposed fetal lungs, can be rescued by maternal dexamethasone administration. As such, we sought to determine whether maternal administration of dexamethasone can rescue the lung immaturity phenotype observed in BPA exposed fetuses.

Therefore, we ***hypothesized that maternal administration of synthetic glucocorticoids rescues the BPA-induced fetal lung immaturity.*** To address this hypothesis, BPA-exposed pregnant mice were administered two courses of dexamethasone injections at E16.5 and E17.5, respectively. We describe in Chapter 3 that maternal dexamethasone administration completely restored the alveolar architecture, such that there was no observable difference in alveolar airspace and septa thickness between control and BPA-exposed fetal lungs. Moreover, dexamethasone treatment also normalized the expression of the type I cell marker in BPA-exposed fetal lungs, indicative of normal type I cell differentiation.

Given that the BPA-induced lung immaturity phenotype closely resembles that of glucocorticoid deficient mice and dexamethasone was able to rescue this lung immaturity phenotype, these findings suggest that prenatal exposure to BPA disrupts fetal lung maturation likely through altering the glucocorticoid signaling pathway. Indeed, a recent study using *in vitro* yeast bioassays shows that BPA acts as a potent glucocorticoid receptor antagonist[31]. Thus, we further ***hypothesized that BPA may disrupt fetal lung maturation by suppressing the glucocorticoid signaling pathway***. To address this hypothesis, we determined if prenatal exposure to BPA resulted in reduced expression of key

glucocorticoid target genes in the fetal lung. We describe in Chapter 3 that the expression of both epithelial sodium channel γ (ENaCγ) and glutathione peroxidase (GPX), two well-known glucocorticoid target genes in the lung[32], was dramatically decreased in BPA-exposed fetal lungs. Collectively, these findings suggest that BPA likely functions as a glucocorticoid receptor antagonist to disrupt fetal lung maturation.

2.4 Specific Aim 3

To determine the precise molecular mechanisms of the adverse effects of BPA on the glucocorticoid signaling pathway.

In Chapter 3 we provide evidence that prenatal exposure to BPA disrupts fetal lung maturation likely through altering the glucocorticoid signaling pathway, but the precise molecular mechanisms remain obscure. Given that BPA diminished the expression of ENaCγ, a well-known glucocorticoid receptor target gene, in fetal lungs, we used this target gene to delineate the molecular pathway through which BPA exerts its effects on lung epithelial cells.

Therefore, we ***hypothesized that BPA may repress ENaCγ expression through inhibition of glucocorticoid receptor signaling in lung epithelial cells.*** To address this hypothesis, the human A549 lung epithelial cell line was used as an *in vitro* model system. As a first step, in Chapter 4 we validated our *in vitro* cell model by demonstrating a robust concentration-dependent suppression of ENaCγ expression following BPA exposure. We also showed that both dexamethasone and siRNA-mediated knockdown of GR expression blocked/abrogated the inhibitory effects of BPA on ENaCγ expression, suggesting that BPA repress ENaCγ expression via inhibition of GR activity. Given that BPA is known to function as a pro-inflammatory factor via the estrogen receptor β (ERβ)[33–35], and a mutual antagonism exists between the pro-inflammatory transcriptional factor NF-κB and GR[36–39], we then explored and provide evidence supporting the notion that BPA acts on ERβ to activate the NF-κB signaling pathway, which in turn leads to diminished GR activity and consequent repression of ENaCγ expression in lung cells. Thus, our present findings reveal a novel BPA signaling pathway that involves ERβ, NF-κB and GR, and suggest a molecular pathway through which BPA may disrupt fetal lung development.

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3 PRENATAL EXPOSURE TO BISPHENOL A DISRUPTS MOUSE FETAL LUNG DEVELOPMENT[1]

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3.1 Introduction

Exposure to endocrine-disrupting chemicals (EDCs) has become a major public concern as a result of its association with a wide range of disorders in humans, including reproductive, neurologic, behavioral, respiratory, and metabolic disorders as well as cancers[1,2]. In particular, bisphenol A (BPA), one of the most prevalent EDCs in the environment, has attracted enormous worldwide attention because BPA exposure has been shown to cause various adverse effects in both humans and experimental animals[3,4]. Humans are routinely exposed to BPA through air and contact with numerous consumer products, including plastic ware, the inner linings of canned food containers, and various paper products, as well as contaminated water and food[5]. Importantly, BPA has been shown to accumulate in human body tissues, resulting in much higher exposure levels than previously thought[6]. Of particular concern is BPA exposure during pregnancy, a critical period during which the developing fetus is especially vulnerable to this EDC[7,8]. Indeed, appreciable amounts of bioactive BPA have been detected in amniotic fluid, umbilical cord blood, and fetal and maternal blood of pregnant women[9,10]. Furthermore, prenatal exposure to BPA is known to affect the development of various fetal organs, including brain[11,12], heart[13], mammary gland[14,15], ovary[16,17], and uterus[18].

Abnormal fetal organ development may account for the adverse health outcomes observed in later life after prenatal BPA exposure[3]. In humans, exposure to BPA during various periods of development is associated with impaired lung function[19] and the development of asthma[20,21] and wheeze[22] in children. Recently high serum levels of BPA have been linked to chronic obstructive pulmonary disease[23]. In addition, perinatal BPA exposure induces asthma in mice[24–26]. More importantly, recent studies suggest that exposure to BPA exclusively during the prenatal period is sufficient to disrupt lung function during later life. For example, prenatal, but not postnatal, BPA exposure has been linked to diminished lung function and chronic wheeze in children[27]. This finding is supported by an animal study that demonstrated that exposure to BPA during gestation, but not lactation, leads to asthma in mice[28]. Collectively, these findings show that prenatal BPA exposure is detrimental to lung function, suggesting that BPA may alter fetal lung development, thus leading to lung dysfunction and diseases in later life. However, evidence in support of this hypothesis is

lacking. Therefore, the goal of the present study was to address this important question using the mouse as an experimental model.

3.2 Materials and Methods

3.2.1 Animal Experiments

The use of animals in this study was approved by the Council on Animal Care at the University of Western Ontario, following the guidelines of the Canadian Council on Animal Care. Breeding pairs of adult C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA, USA). Mice were housed under standard conditions and provided with food and water *ad libitum.* To minimize background exposure to BPA beyond treatment regimen, mice were housed in polystyrene cages and maintained at humidity- and temperature-controlled rooms on a 12 h/12 h light–dark cycle. For experiments, female mice 6 to 8 wk old were placed overnight with males, and pregnancy was determined the next morning by the observation of a vaginal plug. Plugged females were separated from the males and gestational days counted, with the day of the vaginal plug being embryonic day (E) 0.5. Pregnant mice were randomly assigned to receive 1 of the following 4 diets: control diet (phytoestrogen-free food pellets supplemented with 7% corn oil; TD.120176; Harlan Teklad, Madison, WI, USA); control diet supplemented with 5 mg BPA/kg (TD.120465; Harlan Teklad); control diet supplemented with 25 mg BPA/kg (TD.120466; Harlan Teklad); or control diet supplemented with 50 mg BPA/kg (TD.120177, Harlan Teklad). Mice were fed the above diets from E7.5 to E18.5. The gestational age of E7.5 was chosen as the start of the feeding regimen in order to avoid any confounding effects of BPA on embryo implantation. For the rescue studies, saline or dexamethasone (0.4 mg/kg; Omega, Montreal, QC, Canada) was injected subcutaneously on E16.5 and E17.5 to the control and BPA-fed pregnant mice, respectively. At E18.5, animals were euthanized with CO_2, fetuses were recovered by caesarean section, and their weights recorded. In addition, maternal weight, number of pups, and the number of reabsorption sites per uterine horn were noted. Fetal lungs were either fixed in 4% paraformaldehyde or frozen rapidly in liquid nitrogen and stored at −80°C.

3.2.2 Histology

Fetal lungs were fixed in 4% paraformaldehyde in 0.1 M PBS buffer, dehydrated, and embedded in paraffin. Using a rocking microtome, the lungs were carefully sectioned; 5 µm sections were transferred to Superfrost/Plus microscope slides (Fisher Scientific, Whitby, ON, Canada). Sections were stained using a standard hematoxylin and eosin protocol[29]. Selected lung sections were also stained with periodic acid–Schiff (PAS). Stained lung sections were examined with a light microscope. Images were captured at ×20 and ×40 magnifications. For quantitative morphometry, a total of 16 pups from 4 different litters (4 pups/litter) per treatment group (*i.e.,* control and BPA) were analyzed. Alveolar size, alveolar wall thickness, and PAS staining were measured with Image ProPlus 6.0 (Silver Springs, MD, USA) on 10 randomly selected micrographs from each individual section, and a total of 16 sections (1 section was randomly selected per pup) per treatment group were analyzed and counted. All analyses were performed by 2 investigators who were masked to the animal number and experimental group.

3.2.3 Immunohistochemistry

Fetal lung tissue sections were prepared as described above. After deparaffinization and rehydration, tissue sections were incubated sequentially in 3% hydrogen peroxide (Fisher Scientific) for 10 min to reduce endogenous peroxidase activity and then in blocking reagent solution (Background Sniper, catalog BS966; Biocare Medical, Concord, CA, USA) for 10 min to prevent nonspecific background labeling. Tissue sections were incubated in rabbit surfactant protein C (1:200, catalog WRAB-76694; Seven Hills Bioreagents, Cincinnati, OH, USA) overnight at 4°C. Sections were then incubated with biotinylated goat anti-rabbit IgG (1:500; catalog PK-6102; Vector Laboratories, Burlingame, CA, USA) for 30 min, ABC (1:500, catalog PK-6102; Vector Laboratories) for 30 min, and 0.02% 3,3-diaminobenzidene (DAB chromogen, catalog DBC859 H, L10; Biocare Medical) for 2 min. Tissue sections were dehydrated with alcohol and coverslipped with Permount.

3.2.4 Real-time quantitative reverse transcriptase PCR

The relative abundance of various mRNAs was assessed by a 2-step real-time quantitative reverse transcriptase PCR (qRT-PCR), as described previously[29] with the following specific modifications. Briefly, total RNA was extracted from fetal lung tissues using RNeasy Mini Kit (Qiagen, Mississauga, ON, Canada) coupled with on-column DNase digestion with the RNase-Free DNase Set (Qiagen) according to the manufacturer's instructions. One microgram of total RNA was reverse transcribed in a total volume of 20 µl using the High Capacity cDNA Archive Kit (Applied Biosystems, Forest City, CA, USA) following the manufacturer's instructions. For every RT reaction set, 1 RNA sample was set up without reverse-transcriptase enzyme to provide a negative control. Gene transcript levels of glyceraldehyde phosphate dehydrogenase (*GAPDH,* housekeeping gene, the expression level of which was found to be stable across all treatment groups), *aquaporin 5 (AQP5)*, *surfactant protein (SP)-A, SP-B, SP-C, SP-D, versican, midkine*, and *Ki-67* were quantified separately by predesigned and validated TaqMan Gene Expression Assays (Applied Biosystems; Table 3.1) following the manufacturer's instructions. Briefly, gene expression assays were performed with the TaqMan Gene Expression Master Mix (P/N #4369016; Applied Biosystems) and the universal thermal cycling condition (2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C) on the ViiA 7 Real-Time PCR System (Applied Biosystems).

The relative amount of various gene-specific mRNAs in each RNA sample was quantified by the comparative C_t method (also known as the $\Delta\Delta C_t$ method) using the Applied Biosystems relative quantitation and analysis software according to the manufacturer's instructions. For each RNA sample, the amount of gene-specific mRNAs in BPA-exposed fetal lungs is expressed relative to the amount of transcript present in the control lungs.

3.2.5 Western Blot Analysis

Western blot analysis was used to determine various protein levels, as described previously[29]. Briefly, lung tissues were homogenized in 10 volumes of ice-cold 10 mM sodium phosphate buffer, pH 7.0, and containing 0.25 M sucrose. Equal volumes of the homogenates were mixed with SDS gel loading buffer (50 mM Tris-HCl, pH 6.8, 2%

wt/vol SDS, 10% vol/vol glycerol, 100 mM DTT, and 0.1% wt/vol bromophenol blue), and equal concentrations of this mixture were subjected to a standard 10% SDS-PAGE. After electrophoresis, proteins were transferred to PVDF transfer membrane (Amersham Hybond-P, catalog RPN303F; GE Healthcare Lifesciences, Baie D'Urfe, QC, Canada) using a Mini Transfer Apparatus (Bio-Rad, Hercules, CA, USA). Nonspecific antibody binding was blocked with 5% wt/vol milk in TTBS (0.1% vol/vol Tween-20 in Tris-buffered saline) for 1 h at room temperature. Membranes were then hybridized with primary antibody (Ki-67; catalog ab15580, 1:1000 dilution; Abcam, Toronto, ON, Canada; proliferating cell nuclear antigen [PCNA]: catalog 2586, 1:1000 dilution; Cell Signaling Technology, Beverly, MA, USA; caspase-3: catalog 9662, 1:1000 dilution; Cell Signaling Technology; SP-A: catalog AB78173, 1:2000 dilution; Abcam; SP-B: the gift of F. Possmayer, 1:1000 dilution; AQP5: catalog LS-C-172028, 1:1000 dilution; LifeSpan BioSciences, Seattle, WA, USA; *GAPDH,* catalog IMG-3073, 1:5000; Imgenex, Port Coquitlam, British Columbia, Canada) overnight at 4°C. After 3 × 10 min washes with TTBS, the membrane was incubated with the appropriate horseradish peroxidase–labeled secondary antibody, either anti-rabbit (catalog HAF008, 1:500; Fisher Scientific) or anti-mouse (catalog G-202-C, 1: 10,000; Fisher Scientific) for 1 h at room temperature. After another 3 × 10 min washes in TTBS, proteins were detected by chemiluminescence (catalog WBLUR0500, Luminata Crescendo, Western HRP Substrate; Millipore, Etobicoke, ON, Canada) and captured on the VersaDoc Imaging System (Bio-Rad). Densitometry was performed on the images, and the level of various proteins was expressed as a percentage of controls.

3.2.6 Corticosterone Assays

Blood samples were collected from fetuses on E18.5 in heparinized capillary tubes (catalog 22-260-950; Fisher Scientific) between 9 and 11 am, then centrifuged at 2000*g* for 10 min. Blood samples were also collected in the same fashion from another group of fetuses at E15.5. Plasma was then collected and stored at −80°C until use. Levels of corticosterone in plasma samples (fetal plasma from 1 litter were pooled and used as 1 sample) were determined with an ELISA Kit (catalog ab108821; Abcam) following the manufacturer's

instructions. To eliminate interassay variations, all samples were analyzed in triplicate in 1 assay, and the intra-assay coefficient of variation was <5%.

3.2.7 BPA Assays

Blood samples were collected from pregnant mice (n = 12) at E18.5, and plasma was collected and pooled (n = 2–3). Pooled plasma samples were sent to NMS Labs (Willow Grove, PA, USA), where unconjugated (free) BPA was measured by GC-MS.

3.2.8 Statistical Analysis

Results are presented as means ± SEM of 4 to 6 different litters, as indicated. Data were analyzed by 1-way ANOVA followed by Tukey's *post hoc* test or Student's t test, as indicated. Significance was set at $P < 0.05$.

Table 3.1. TaqMan® gene expression assays for the mouse genes analyzed.

Gene Name	**Assay ID**
AQP5	Mm00437579_m1
SP-A	Mm00499170_m1
SP-B	Mm00455681_m1
SP-C	Mm00488144_m1
SP-D	Mm00486060_m1
GAPDH	Mm99999915_g1

3.3 Results

3.3.1 Effects of BPA on fetal body weight and litter size

As a first step in exploring the effects of BPA on fetal organ development, we assessed changes in fetal body weight and litter size at E18.5. There was no difference in either body weight (**Fig. 3.1 A**) or litter size (**Fig. 3.1 B**) between control and BPA-exposed fetuses, irrespective of BPA dose.

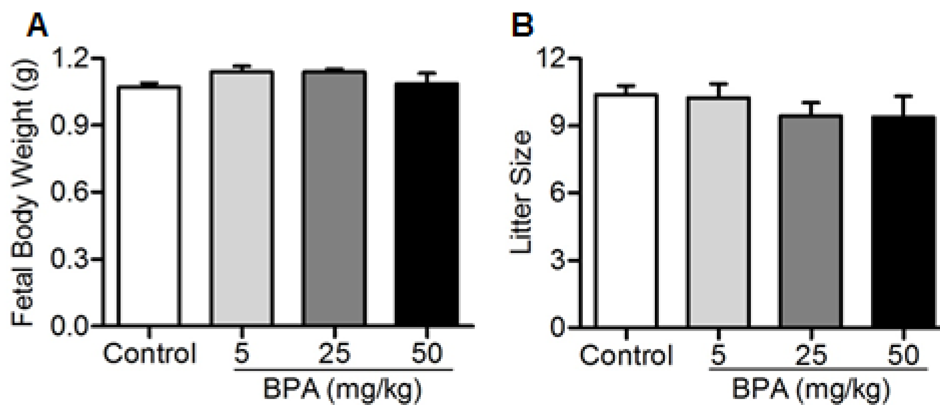

Figure 3.1: BPA does not affect fetal body weight or litter size. Pregnant mice were fed a control diet (phytoestrogen free food pellets) or the control diet supplemented with 5, 25, or 50 mg BPA/kg food pellets from embryonic day (E) 7.5 to E18.5. On E18.5, mice were sacrificed, fetal body weight (A) and litter size (B) were recorded. Data are presented as means ± SEM (n=6 litters).

3.3.2 BPA severely impairs fetal lung maturation

To determine whether prenatal BPA exposure affects structural maturation of the fetal lung, standard histologic analysis was performed on fetal lungs collected at E18.5 from control and BPA (5, 25, and 50 mg BPA/kg diet)-fed pregnant mice. We found that the overall organization of fetal lungs (*i.e.,* 4 right lobes and 1 left lobe flanking the heart) was not altered by BPA. At a microscopic level, both subjective observations (**Fig. 3.2 A**) and quantitative morphometric measurements (**Fig. 3.2 B, C**) revealed diminished alveolar airspace (15% of control) and increased alveolar septa thickness (172% of control) in fetal lungs exposed to 25 and 50 mg BPA/kg diet compared to control and 5 mg/kg BPA diet groups. These characteristics are hallmarks of severe lung immaturity, closely resembling the lung immaturity phenotype of several knockout mouse models[30–32]. Given that fetal lungs exposed to 25 and 50 mg BPA/kg diet displayed similar defects in lung maturation, subsequent biochemical analysis was performed only on lungs from fetuses exposed to the 25 mg BPA/kg diet.

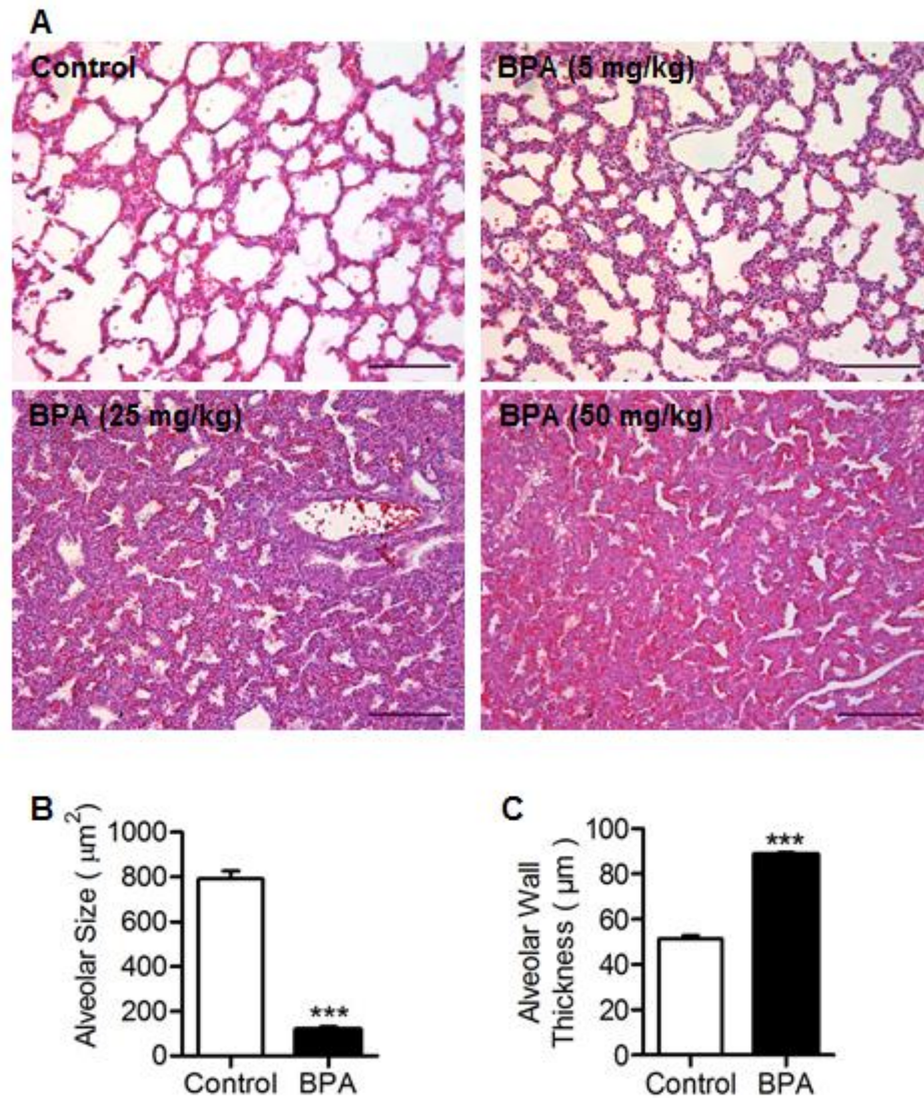

Figure 3.2: BPA severely impairs fetal lung maturation. Pregnant mice were fed a control diet (phytoestrogen free food pellets) or the control diet supplemented with 5, 25, or 50 mg BPA/kg food pellets from embryonic day (E) 7.5 to E18.5. On E18.5, mice were sacrificed, and fetal lungs were collected, fixed and subjected to standard histological analysis. Representative fetal lung histology from mice fed the control diet, the control diet supplemented with 5 mg BPA/kg, 25 mg BPA/kg, and 50 mg BPA/kg are shown in (A). Morphometric data of alveolar size and alveolar wall thickness are shown in (B and C). Data are presented as mean ± SEM (****P*<0.001; n=6 litters). Scale bar, 100 µm.

3.3.3 BPA exposure levels

To confirm that the dose of BPA (25 mg/kg diet) was relevant to humans, we measured maternal plasma levels of unconjugated (free) BPA at E18.5 and found them to be in the range of 1.2 to 2.6 ng/ml (mean 1.7 ng/ml).

3.3.4 BPA increases cytoplasmic glycogen content without affecting proliferation or apoptosis of alveolar epithelial cells

To determine whether the thickened alveolar septa was a result of altered epithelial cell maturation, we compared cytoplasmic glycogen content, a well-known marker of fetal lung epithelial cell maturation[32–35], between control and BPA-exposed fetuses. Intracellular glycogen content was measured by staining fetal lung sections with PAS. We found that fetal lungs from BPA-exposed mice displayed a significant increase (~50%) in PAS staining (**Fig. 3.3 A, B**). To further investigate whether changes in epithelial cell proliferation or apoptosis also contributed to the thickened alveolar septa, we examined expression levels of markers of proliferation and apoptosis. We showed that both mRNA and protein levels of Ki-67 as well as protein levels of PCNA and caspase-3 were not altered by BPA (**Fig. 3.4**).

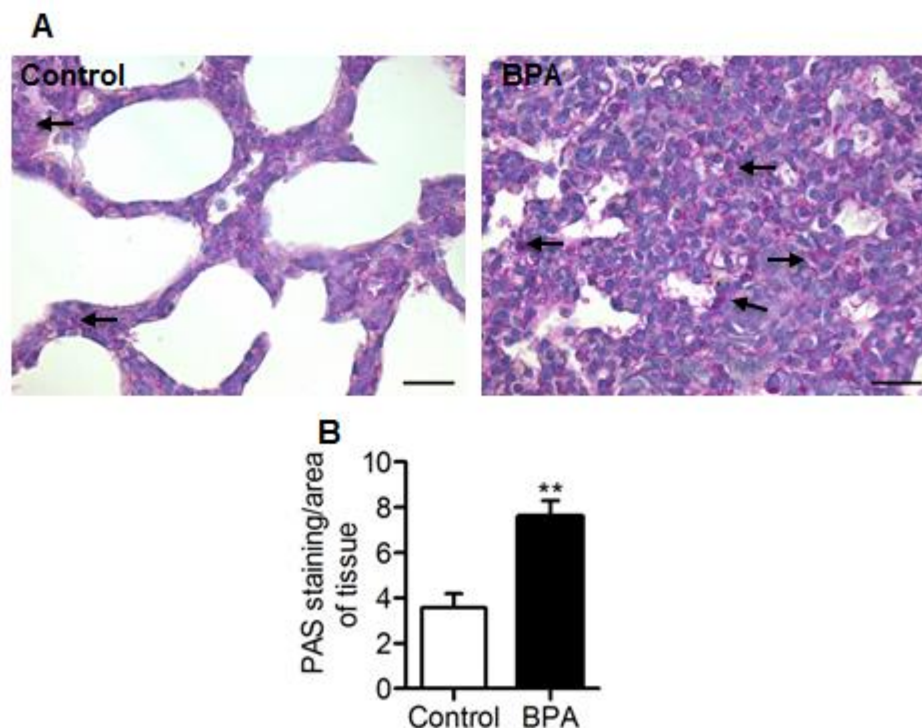

Figure 3.3: BPA increases cytoplasmic glycogen deposition. Pregnant mice were fed a control diet (phytoestrogen free food pellets) or the control diet supplemented with 25 mg BPA/kg food pellets from embryonic day (E) 7.5 to E18.5. On E18.5, mice were sacrificed, and fetal lungs were collected, fixed and subjected to standard PAS staining. Representative PAS staining of cytoplasmic glycogen deposition, as indicated by arrows, in fetal lung sections from mice fed the control diet and the control diet supplemented with 25 mg BPA/kg are shown in (A). Morphometric data of cytoplasmic PAS staining is shown in (B). Data are presented as mean ± SEM (***P*<0.01; n=6 litters). Scale bar, 20 µm

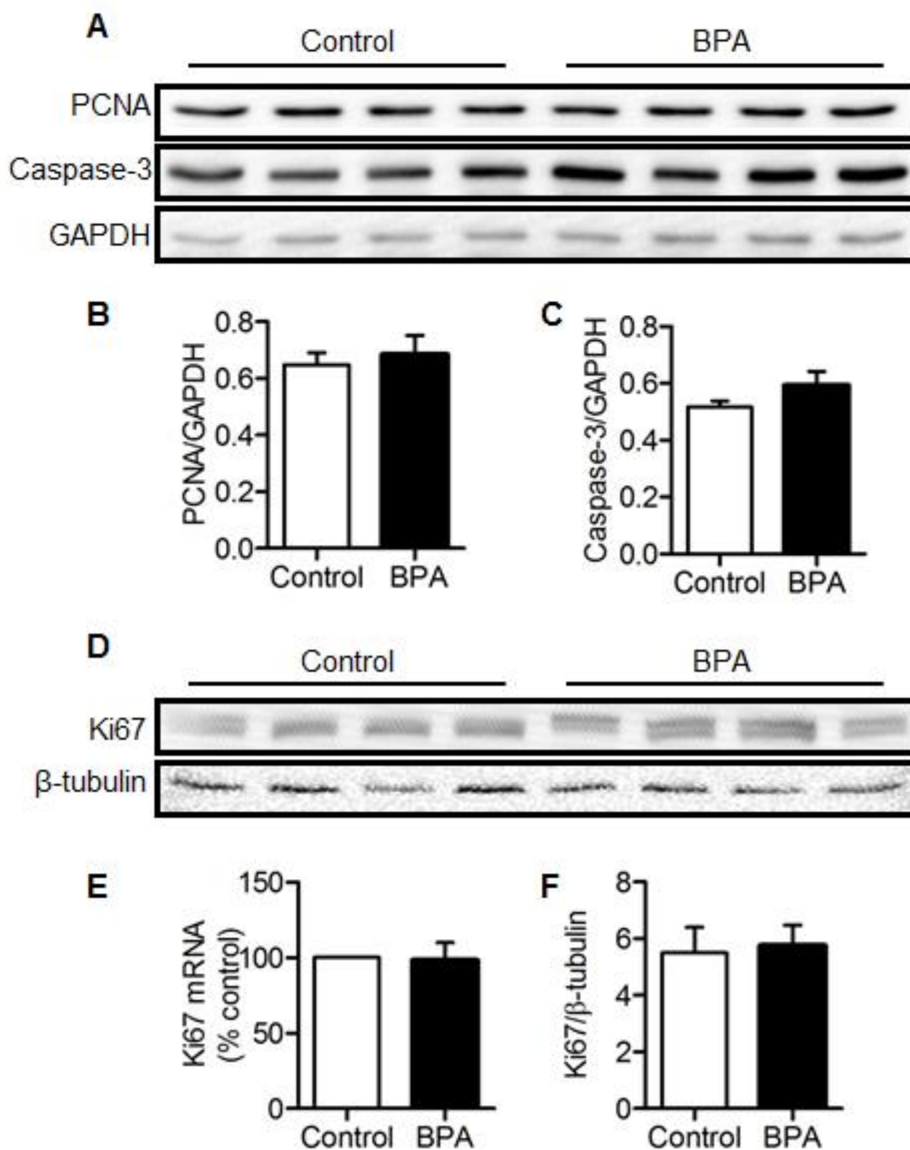

Figure 3.4: BPA does not alter the expression of proliferation and apoptosis marker proteins. Pregnant mice were fed a control diet (phytoestrogen free food pellets) or the control diet supplemented with 25 mg BPA/kg food pellets from embryonic day (E) 7.5 to E18.5. On E18.5, mice were sacrificed, and fetal lungs were collected, flash frozen in liquid nitrogen, and stored at -80°C. Levels of PCNA protein, Ki67 protein (A, B and D; proliferation marker) and caspase-3 protein (A and C; apoptosis marker) were determined by standard western blot analysis and immunohistochemistry. Data are presented as means ± SEM (n=6 litters). Scale bar, 20 µm.

3.3.5 BPA disrupts alveolar epithelial type I cell differentiation

To define the precise cellular defects in the BPA-induced lung immaturity phenotype, we measured alveolar epithelial types I and II cell-specific markers, and used them as indicators of epithelial cell differentiation. We found that both mRNA and protein levels of SP-A, -B, -C, and -D type II cell markers[36,37], were similar between control BPA-exposed fetal lungs (**Fig. 3.5**). In marked contrast, both mRNA and protein levels of AQP5, a water channel protein whose expression is restricted to type I cells during lung development[38,39], were dramatically reduced in BPA-exposed fetal lungs (**Fig. 3.6**).

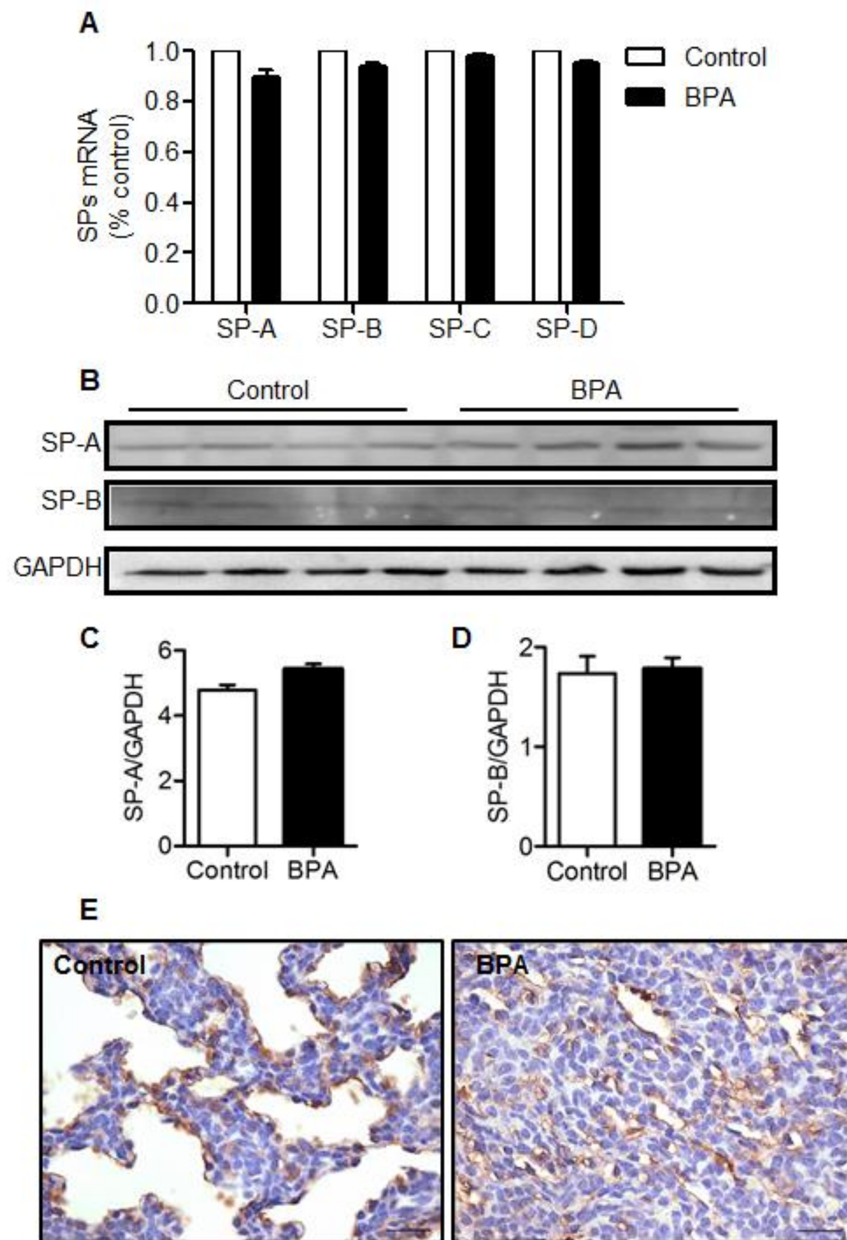

Figure 3.5: BPA does not affect alveolar epithelial type II cell differentiation. Pregnant mice were fed a control diet (phytoestrogen free food pellets) or the control diet supplemented with 25 mg BPA/kg food pellets from embryonic day (E) 7.5 to E18.5. On E18.5, mice were sacrificed, and fetal lungs were collected, flash frozen in liquid nitrogen, and stored at -80°C. The mRNA (A) and protein (B-E) levels of surfactant proteins (SP-A, SP-B, SP-C, and SP-D) were determined by qRT-PCR, western blotting and immunohistochemistry (SP-C), respectively. Data are presented as means ± SEM (n=6 litters). Scale bar, 20 μm.

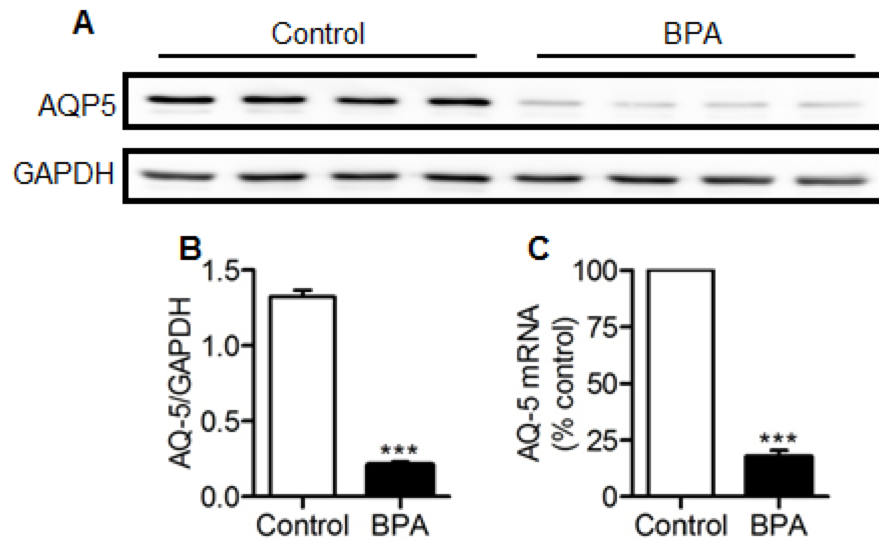

Figure 3.6: BPA disrupts alveolar epithelial type I cell differentiation. Pregnant mice were fed a control diet (phytoestrogen free food pellets) or the control diet supplemented with 25 mg BPA/kg food pellets from embryonic day (E) 7.5 to E18.5. On E18.5, mice were sacrificed, and fetal lungs were collected, flash frozen in liquid nitrogen, and stored at -80°C. Levels of AQP5 protein (A and B) and mRNA (C) were determined by qRT-PCR and western blotting, respectively. Data are presented as means ± SEM (****P*<0.001; n=6 litters).

3.3.6 BPA deceases the expression of epithelial sodium channel γ and glutathione peroxidase but not versican and midkine

To determine whether BPA interferes with glucocorticoid signaling, we first examined changes in the expression of epithelial sodium channel γ (ENaCγ) and GPX, 2 well-known glucocorticoid target genes in the lung[40]. We showed that levels of both ENaCγ and glutathione peroxidase (GPX) proteins were significantly decreased in BPA-exposed fetal lungs compared to controls (**Fig. 3.7A–C**). We also examined the expression of another 2 glucocorticoid target genes, but we found that mRNA levels of both versican and midkine were not different between control and BPA-exposed fetal lungs (**Fig. 3.7D**).

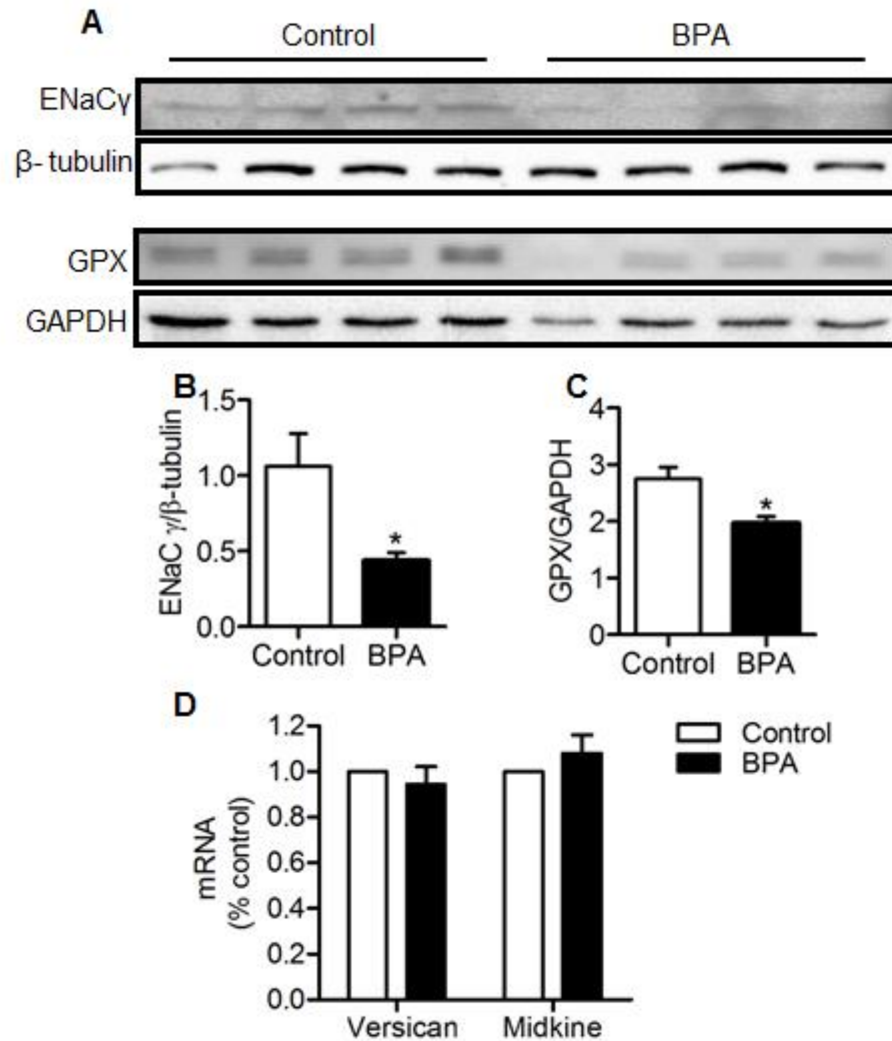

Figure 3.7: BPA decreases the expression of glucocorticoid target genes. Pregnant mice were fed a control diet (phytoestrogen free food pellets) or the control diet supplemented with 25 mg BPA/kg food pellets from embryonic day (E) 7.5 to E18.5. On E18.5, mice were sacrificed, and fetal lungs were collected, flash frozen in liquid nitrogen, and stored at -80°C. Levels of ENaCγ (A and B), GPX (A and C), Versican (D), and Midkine (D) proteins were determined by western blotting. Data are presented as means ± SEM (*P<0.05; n=6 litters).

3.3.7 BPA does not alter fetal plasma levels of corticosterone

We then examined changes in fetal plasma corticosterone levels. We found that plasma levels of corticosterone were similar between control and BPA-exposed fetuses at E18.5 (**Fig. 3.8A**). To ascertain whether changes in corticosterone levels may precede the gestational age at which the lung immaturity phenotype was observed, we also measured fetal plasma corticosterone levels at E15.5. Our results showed that BPA did not alter corticosterone levels at this gestational age (**Fig. 3.8B**).

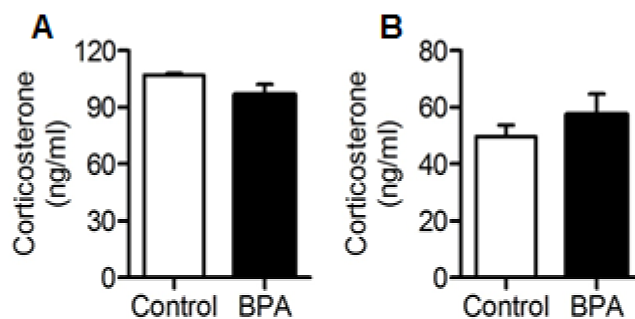

Figure 3.8: BPA does not alter fetal plasma levels of corticosterone. Pregnant mice were fed a control diet (phytoestrogen free food pellets) or the control diet supplemented with 25 mg BPA/kg food pellets from embryonic day (E) 7.5 to E15.5 or E18.5. On E15.5 and E18.5, mice were sacrificed, and blood samples collected from fetuses. Plasma was then collected, and stored at -80°C until use. Levels of corticosterone at E18.5 (A) and E15.5 (B) were determined in fetal plasma samples using an ELISA Kit. Data are presented as means ± SEM (n=6 litters).

3.3.8 Maternal dexamethasone administration rescues the lung immaturity phenotype

To provide further insight into the possibility that BPA interferes with glucocorticoid signaling, we determined whether synthetic glucocorticoid administration would rescue the BPA-induced lung immaturity phenotype. To do so, we treated BPA-exposed pregnant mice with two courses of dexamethasone injections at E16.5 and E17.5, respectively. We showed that maternal dexamethasone administration completely restored the alveolar architecture such that there was no observable difference in alveolar airspace and septa thickness between control and BPA-exposed fetal lungs (**Fig. 3.9A**). Moreover, dexamethasone treatment also normalized AQP5 protein expression in BPA-exposed fetal lungs (**Fig. 3.9B, C**).

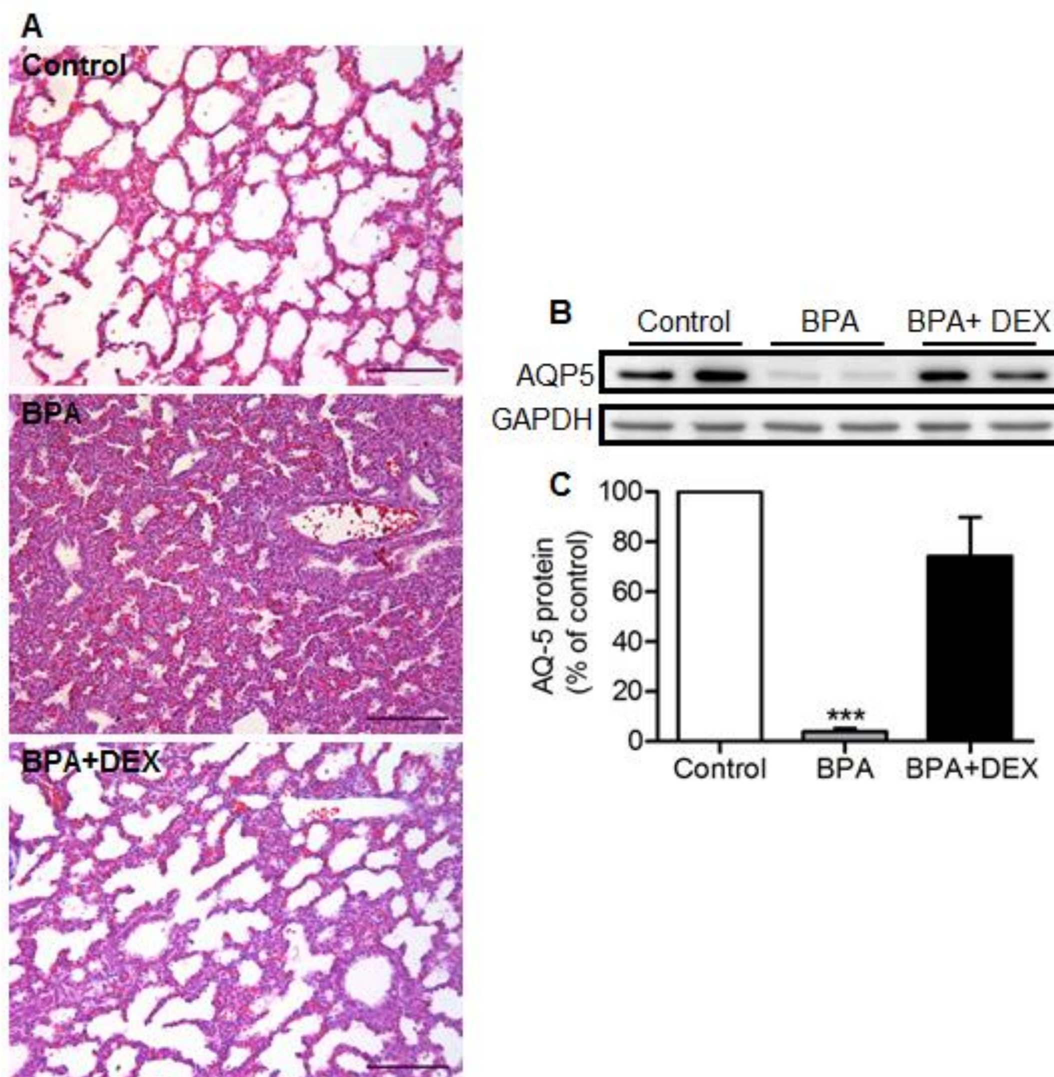

Figure 3.9: Maternal dexamethasone administration rescues the lung immaturity phenotype. Pregnant mice were fed a control diet (phytoestrogen free food pellets) or the control diet supplemented with 25 mg BPA/kg food pellets from embryonic day (E) 7.5 to E18.5. On E16.5 and E17.5, control and BPA fed mice were injected subcutaneously with saline and dexamethasone (0.4 mg/kg), respectively. On E18.5, mice were sacrificed, fetal lungs were collected, fixed, and subjected to standard histological analysis, or flash frozen in liquid nitrogen, and stored in -80°C. Representative fetal lung histology from mice fed the control diet, the control diet supplemented with 25 mg BPA/kg, and the control diet supplemented with 25 mg BPA/kg + dexamethasone are shown in (A). Protein levels of AQP5 (B and C) were determined by standard western blot analysis. Data are presented as mean ± SEM (***P<0.001; n=6 litters). Scale bar, 100 µm.

3.4 Discussion

Numerous studies have shown that BPA permanently alters organ growth and development, ultimately leading to tissue dysfunction and various diseases later in life[3,4,41]. Although developmental exposure to BPA is linked to impaired lung function and the development of asthma in both humans and experimental animals, to our knowledge, the role of BPA in fetal lung development had never been explored in either human clinical/epidemiologic studies or animal models. In the present study, we demonstrate that prenatal exposure to environmentally relevant doses of BPA *via* maternal diet severely impairs fetal lung maturation in the mouse. We also provide evidence suggesting that these effects of BPA are mediated at least in part through the glucocorticoid-signaling pathway. Thus, our present findings highlight the potential adverse effects of BPA on human fetal lung maturation and suggest a fetal origin for BPA-induced lung dysfunction and diseases. However, we recognize that prenatal exposure to BPA in humans likely contributes to a small proportion of cases of abnormal fetal lung development in instances where fetuses are exposed to high levels of BPA during critical periods of development.

The BPA dosages used in our study (5, 25, and 50 mg/kg diet; equivalent to 1, 5, and 10 mg BPA/kg body weight/d) were designed with the maximal dosage (*i.e.,* 10 mg/kg/d) to mimic that used in a previous study on A^{vy} mice in which BPA shifted the coat color and altered the methylation status of the *Agouti* gene without any adverse effects[42]. Furthermore, these dosages were also several orders of magnitude below the lowest observable adverse effect level (LOAEL) for rodents (50 mg/kg/d), as established by the U.S. Environmental Protection Agency[43]. Importantly, the middle dose produced an average maternal plasma BPA concentration of 1.7 ng/ml, which is at the lower end of the range (0.5–22.3 ng/ml) reported in pregnant women in the United States[44]. This concentration is 10 times lower than the estimated maximal concentration (20 ng/ml, which is estimated to be maximal because pregnant mice were exposed for a total of 11 d in the present study *vs.* 28 d in the previous study) based on one previous mouse study in which the same BPA-containing food pellet (5 mg/kg; equivalent to 1 mg BPA/kg body weight/d) was fed before and throughout pregnancy and lactation[11]. Although the precise reasons for our apparent underestimation of plasma BPA concentrations are unclear, it is possible that

BPA degradation may be a contributing factor, because maternal plasma samples were stored at −80°C for over 1 year before being analyzed for BPA concentrations. Nevertheless, even the estimated maximal maternal BPA concentration of 20 ng/ml falls within the range of those reported previously in pregnant women, indicating that the dosage used in the present study is relevant to humans.

Previous studies have shown that prenatal BPA exposure *via* various routes and at different dosages can lead to fetal growth restriction in animal models, although not all studies demonstrated such an effect[4]. In the present study, we found no effect of BPA on fetal body weight or litter size, regardless of the dosage. This demonstrates that the BPA dosages used in this study have no adverse effects on overall fetal growth morphometry. We then examined the effects of BPA on the structural maturation of fetal lung at E18.5, a critical organ essential for extrauterine survival and a time point at which all previous studies on fetal lung maturation were carried out[31–34]. By standard histologic analysis, we showed that BPA at both 25 and 50, but not 5, mg/kg diet severely impaired fetal lung maturation, as evidenced by diminished alveolar airspace and thickened alveolar septa, reminiscent of the impaired lung maturation phenotype of several knockout mouse models. These include glucocorticoid receptor (GR)[30–33]; corticotrophin-releasing hormone (CRH)[31]; critical signal transduction proteins, such as extracellular signal–regulated kinase 3 (Erk3)[32]; differentiation genes of lung type I cells, such as T1α[45], and several transcription factors that play key roles in development such as hypoxia-inducible factor (HIF)-1α[35], HIF-2α[46], and specificity protein 3 (Sp3)[47]; and c/ebpα[34].

To ascertain whether the thickened alveolar septa were a result of altered epithelial cell maturation, we examined cytoplasmic glycogen levels in fetal lung sections from both control and BPA-exposed mice. BPA-exposed fetal lungs showed a ∼50% increase in glycogen content, a well-known indicator of immature epithelial cells and the level of which has also been shown to be elevated in GR[33], Erk3[32], HIF-1α[35], and c/ebpα[34] knockout mice. We then determined whether altered proliferation and/or apoptosis contributed to the observed increase in alveolar septal wall thickness. Our results suggested that prenatal exposure to BPA unlikely affected epithelial cell proliferation or apoptosis because both mRNA and protein levels of Ki-67 as well as protein levels of PCNA and caspase-3

(universal markers of cell proliferation and apoptosis, respectively) were similar between control and BPA-exposed fetal lungs. Our present findings also corroborated studies that reported similar findings using mice deficient in the cell cycle inhibitors *p21/p57*[48]. However, our findings deviate from those reported in GR-null mice, which showed hyperproliferation in the fetal lung mesenchyme component[49].

The functional units of lungs are composed of alveolar epithelial type I and type II cells, which are responsible for gas exchange and surfactant production, respectively[50]. Type II cells, derived from immature pretype II cells, are considered the progenitors of both epithelial cell types, capable of proliferation and differentiation into type I cells[50]. The type II cells are marked by the expression of SP-A, SP-B, SP-C, and SP-D[36,37]. We showed that prenatal BPA exposure did not alter the expression of any SPs, indicating that type II cell differentiation was not affected. Similar findings were also reported in T1α[45], Sp3[47], and sorting nexin 5 (Snx5)-null mice[51], which displayed the same lung immaturity phenotype as observed in the present study. Having established the lack of an effect of BPA on type II cell differentiation, we then examined the status of type I cell differentiation. We did so by measuring the expression level of AQP5, a type I cell-specific marker and a key water channel protein involved in the transcellular movement of water across the type I cells[38,39]. We showed that levels of AQP5 protein were dramatically decreased in BPA exposed fetal lungs, indicative of impaired differentiation of type I cells. This contention is supported by a previous study showing a similar lung immaturity with disrupted type I cell differentiation in mice deficient in the type I cell differentiation gene *T1α*[45]. Decreased AQP5 expression, and by inference impaired type I cell differentiation, was also observed in fetal lungs of several mutant mouse models, including a locked-in mutant mouse model with a disrupted thyroid hormone receptor corepressor function[52], GR[33] and Snx5[51]-deficient mice, and mice harboring a silencing mutation in the GATA-6 transcription factor[53]. Taken together, these findings demonstrate that prenatal exposure to BPA impairs type I, but not type II, epithelial cell differentiation.

Given that glucocorticoids play a key role in fetal lung maturation[40] and that our BPA-induced lung immaturity phenotype closely resembles that of glucocorticoid deficient (*i.e.,* $CRH^{-/-}$ and $GR^{-/-}$) mouse models[31,33], we hypothesized that BPA disrupts fetal lung

maturation by suppressing glucocorticoid signaling. As a first step in testing this hypothesis, we determined whether prenatal exposure to BPA resulted in reduced expression of key glucocorticoid target genes in the lung. We showed that the expression of both ENaCγ and GPX was significantly decreased in BPA-exposed fetal lungs. This repression can be achieved by either decreasing circulating levels of corticosterone or antagonizing glucocorticoid actions. Our examination found no evidence to support the first possibility because fetal plasma levels of corticosterone were not altered at either E18.5, when the lung immaturity phenotype was observed, or E15.5, a critical time point just before terminal lung differentiation. More importantly, maternal dexamethasone administration, a common intervention in managing high-risk pregnancies with threatened preterm delivery to promote fetal lung maturation[54], rescued the lung immaturity phenotype and normalized AQP5 protein expression. Thus, our data support the possibility that BPA functions as a glucocorticoid antagonist to disrupt fetal lung maturation. Indeed, one recent study using *in vitro* yeast bioassays found that BPA acts as a GR antagonist, with a drug concentration causing 50% inhibition of 67 μM[55]. However, the lack of a change in expression of cell proliferation markers as well as some of the glucocorticoid target genes (*i.e.,* versican and midkine) in the present study is not entirely compatible with a glucocorticoid-mediated signaling pathway because fetal lungs from GR-null mice exhibit hyperproliferation in the mesenchyme component[49]. This suggests that the BPA-induced lung immaturity phenotype is partially mediated through glucocorticoid signaling. Furthermore, given that circulating levels of corticosterone in fetal mice increase dramatically at E16.5 to drive the final stages of fetal lung maturation[56], we anticipate that the BPA-induced lung immaturity phenotype would be evident before E18.5 (*e.g.,* E16.5), similar to that reported in GR-null mice[30]. Obviously, future studies will be required to confirm this as well as to provide definitive evidence in support of BPA acting as a glucocorticoid antagonist in fetal lung cells.

In conclusion, the present study demonstrated that BPA disrupts fetal lung maturation partially *via* the glucocorticoid signaling pathway. Although the long-term consequences of our present findings remain to be determined, they suggest that BPA-induced lung dysfunction and diseases in later life, as reported previously in mice and humans, may have a fetal origin. Furthermore, because BPA pharmacokinetics (metabolism and clearance) is

similar between humans and mice[57], our present findings also underscore the potential impact of BPA exposure during pregnancy on fetal lung development in humans.

3.5 References

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4 BISPHENOL A SUPRESSES GLUCOCORTICOID TARGET GENE EXPRESSION VIA A NOVEL ERβ-NF-kB-GR SIGNALING PATHWAY IN LUNG EPITHELIAL CELLS[2]

[2] The material in this chapter is based on a manuscript accepted (with minor revisions) to *Archives of Toxicology*: Hijazi A, Guan H, and Yang K. Bisphenol A suppresses glucocorticoid target gene expression via a novel ERβ-NF-κB-GR signaling pathway in lung epithelial cells. (2016)

4.1 Introduction

Exposure to endocrine disrupting chemicals (EDCs) has been associated with a range of human diseases including cancer, diabetes, obesity, and decreased fertility[1,2]. Bisphenol A (BPA), one of the most prevalent EDCs in the environment, has gained world-wide attention due to growing evidence of its association with adverse health effects in humans and experimental animal models[3,4]. BPA is an industrial chemical used to manufacture polycarbonate plastics, epoxy resins lining the inside of food and beverage containers, thermal print papers and composites used in dental sealants[5]. The pervasiveness of BPA is such that it is detected in the air, water and soil; making human exposure to BPA unavoidable[5]. Biomonitoring studies have detected BPA at nanomolar concentrations in human body fluids such as serum, milk, saliva, and urine collected from all over the world[5,6]. Importantly, BPA has been shown to accumulate in human body tissues, thus exposure levels are much higher than previously thought[7]. Emerging evidence suggests that BPA may influence multiple endocrine-related signaling pathways during development, resulting in adverse effects on various organs including the brain[8,9], heart[10], mammary gland[11,12], lungs[13], liver[14], ovary[15,16], and uterus[17].

We recently showed that prenatal exposure to BPA severely impaired fetal lung maturation in the mouse, likely via the glucocorticoid (GC) signaling pathway[13]. Other studies have shown that early life exposure to BPA interferes with lung developmental processes in humans and animal models, resulting in abnormal lung structure and function later in life. In children, exposure to BPA during various periods of development is associated with impaired lung function[18] and the development of asthma[19,20] and wheeze[21]. In addition, high serum levels of BPA have been linked to chronic obstructive pulmonary disease (COPD)[22]. More importantly, recent studies suggest that exposure to BPA exclusively during the prenatal period is sufficient to disrupt lung function later in life. For example, prenatal, but not postnatal, BPA exposure has been linked to diminished lung function and chronic wheeze in children[23]. This finding is supported by an animal study demonstrating that exposure to BPA during gestation, but not lactation, leads to asthma in mice[24]. Collectively, these findings show that prenatal exposure to BPA is detrimental to lung development and function, however, the underlying mechanism remains elusive.

Considering the critical role of the glucocorticoid receptor (GR) during fetal lung development and the evidence that BPA can act as a GR antagonist, the present study was undertaken to examine how BPA alters the GC signaling pathway using a human epithelial cell line as a model system.

4.2 Materials and Methods

4.2.1 Reagents

Bisphenol A was purchased from Sigma-Aldrich Canada Ltd. (>99% purity; CAS 80-05-7; Oakville, ON) and dissolved in ethanol to prepare a 10 mM stock solution, and stored at -20°C. BAY 11-7082 was purchased from Millipore (cat. no. 196871) and dissolved in DMSO to prepare a 100 mM stock, and stored at -20°C. Dexamethasone was purchased from Stemcell Technologies (cat. no. 05407) and dissolved in ethanol to a concentration of 4 mg/mL, and stored at -20°C.

4.2.2 Cell culture

A549 cells, an alveolar cell carcinoma-derived cell line, were obtained from the American Type Culture Collection (Manassas, VA, USA). A549 lung cells were cultured in standard growth medium consisting of RPMI 1640 media (Invitrogen), 10% fetal bovine serum (FBS; Sigma), and 100 IU penicillin and 100 µg/mL streptomycin (Invitrogen). All cultures were maintained in a humidified incubator at 37°C and 5% CO_2. Growth medium was replaced every other day.

4.2.3 Real time quantitative RT-PCR

The relative abundance of various mRNAs was assessed by a two-step real-time quantitative RT-PCR (qRT-PCR), as described previously[25] with the following specific modifications. Briefly, total RNA was extracted from cells using RNeasy Mini Kit (QIAGEN Inc., Mississauga, ON) coupled with on-column DNase digestion with the RNase-Free DNase Set (QIAGEN) according to the manufacturer's instructions. 1 µg of total RNA was reverse-transcribed in a total volume of 20 µL using the High Capacity cDNA Archive Kit (Applied Biosystems, Forest City, CA) following the manufacturer's instructions. For every RT reaction set, one RNA sample was set up without reverse-

transcriptase enzyme to provide a negative control. Gene transcript levels of *GAPDH* (housekeeping gene whose expression level was found to be stable across all treatment groups), *IκB-α*, *p65*, and *ENaCγ* were quantified separately by pre-designed and validated TaqMan® Gene Expression Assays (Applied Biosystems; Table 4.1) following the manufacturer's instructions. Briefly, gene expression assays were performed with the TaqMan® Gene Expression Master Mix (Applied Biosystems; P/N #4369016) and the universal thermal cycling condition (2 minutes at 50°C and 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C) on the ViiA^{TM} 7 Real-Time PCR System (Applied Biosystems).

The relative amounts of various gene specific mRNAs in each RNA sample was quantified by the comparative C_T method (also known as $\Delta\Delta$ C_T method) using the Applied Biosystems relative quantitation and analysis software according to the manufacturer's instructions. For each experiment, the amount of various gene specific mRNAs under different treatment conditions is expressed relative to the amount of transcript present in the untreated control.

4.2.4 Western blot analysis

Western blot analysis was used to determine various protein levels, as described previously[26]. Briefly, A549 cells were lysed in SDS Sample Buffer (62.5 mM Tris–HCl, pH 6.8, 2% w/v SDS, 10% v/v glycerol, 50 mM DTT and 0.01% w/v bromophenol blue). Equal concentrations of the whole cell lysates, or cytosolic and nuclear extracts, were subjected to a standard 10% SDS-PAGE. After electrophoresis, proteins were transferred to PVDF transfer membrane (Amersham Hybond-P, cat. no. RPN303F, GE Healthcare Lifesciences, Baie D'Urfe, QC) using a Bio-Rad Mini Transfer Apparatus. Nonspecific antibody binding was blocked with 5% wt/vol milk in TTBS (0.1% vol/vol Tween-20 in TBS) for 1 h at room temperature. Membranes were then hybridized with the appropriate primary antibody (Table 4.2) overnight at 4°C. The membranes were probed with the horseradish peroxidase-labeled secondary anti-rabbit antibody for 1 h at room temperature the following day. Proteins were detected by chemiluminescence (cat. no. WBLUR0500, Luminata Crescendo, Western HRP Substrate; Millipore, Etobicoke, ON) and visualized

using the VersaDoc Imaging System (BioRad, UK). Densitometry was performed on the images, and the level of various proteins was expressed as a percentage of controls.

4.2.5 siRNA-mediated knockdown of GR expression

To provide direct evidence for the involvement of the GR in mediating the effects of BPA on ENaCγ expression, a siRNA-mediated knockdown approach was utilized[27]. Briefly, A549 cells were plated on 6-well plates and cultured under standard conditions for 48 h. Cells were then transfected with 20 nM of siRNA targeting human GR (siGENOME SMARTpool- Human) in Opti-MEM I medium (Invitrogen) containing Lipofectamine™ 3000 (Invitrogen), following the manufacturer's instructions. Cells were also transfected in an identical manner with 20 nM of the negative control #1 siRNA (cat. no. 4624, Ambion) or with the transfection agent alone to serve as controls. At 48 h post-transfection, cells were collected for western blot analysis. Alternatively, 36 h or 30 h after transfection, cells were treated for 12 h or 24 h with 10 nM of BPA, respectively.

4.2.6 Statistical Analysis

Results are presented as mean ± SEM of four independent experiments, as indicated. Data were analyzed using one-way ANOVA followed by Tukey's post-hoc test, or Student's t test as indicated. Significance was set at $p<0.05$.

Table 4.1. TaqMan® gene expression assays for the human genes analyzed.

Gene Name	Assay ID
I-κBα	Hs00153283_m1
p65	Hs00153294_m1
ENaCγ	Hs00168918_m1
GAPDH	Hs02758991_g1

Table 4.2. Antibodies for the proteins analyzed.

Antibody	Catalogue No.	Dilution	Company
GR	sc-8992	1:1,000	Santa Cruz Biotechnology, Inc., Santa Cruz. CA
Epithelial sodium channel-gamma	ab3468	1:1,000	Abcam, Toronto, ON
NF-kB p65	sc-372	1:1,000	Santa Cruz Biotechnology, Inc., Santa Cruz. CA
IκB-α	sc-371	1:1,000	Santa Cruz Biotechnology, Inc., Santa Cruz. CA
Hsp90	ab178854	1:5,000	Abcam, Toronto, ON
ERβ	sc-8974	1:1,000	Santa Cruz Biotechnology, Inc., Santa Cruz. CA
ERα	sc-543	1:500	Santa Cruz Biotechnology, Inc., Santa Cruz. CA
GAPDH	IMG-3073	1:5,000	IMGENEX, Port Coquitlam, BC
Histone H3	06-755	1:5,000	Millipore, Temecula, CA
β-Tubulin	IMG-5810	1:1,000	IMGENEX, Port Coquitlam, BC
HRP-labeled anti-rabbit Antibody	HAF008	1:1,000	R&D Systems, Minneapolis, MN, USA

4.3 Results

4.3.1 Concentration-dependent effects of BPA on ENaCγ expression

Given that BPA diminished the expression of ENaC**γ**, a well-known GR target gene[28], in fetal lungs, we used this GR target gene to delineate the molecular pathway through which BPA exerts its effects on lung cells. As shown in **Fig. 4.1**, treatment with increasing concentrations of BPA (1–1000 nM) for 24 h resulted in a concentration-dependent decrease in levels of ENaC**γ** protein.

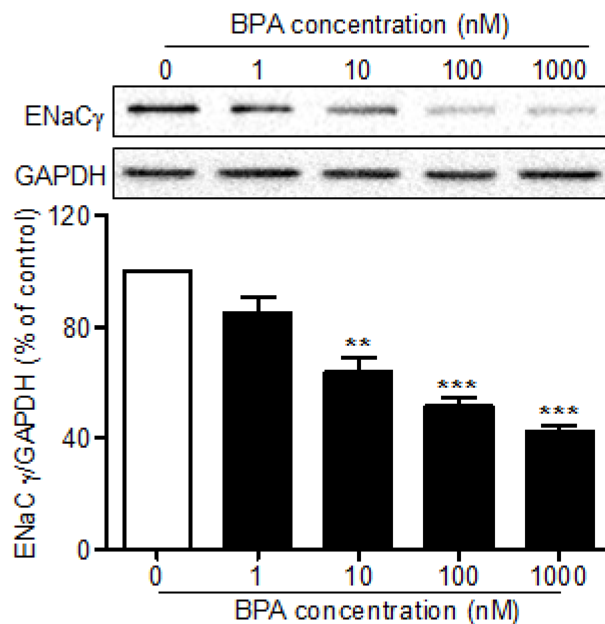

Figure 4.1: Concentration-dependent effects of BPA on ENaCγ expression. A549 cells were maintained for 4 h in serum-free medium and then treated with increasing concentrations of BPA (1–1000 nM) for 24 h. At the end of treatment, levels of ENaCγ protein were determined by western blotting. Data are presented as mean ± SEM of four independent experiments (**P<0.01, ***P<0.001 vs. control).

4.3.2 Effects of dexamethasone and siRNA-mediated knockdown of GR on BPA inhibition of ENaCγ expression

As a first step in determining if BPA repression of ENaCγ expression occurs via inhibition of GR activity, we treated cells with BPA in the presence and absence of dexamethasone, a potent GR agonist. We first confirmed dexamethasone significantly increased both ENaCγ mRNA and protein in A549 cells (Fig. 4.2A and 2B). We then showed that treatment with dexamethasone counteracts the effects of BPA on both ENaCγ mRNA and protein (Fig. 4.2A and 2B). However, since the expression level ENaCγ is much lower than with dexamethasone alone, this suggests BPA may be a non-competitive inhibitor of the GR. To provide definitive evidence for the involvement of GR in mediating the inhibitory effects of BPA on ENaCγ, we conducted siRNA-mediated knockdown of GR expression. We found that transient transfection of A549 cells with siRNA targeting GR resulted in 86% reduction in the levels of GR protein without altering levels of GAPDH protein (**Fig. 4.2C**). Furthermore, transfection with a negative control siRNA (i.e., scrambled siRNA) did not affect levels of GR protein or GAPDH protein, demonstrating the specificity of the siRNA-mediated knockdown of GR in A549 cells. Having established the efficacy of siRNA-mediated knockdown of GR expression, we then determined the effects of BPA on ENaCγ expression in these cells. We showed that GR knockdown resulted in the complete loss of the ability of BPA to inhibit ENaCγ expression (**Fig. 4.2D**).

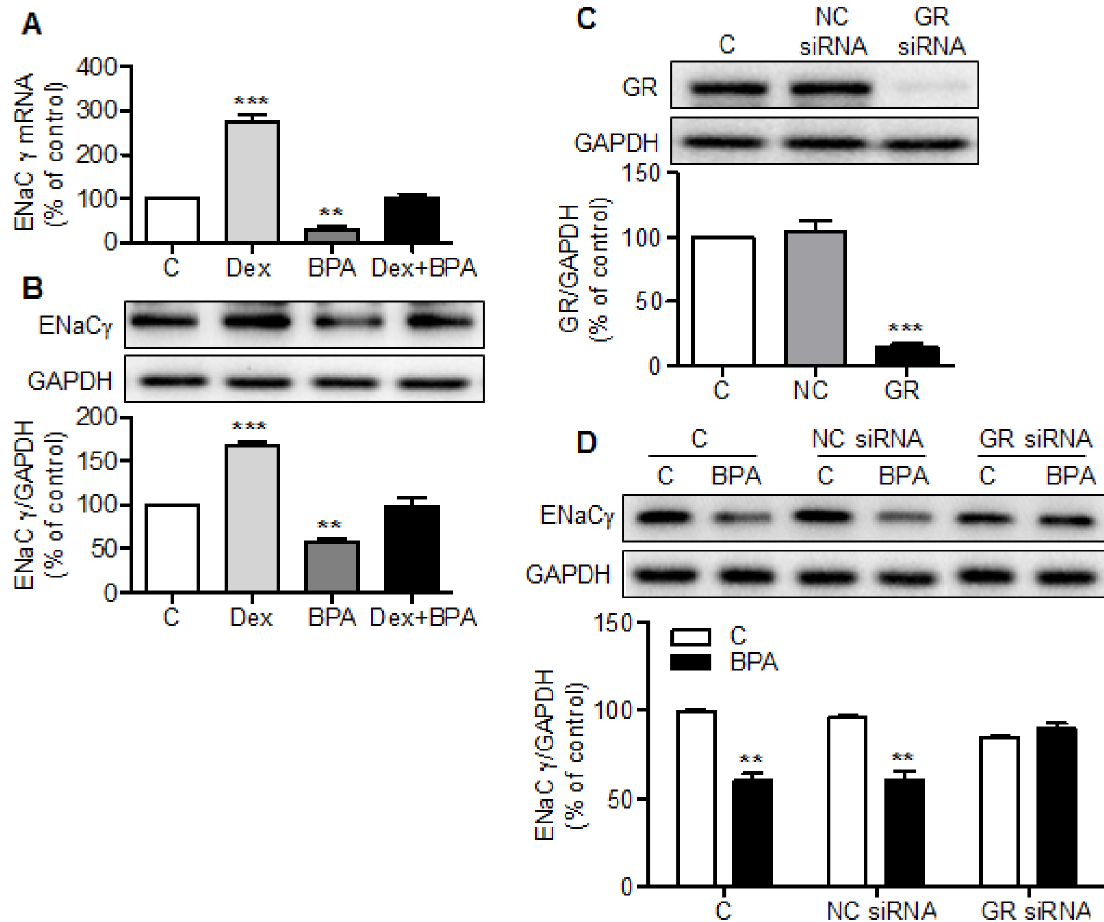

Figure 4.2: Dexamethasone and siRNA knockdown of GR reverse the inhibitory effects of BPA on ENaCγ expression. A549 cells were maintained for 4 h in serum-free medium and then treated with either 10 nM BPA, 100 nM Dex or co-treated with 10 nM BPA and 100 nM Dex for 24 h. At the end of treatment, levels of ENaCγ mRNA (A) and ENaCγ protein (B) were determined by qRT-PCR and western blotting, respectively. A549 cells were transfected with 20 nM of GR siRNA, 20 nM negative control (i.e., scrambled) siRNA or the transfection agent alone to serve as control. Forty-eight hours after transfection, cells were lysed, and levels of GR protein were determined by western blotting (C). GAPDH was used as a control to show the specificity of siRNA mediated knockdown of GR. Alternatively, 24 h after transfection, cells were treated for 24 h with or without 10 nM of BPA (D). At the end of treatment, levels of ENaCγ protein were determined by western blot analysis. Data are presented as mean ± SEM of four independent experiments (**P<0.01, ***P<0.001 vs. control).

4.3.3 Effects of BPA on the selected key components of GR signaling pathway

To determine the precise mechanism by which BPA represses GR activity, we analyzed several key components of the GR signaling pathway. Given that HSP90 is associated with unbound GR in the cytoplasm[29], we reasoned that altered expression of HSP90 might be one of the mechanisms by which BPA influences GR activity. As shown in **Fig. 4.3A and 4.3B**, levels of HSP90 protein were not altered by BPA treatment for either 12 h or 24 h. We also measured and found no changes in the level of GR protein following BPA treatment (**Fig. 4.3C and 4.3D**). We then determined GR nuclear translocation by measuring GR protein levels in cytosolic and nuclear fractions following treatment with BPA. As shown in **Fig. 4.3E and 4.3F**, levels of GR protein in cytosolic fraction were significantly increased while those in nuclear fraction were significantly decreased following BPA treatment.

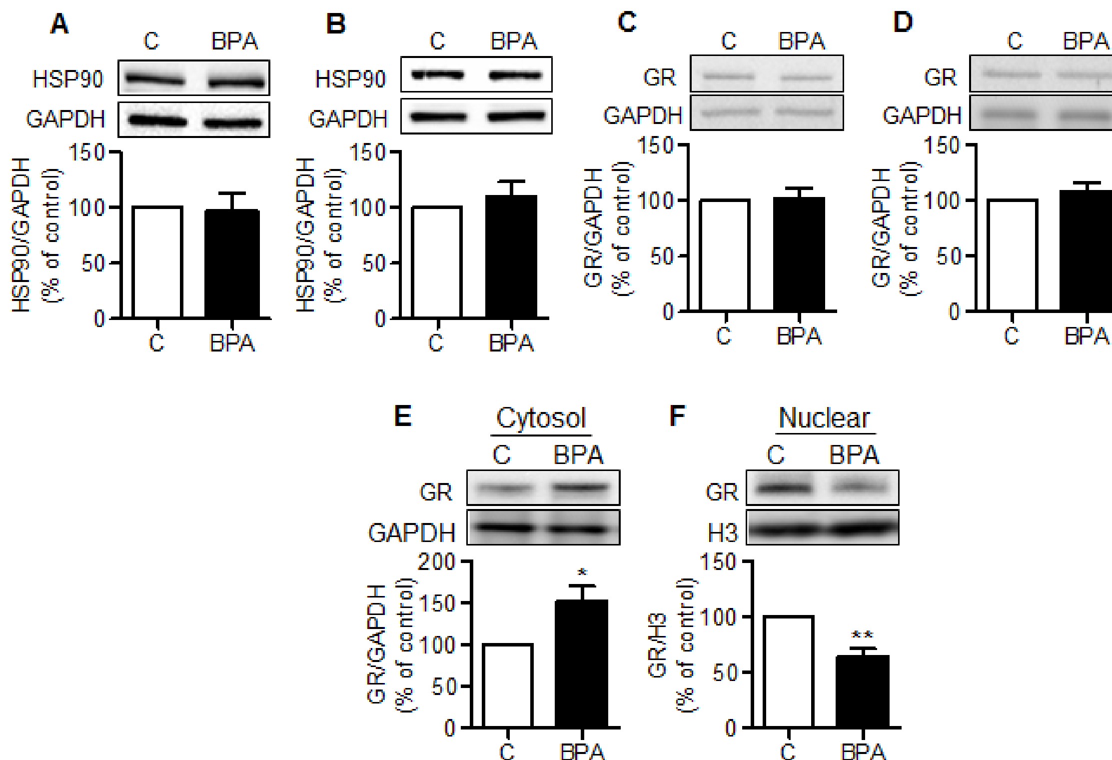

Figure 4.3: Effects of BPA on the GR signaling pathway. A549 cells were maintained for 4 h in serum-free medium and then treated with 10 nM BPA for either 12 h (A) or 24 h (B), after which levels of HSP90 protein were determined by western blot analysis. A549 cells were maintained for 4 h in a serum-free medium and then treated with 10 nM BPA for either 12 h (C) or 24 h (D), after which levels of GR protein were determined by western blot analysis. A549 cells were maintained for 4 h in a serum-free medium and then treated with 10 nM BPA for 12 h, at the end of the treatment cytosolic (E) and nuclear extracts (F) were subjected to western blotting for GR protein. Data are presented as mean ± SEM of four independent experiments (*P<0.05, **P<0.01 vs. control).

4.3.4 Effects of BPA on NF-κB expression and activity

Since a mutual antagonism exists between GR and NF-κB, and BPA is known to activate NF-κB[30–37], we hypothesized that BPA inhibits GR activity via induction of NF-κB. As a first step in testing this hypothesis, we measured NF-κB expression and activation following treatment with BPA for 12 and 24 h. We found that treatment with BPA for 12 h (**Fig. 4.4A and 4.4C**) but not 24 h (**Fig. 4.4B and 4.4D**) resulted in an increase in levels of both NF-κB (p65) mRNA and protein. Since NF-κB activation requires translocation of the p65 subunit to the nucleus, we measured p65 protein level in cytosolic and nuclear fractions following treatment with BPA for 12 h. We observed that BPA treatment significantly increased levels of p65 protein in both cytosolic and nuclear fractions (**Fig. 4.4E and 4.4F**). To provide functional evidence for the involvement of NF-κB activation in mediating BPA-induced inhibition of GR activity, we treated cells with BPA in the presence and absence of BAY 11-7082, an irreversible inhibitor of NF-κB activation. We showed that BAY 11-7082 abrogated the inhibitory effects of BPA on ENaCγ expression (**Fig. 4.4G**).

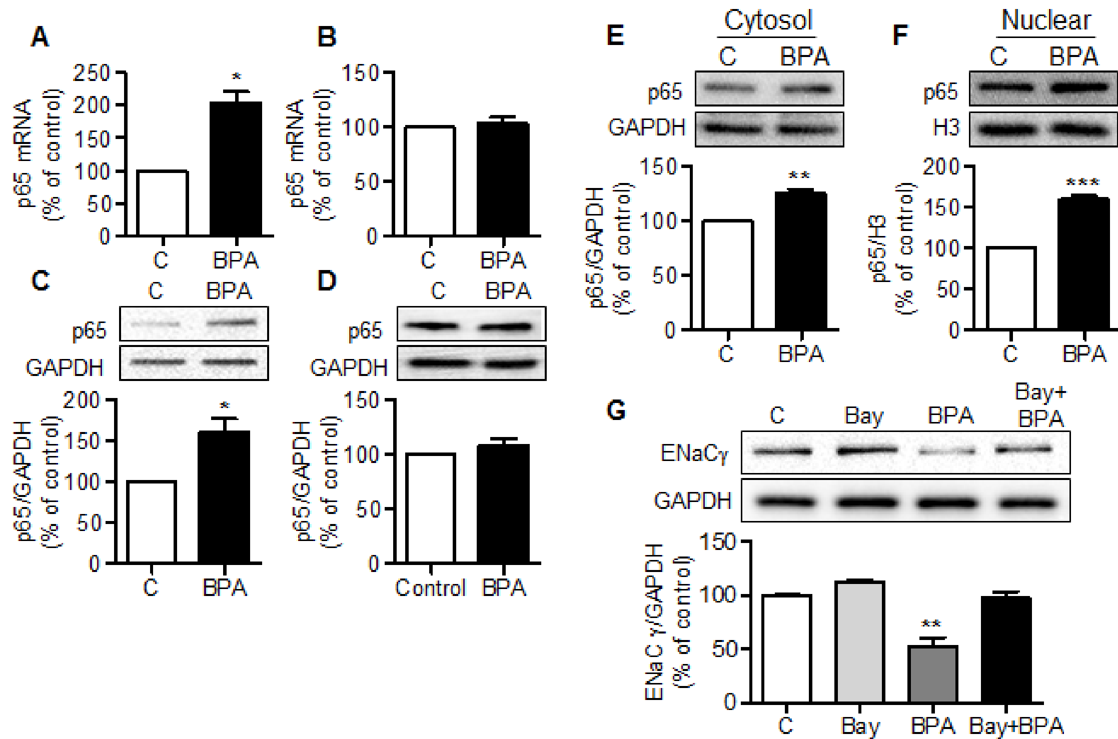

Figure 4.4: Involvement of NF-κB (p65) in BPA repression of ENaCγ expression. A549 cells were maintained for 4 h in serum-free medium and then treated with 10 nM BPA for either 12 h or 24 h. At the end of treatment, levels of p65 mRNA (A and B) and p65 protein (C and D) were determined by qRT-PCR and western blotting, respectively. A549 cells were maintained for 4 h in serum-free medium and then treated with 10 nM BPA for 12 h, at the end of the treatment cytosolic and nuclear extracts were subjected to western blotting for p65 protein (E and F). A549 cells were maintained for 4 h in serum-free medium and then treated with either 10 nM BPA, 10 μM BAY 11-7082 or pretreated for 1 h with 10 μM BAY 11-7082 and were then treated with 10 nM BPA for 24 h. At the end of treatment, levels of ENaCγ protein were determined by western blot analysis. Data are presented as mean ± SEM of four independent experiments (*P<0.05, **P<0.01, ***P<0.001 vs. control).

4.3.5 Effects of BPA on IκB-α expression

The translocation of NF-κB to the nucleus is critically controlled through binding with its inhibitory protein (IκB-α), and IκB-α expression is in turn induced by NF-κB activation in a classic negative feedback fashion[38–40]. We found that levels of IκB-α mRNA and protein were significantly decreased and increased following BPA treatment for 12 h (**Fig. 4.5A and 4.5C**) and 24 h (**Fig. 4.5B and 4.5D**), respectively.

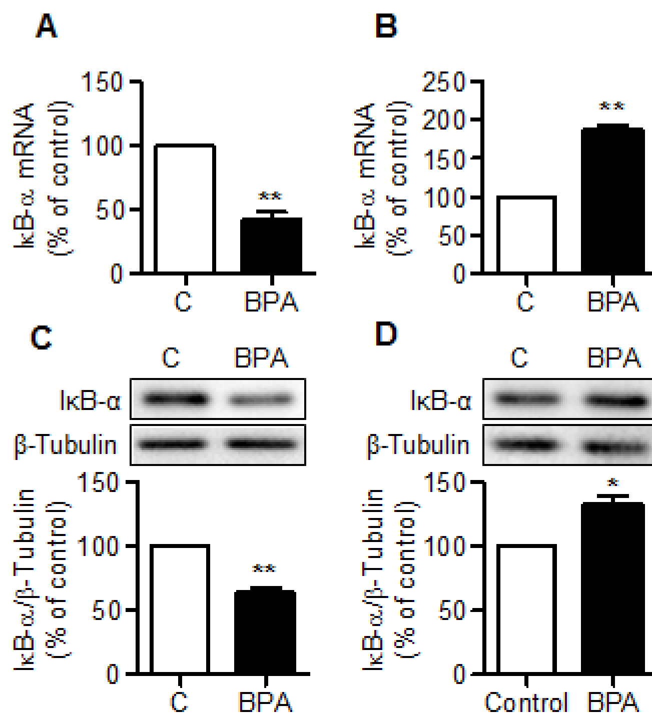

Figure 4.5: Effects of BPA on IκB-α expression. A549 cells were maintained for 4 h in serum-free medium and then treated with 10 nM BPA for either 12 h or 24 h. At the end of treatment, levels of IκB-α mRNA (A and B) and IκB-α protein (C and D) were determined by qRT-PCR and western blotting, respectively. For measuring IκB-α protein expression, β-Tubulin was used as a loading control since IκB-α (molecular weight of 35-41 kDa) and GAPDH (molecular weight of 37 kDa) have similar molecular weights. Data are presented as mean ± SEM of four independent experiments (*P<0.05, **P<0.01 vs. control).

4.3.6 Effects of ICI 182780 on BPA-mediated NF-κB expression and activation – involvement of ERβ

Since BPA can act as an inflammatory inducer via the estrogen receptor (ER)[34,35], we investigated the involvement of ER in mediating the effects of BPA on the NF-κB signaling pathway. As shown in Fig. 6, treatment with the ER-specific antagonist ICI 182780 (10 μM) completely blocked BPA-induced changes in levels of both p65 protein (**Fig. 4.6A**) and IκB-α protein (**Fig. 4.6B**). To link BPA activation of ER to BPA-induced repression of ENaCγ expression, we determined the effects of ICI 182780 and found that treatment with this ER antagonist abrogated the inhibitory effects of BPA on ENaCγ expression (**Fig. 4.6C**). Given that ICI 182780 does not discriminate ERα and ERβ, we examined and found that only ERβ protein is detected under basal conditions in A549 cells (**Fig. 4.6D**). To provide evidence for the hierarchy of BPA signaling (i.e., ER-mediated NF-κB is upstream of GR), we determined the effects of siRNA mediated knockdown of GR expression on BPA-induced p65 expression (**Fig. 4.7**). As shown in **Fig. 4.7**, BPA was equally effective in increasing levels of p65 protein in cells transfected with and without GR siRNA.

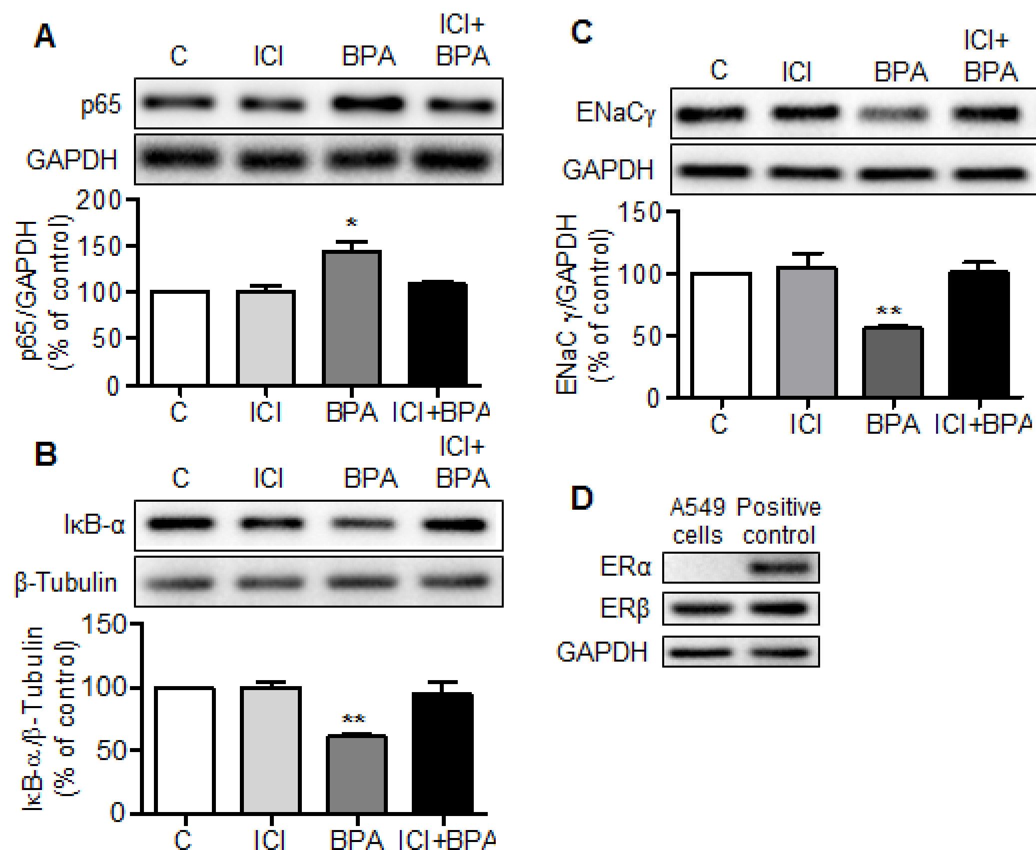

Figure 4.6: Effects of the Estrogen Receptor-β pathway on BPA mediated NF-κB (p65) expression and activation. A549 cells were maintained for 4 h in serum-free medium and then treated with either 10 nM BPA, 100 nM ICI 182780 or pretreated for 1 h with 100 nM ICI 182780 and were then treated with 10 nM BPA for 12 h or 24 h. At the end of treatment, levels of p65 (A), IκB-α (B) and ENaCγ (C) protein were determined by western blot analysis. Western blot analysis performed to examine the expression of estrogen receptor subunits (ERα and ERβ) in A549 cells (D). Data are presented as mean ± SEM of four independent experiments (*$P<0.05$, **$P<0.01$ vs. control).

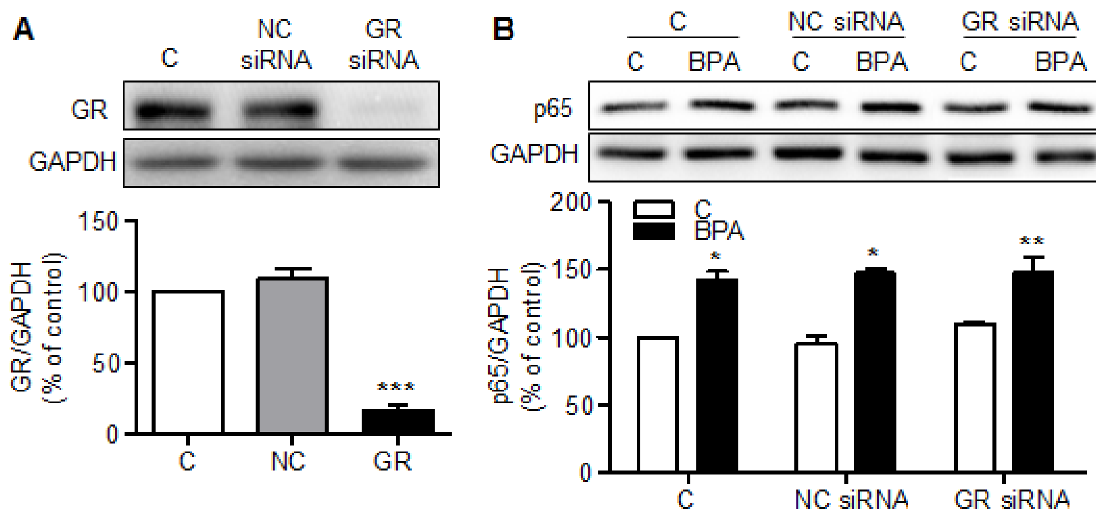

Figure 4.7: GR siRNA does not reverse the effects of BPA on p65 expression. A549 cells were transfected with 20 nM of GR siRNA, 20 nM negative control (i.e., scrambled) siRNA or the transfection agent alone to serve as control. Forty-eight hours after transfection, cells were lysed, and levels of GR protein were determined by western blotting (A). GAPDH was used as a control to show the specificity of siRNA mediated knockdown of GR. Alternatively, 36 h after transfection, cells were maintained for 4 h in serum-free medium and then treated for 12 h with or without 10 nM of BPA (B). At the end of treatment, levels of p65 protein were determined by western blot analysis. Data are presented as mean ± SEM of four independent experiments (*P<0.05, **P<0.01, ***P<0.001 vs. control).

4.4 Discussion

During fetal life, the GC-signaling pathway is essential in the development of major organs such as the brain, lung, liver and kidneys[41]. We recently demonstrated that prenatal exposure to environmentally relevant doses of BPA severely impairs fetal lung maturation in the mouse[13]. We also provided evidence that these effects are likely mediated through the GC signaling pathway, however, the precise molecular mechanisms remained obscure[13]. The present study demonstrates that BPA acts on ERβ to activate the NF-κB signaling pathway, which in turn leads to diminished GR activity and consequent repression of ENaC**γ** expression in lung epithelial cells. Thus, our present findings reveal a novel BPA signaling pathway that involves ERβ, NF-κB and GR.

The epithelial sodium channel (ENaC) is a universally recognized GC-target gene, as there is extensive evidence that ENaC expression can be regulated by glucocorticoids, particularly in the lungs[28]. Several studies demonstrate that dexamethasone increases the expression of all three ENaC subunits in a time-dependent manner in A549 cells[42,43]. In addition, GR knockout mouse studies measured the expression of the three ENaC subunits as an indication of ablated GR response in the lungs[44,45]. Interestingly, although ENaCγ expression is significantly reduced in the lung of GR-null mice, expression of the ENaC α and β subunits is relatively unaltered compared with the lungs of WT mice[44,45]. Considering that our previous study in mice showed that the expression of ENaCγ was significantly decreased in BPA-exposed fetal lungs, we investigated the effects of nanomolar concentrations of BPA on the expression of ENaCγ in lung epithelial cells. We show a robust concentration-dependent suppression of ENaC**γ** expression following BPA exposure. Further, we showed that dexamethasone blocked the inhibitory effects of BPA on ENaCγ mRNA and protein expression, suggesting that BPA may function to suppress GR signaling. Importantly, siRNA-mediated knockdown of GR expression completely abrogated the inhibitory effects of BPA on ENAC**γ** expression. Taken together, these findings demonstrate that BPA suppresses ENaC**γ** expression via inhibition of GR activity.

In the absence of GC stimulation, GR resides in the cytosol complexed with a variety of proteins, particularly HSP90[46,47]. The binding of GC to the cytoplasmic GR results in its release from its chaperone proteins and rapid nuclear translocation[47]. Within the nucleus,

GR induces transcription of genes by binding to specific DNA elements (GREs) at the regulatory sequences of GR-target genes[47]. To determine the mechanism by which BPA represses GR function, we analyzed several key components of the GR signaling pathway. HSP90 association with GR is essential for maintaining the receptor in a high affinity binding state. Studies *in vitro* show that GR immediately loses its ability to bind to GCs when stripped of HSP90[48,49]. Considering the important role of HSP90 in retaining GR activity, we postulated that changes in HSP90 protein expression would lead to a decrease in GR affinity and GC resistance. Our results suggested that this was unlikely as protein levels of HSP90 were similar between control and BPA treated cells. Next, we measured the levels of GR protein in lung cells following BPA treatment, and found that BPA treatment did not alter the level of GR protein. We then investigated whether BPA inhibited GR activity through inhibition of GR nuclear translocation, and found a significant decrease in GR protein in the nuclear fraction of cells treated with BPA. Taken together, these results suggest that BPA likely inhibits GR activity through decreasing GR nuclear translocation.

The nuclear factor-κB (NF-κB) transcription factor is a heterodimeric protein that comprises a p50 and a transcriptionally active p65 subunit[50]. NF-κB is a central mediator of the inflammatory process and a key participant in innate and adaptive immune responses. Recently, BPA has been shown to function as an inflammatory inducer, since BPA activates the NF-κB pro-inflammatory pathway in various cell types[30–37]. A mutual antagonism exists between GR and NF-κB, whereby NF-κB represses GR and *vice versa*[50,51]. This mutual antagonism is mediated solely through the p65 subunit of NF-κB[50,51]. Studies have indicated several mechanisms by which the physical interaction of NF-κB and GR can lead to mutual antagonism. First, in the cytoplasm, the direct interaction between activated NF-κB and GR has been reported as one of the mechanisms impairing GR nuclear translocation[52]. Second, in the nucleus[53,54], NF-κB and GR may compete for co-activators and/or by disturb their interaction with the basal transcription machinery and thus inhibit transcription of target genes[50,53,54]. We therefore postulated that the inhibitory effects of BPA on GR activity may be a result of NF-κB activation (p65) by BPA. As a first step in testing this hypothesis, we examined both the expression and activation of NF-κB (p65) in A549 cells following BPA treatment. We found that BPA significantly

increased NF-κB expression and activity, suggesting that BPA induced NF-κB activation in this cell line. In order to examine the role of NF-κB in the repression of GR-mediated gene activation following BPA treatment, we used BAY 11-7082, a selective and irreversible inhibitor of NF-κB activation. We showed that BAY 11-7082 abrogated the inhibitory effects of BPA on ENaCγ expression, indicating the involvement of NF-κB in BPA-mediated repression of GR activity.

It is well established that the activation of NF-κB is controlled by proteosomal degradation of its inhibitory subunit, IκB-α, which binds to and sequesters it in the cytosol, preventing the interaction of NF-κB with DNA to induce gene expression[38,39]. The IκB family of proteins each have slightly different functions, but most are directly upregulated by NF-κB activation[38,39]. IκB-α's unique role in NF-κB regulation is indicated by the constitutive nuclear activation of NF-κB in hematopoietic tissues from IκB-α knockout mice[55]. Moreover, constitutive NF-κB activation in Hodgkin's disease-derived cell lines is a consequence of defective IκB-α[56]. To ascertain whether increased NF-κB activity were a result of altered IκB-α expression, we examined IκB-α mRNA and protein expression in BPA-treated cells. BPA treatment for 12 h resulted in a significant decrease in both IκB-α mRNA and protein expression. Furthermore, since IκB-α expression is expected to increase with prolonged NF-κB activation, we next examined mRNA and protein expression following BPA exposure for 24 h. We showed that levels of IκB-α mRNA and protein were significantly increased following BPA exposure for 24 h, indicative of prolonged NF-κB activation in A549 cells.

The ability of estrogen to modulate inflammatory response via interaction with ERs (ERα and ERβ) has been extensively established[57]. BPA is known to mimic estrogen properties in cells through binding to both ERα and ERβ, with a 10-fold higher affinity for ERβ[58]. To examine the involvement of ER in BPA-induced NF-κB activation, we treated cells with BPA in the presence and absence of the ER-specific antagonist ICI 182780. We showed that ICI 182780 abrogated the effects of BPA on NF-κB (p65) and IκB-α, suggesting that ER is involved in mediating BPA-induced activation of the NF-κB signaling pathway. To link ER to BPA inhibition of ENaCγ expression, we examined and found that treatment of A549 cells with ICI 182780 completely abrogated the inhibitory effects of BPA on ENaCγ

expression. Given that ERα is not expressed in the A549 cell line, our data suggest that ERβ is involved in mediating BPA-induced activation of the NF-κB signaling pathway.

In conclusion, the present study demonstrates for the first time that BPA acts on ERβ to activate the NF-κB signaling pathway, which in turn leads to diminished GR activity and consequent repression of ENaCγ expression in lung epithelial cells. Thus, the present study reveals a novel BPA signaling pathway that can be exploited for developing inventions aimed at preventing and/or reversing BPA-induced organ dysfunction and diseases. Furthermore, if these findings could be extrapolated into an *in vivo* setting in humans, they would suggest that GC treatment of respiratory disorders, such as asthma, might result in GC resistance in cases where these disorders are caused by BPA exposure.

4.5 References

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5 DISCUSSION AND CONCLUSIONS

5.1 Summary

The previous chapters describe a series of *in vivo* and *in vitro* experiments aimed to elucidate the adverse effects of BPA on fetal lung development. In these experiments, I examined the effects of prenatal exposure to BPA on fetal lung maturation, and defined the molecular pathway through which BPA exerts its effects. I showed that exposure to environmentally relevant doses of BPA severely impairs fetal lung maturation in the mouse. This lung immaturity phenotype was characterized by defects in type I but not type II epithelial cell differentiation. I also demonstrated that intervention with the synthetic glucocorticoid dexamethasone rescues the lung immaturity phenotype. Finally, I determined that BPA disrupts glucocorticoid signaling in lung epithelial cells through a novel molecular pathway that involves ERβ-mediated activation of the key pro-inflammatory transcription factor NF-κB. Collectively, these findings demonstrate that developmental exposure to environmentally relevant doses of BPA disrupts fetal lung maturation through inhibition of the glucocorticoid signaling pathway. This study provides evidence for a fetal origin of the previously reported BPA-induced lung dysfunction and diseases in humans and in experimental animal models, thus supporting my hypothesis. Furthermore, it identifies a novel molecular pathway of BPA actions involving the ERβ-mediated activation of NF-κB and subsequent inhibition of GR activity in lung epithelial cells.

5.1.1 Doses of BPA Used in our *in vivo* Experiments

The BPA doses used in our *in vivo* experiments (5, 25, and 50 mg/kg diet; equivalent to 1, 5 and 10 mg BPA/kg body weight/day) were several orders of magnitude below the lowest observable adverse effect level (LOAEL) for rodents (50 mg/kg/day), as established by the U.S. Environmental Protection Agency[1]. Furthermore, one previous mouse study in which the same BPA-containing food pellet (5 mg/kg diet; equivalent to 1 mg BPA/kg body weight/day) was fed before and throughout pregnancy and lactation produced average maternal plasma (unconjugated) BPA concentrations of 3.5 ng/mL[2]. Based on this study I estimated a maximal maternal plasma BPA concentration of 20 ng/mL for the middle dose (25 mg/kg diet; which is estimated to be maximal because pregnant mice were exposed for

a total of 11 days in our study *vs*. 28 days in the previous study). Upon measurement, the middle dose (25 mg/kg diet) produced average maternal plasma BPA concentrations of 1.7 ng/mL in our mouse model, which are at the lower end of the range (0.5–22.3 ng/mL) reported in pregnant women in the U.S[3]. Although the precise reasons for our apparent underestimation of plasma BPA concentrations are unclear, it is possible that BPA degradation may be a contributing factor, as maternal plasma samples were stored at -80°C for over 1 year before being analyzed for BPA concentrations. Nevertheless, even the estimated maximal maternal BPA concentration of 20 ng/mL falls within the range of those reported previously in pregnant women, indicating that the dosage used in the present study is relevant to humans. Moreover, toxicokinetic studies that measure plasma levels of unconjugated BPA following oral administration indicate that internal exposure to unconjugated BPA in rodents, non-human primates, and humans are remarkably similar[4]. This is important as it suggests that the mouse is a valid predictor of the consequences of BPA exposure in primates, including humans, and highlights the potential impact of BPA exposure during pregnancy on fetal lung development.

5.1.2 Prenatal Exposure to BPA Severely Impairs Fetal Lung Maturation

Exposure to environmental chemicals during critical periods of development may produce lifelong structural and functional alterations that become apparent only later in life. Evidence is accumulating that disruption of lung development may be caused by some environmental chemicals at environmentally relevant doses. Nonetheless, there is a paucity of literature evaluating the impact of early-life exposure to environmental chemicals on lung structure and function. In recent years, the adverse effects of BPA on human lung function and associated lung diseases has become increasingly recognized. Epidemiological studies indicate that early life exposure to BPA is associated with impaired lung function[5] and the development of asthma[6,7] and wheeze[8,9] in children. In addition, high serum levels of BPA have been linked to chronic obstructive pulmonary disease (COPD)[10]. Animal studies show that perinatal exposure to BPA induces asthma in adult mice[11–14]. Collectively, these findings demonstrate that early life exposure to BPA is

detrimental to lung function, and suggest that BPA may alter fetal lung development, thus potentially cause or contribute to lung diseases later in life.

In Chapter Three, a fetal origin for the BPA-induced lung diseases in adult life was explored by examining the hypothesis that prenatal exposure to environmentally relevant doses of BPA impairs fetal lung maturation in mice. These experiments demonstrate that prenatal exposure to BPA severely impairs fetal lung maturation, as evidenced by the diminished alveolar airspace and thickened alveolar septa, which are hallmarks of lung immaturity. Concomitantly, the expression of the type I cell marker was dramatically reduced in BPA-exposed fetal lungs.

This finding is supported by studies of other environmental chemicals that exert adverse effects on the developing lungs. As summarized in Table 5.1, nitrofen[15,16], 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD)[17], nicotine[18,19], arsenic[20], and di(2-ethylhexyl) phthalate (DEHP)[21,22] impair lung development by disrupting essential processes such as branching morphogenesis, alveolarization, and cellular differentiation. Alarmingly, epidemiologic studies evaluating lung function in individuals exposed prenatally to these environmental chemicals report increased incidence of chronic lung dysfunctions and diseases such as COPD, asthma, and lung cancer[23], similar to the BPA-linked pathologies mentioned above.

Table 5.1. Environmental chemicals that can alter lung development by disrupting essential processes such as branching morphogenesis, alveolarization, and cellular differentiation that are essential for proper lung function after birth.

Environmental Chemical	Structural or Functional Impact	Possible Clinical Implications
Nitrofen	Decreased branching; altered smooth muscle, surfactant, and alveolar septation[14, 15]	Pulmonary hypoplasia & immature lung[22]
TCDD	Delayed lung development, decreased total lung space, increased septal area[16]	Chronic bronchitis, decreased functional capacity & COPD[22]
Nicotine	Slower septal formation, decreased number of alveoli, increased alveolar volume[17, 18]	Decreased functional capacity & emphysematous changes[22]
Arsenic	Altered branching and cell migration, decreased elasticity and structural support[19]	Bronchiectasis, airway hyperreactivity & lung cancer[22]
DEHP	Thickened septa, fewer/more dilated airspaces, increased type II cells[20, 21]	Bronchopulmonary dysplasia, altered lung mechanics, altered surfactant regulation & asthma[22]

Central signaling pathways involved in lung development are highly conserved[24], thus any disruptions in these pathways, including endogenous hormones, pharmaceuticals, and environmental chemicals, may alter fetal lung development. Moreover, a variety of transcription factors and nuclear receptors essential to these processes are susceptible to interference during critical developmental stages. Knockout mouse models have been instrumental in highlighting the importance of some signaling pathways, including glucocorticoid receptor (GR)[25,26], corticotrophin releasing hormone (CRH)[27], critical signal transduction proteins such as extracellular signal-regulated kinase 3 (Erk3)[28], differentiation genes of lung type I cells such as T1α[29], and several transcription factors that play key roles in development such as hypoxia-inducible factor 1α (HIF1α)[30], HIF-2α[31], Sp3[32], and C/EBPα[33]. Mice deficient in these genes suffer from severe lung immaturity that is reminiscent of the BPA-induced lung immaturity phenotype. Therefore, it is likely that BPA disrupts fetal lung maturation by altering essential signaling pathways in lung cells. Indeed, our data suggests that BPA functions likely by inhibiting the glucocorticoid signaling pathway in lung cells.

5.1.3 BPA Disrupts Fetal Lung Maturation by Suppressing Glucocorticoid Signaling

In Chapter Three, I showed that maternal administration of dexamethasone, which is a common intervention in high risk pregnancies with threatened preterm delivery[34], rescued the lung immaturity phenotype and normalized type I cell differentiation. Given that the BPA-induced lung immaturity phenotype closely resembles that of glucocorticoid deficient (i.e. $CRH^{-/-}$ and $GR^{-/-}$) mouse models[25–27] and that maternal dexamethasone administration rescues this condition, I hypothesized that BPA disrupts fetal lung maturation by suppressing glucocorticoid signaling in fetal lungs. During the last stages of fetal lung development, the withdrawal of lung liquid from the airways at birth is an important process allowing the transition to air breathing[35]. Membrane channels that promote the unidirectional movement of water, such as the epithelial sodium channel (ENaC), are essential in fetal lungs, and these channels have been reported as targets for GC induction in the lungs before birth[36]. Indeed, there is extensive evidence that ENaC expression and function can be regulated by glucocorticoids, particularly in the lungs[36–38]. Several studies

demonstrate that all three ENaC subunits (α, β and γ) are rapidly induced by dexamethasone *in vitro* in the type II epithelial A549 cell line[36,37]. In addition, GR-null mice have reduced expression of ENaCγ in their lungs before birth[26,38]. Interestingly, although ENaCγ expression is significantly reduced in the lung of GR-null mice, expression of the ENaC α and β subunits is unaltered[26,38]. Another well-known GR-target gene expressed in the lungs is glutathione peroxidase (GPX)[39,40]. GPX belongs to the enzyme family with peroxidase activity whose main biological role is to protect tissue from oxidative damage[40]. As shown in Chapter Three, the expression of ENaCγ and GPX was significantly decreased in BPA-exposed fetal lungs. This suggests that the adverse effects of BPA on fetal lung development are likely a result of inhibition of glucocorticoid signaling. This inhibition can be achieved by either decreasing circulating levels of corticosterone and/or antagonizing glucocorticoid actions in the fetal lungs. I found no evidence to support the first possibility, as fetal plasma levels of corticosterone were not altered at either E18.5, when the lung immaturity phenotype was observed, or E15.5, a critical time point just before terminal lung differentiation. Collectively, these findings suggest that BPA may suppress GC signaling which results in impaired fetal lung maturation. A recent study using *in vitro* yeast bioassays found that BPA acts as a GR antagonist, with a drug concentration causing 50% inhibition of 67 μM[41]. However, the precise molecular mechanisms of this antagonistic relationship remained obscure.

5.1.4 BPA Alters the Expression of ENaCγ by Inhibiting the Glucocorticoid Signaling Pathway

Next, I investigated the precise molecular mechanisms by which developmental exposure to BPA inhibits glucocorticoid signaling. Since expression of ENaCγ, a well-known GR target gene, was diminished in BPA-exposed fetal lungs, I tested the hypothesis that BPA inhibits ENaCγ expression through repression of the glucocorticoid signaling pathway using the A549 lung epithelial cell line as an *in vitro* model system. I first validated the model by demonstrating a robust concentration-dependent suppression of ENaCγ expression following BPA exposure, analogous to the *in vivo* results. I then showed that both dexamethasone and siRNA-mediated knockdown of GR expression blocked the

inhibitory effects of BPA on ENaCγ expression, suggesting that BPA repressed ENaCγ expression via inhibition of GR activity.

To determine the precise molecular mechanism by which BPA represses GR activity, I analyzed various aspects of the GR signaling pathway. Glucocorticoid binding to the cytoplasmic GR results in its release from its chaperone proteins, and rapid nuclear translocation[42]. Analysis of this pathway revealed that although BPA did not alter levels of either HSP90 protein or GR protein, it reduced GR nuclear translocation. Taken together, these results suggest that BPA inhibits GR activity by diminishing GR nuclear translocation.

NF-κB is a pro-inflammatory transcription factor that plays a key role in amplifying inflammatory and immune responses[43]. NF-κB is a heterodimeric protein composed of a p50 and p65 subunit, with p65 primarily responsible for the transactivation function[44]. Under unstimulated conditions, NF-κB resides in the cytoplasm in an inactive form due to its association with its inhibitory protein (IκB-α). Following stimulation of cells, IκB-α undergoes proteasomal degradation, allowing NF-κB to be released, and its rapid nuclear translocation[43,44]. Recently, BPA has been shown to function as an inflammatory inducer as it induces NF-κB expression and activation in various cell types[45–51]. A mutual antagonism exists between GR and NF-κB, whereby NF-κB represses GR and *vice versa*[44,52–54]. Furthermore, this mutual antagonism is mediated through the p65 subunit of NF-κB. Several mechanisms were identified to explain this antagonistic relationship. First, the direct interaction between activated NF-kB and GR in the cytoplasm has been shown to impair GR nuclear translocation[55,56]. Second, in the nucleus, NF-κB and GR compete for co-activators and/or NF-κB can disturb the interaction of GR with its basal transcription machinery thus inhibiting transcription of target genes[44,54,57,58]. Therefore, I determined whether BPA stimulates NF-κB expression, which, in turn, would inhibit GR activity. I found that treatment with BPA resulted in a significant increase in the expression and activation of NF-κB (p65) while decreasing mRNA and protein expression of its inhibitory protein, IκB-α. Moreover, inhibition of NF-κB activation by the specific inhibitor BAY 11-702 abrogated the inhibitory effects of BPA on ENaCγ expression. Together, these findings

suggest that BPA represses ENaCγ expression in A549 lung cells via induction of the NF-κB signaling pathway, and consequent inhibition of GR activity.

The ability of estrogen to modulate inflammatory response via ERs (ERα and ERβ) has been extensively established[59,60]. Furthermore, recent studies show that BPA can activate the NF-κB pro-inflammatory pathway via ERs[49,50]. As a first step in examining if ER is involved in mediating BPA-induced NF-κB expression and activation, A549 cells were treated with BPA in the presence and absence of the ER antagonist ICI 182780. As shown in Chapter Four, ICI 182780 blocked the effects of BPA on NF-κB (p65) and IκB-α, suggesting that ERβ (although the ER antagonist does not discriminate the two nuclear ERs, ERα is undetectable in the A549 cell line) mediates the effects of BPA on the NF-κB signaling pathway. As induction of the NF-κB signaling pathway in BPA treated cells inhibits GR activity, I reasoned that if BPA signals via ERβ to activate the NF-κB signaling, the addition of ICI 182780 should block BPA inhibition of ENaCγ expression. Indeed, ENaCγ expression was restored to control levels in the presence of the ER antagonist, providing further support for the notion that BPA acts on ERβ to induce the NF-κB signaling pathway, which in turn inhibits the GR activity.

Taken together, these findings suggest that BPA activates the pro-inflammatory NF-κB signaling pathway via ERβ, leading to interference with the glucocorticoid signaling pathway in lung cells. Glucocorticoids are the most commonly prescribed drugs for the treatment of chronic inflammatory conditions of the respiratory tract[61]. If the present findings could be extrapolated to the human, it is tempting to speculate that exposure to BPA may contribute to glucocorticoid resistance/insensitivity, especially in patients with chronic airway inflammation, such as asthma.

5.2 Future Directions

I have provided evidence for a fetal origin for BPA-induced lung dysfunction and diseases that are observed previously in adult life. Also, I have identified a novel molecular mechanism by which BPA exerts its adverse effects on lung cells. Upon collectively analyzing these findings, it is critical to recognize that there are a number of important questions that remain to be resolved, some of which are discussed below.

5.2.1 The Effects of BPA on Primary Human Bronchial Epithelial Cells (HBECs)

To determine whether the disruptive effects of BPA that were observed in the adenocarcinomic human lung A549 cell line, can be replicated in a primary human lung cell model.

Lung primary epithelial cells consist of mixed cell types, such as goblet cells, basal cells, or ciliated epithelial cells, and are thus preferred as a good representative *in vitro* model to capture the intrinsic properties of the *in vivo* lung. Well-established primary cell models are useful in investigating properties of intact organs under normal or diseased conditions as these cells are likely to be more physiologically relevant to *in vivo* organs, compared to cancer-derived cell lines. However, the disadvantage of using primary cell cultures is these cell types have a short life span and require specialized culture skills for expansion in culture. Therefore, studies are usually conducted in immortalized cancer-derived cell lines prior to experimenting in a primary cell model.

Exposure of the lung A549 cell line, an alveolar cell carcinoma-derived cell line that retains some features of the type II alveolar epithelial cells, to nanomolar concentrations of BPA resulted in the activation of the NF-kB pro-inflammatory pathway via ERβ, leading to inhibition of the glucocorticoid signaling pathway. Given that the primary lung cell model is more physiologically relevant to the human lung, it is imperative to determine whether the observed effects of BPA are operating in primary cells. If BPA does indeed inhibit glucocorticoid signaling in primary cells, this will have significant implications on human exposure to BPA, especially in cases where BPA exposure occurs through inhalation[62,63].

More importantly, it is estimated that 30% of the normal healthy population is resistant to treatment with glucocorticoids[64]. If BPA results in glucocorticoid resistance/insensitivity in human lung cells, response to steroid treatment may become a major problem for many people, especially those who suffer from a chronic inflammatory lung disease, such as asthma.

5.2.2 To Determine Whether Aspects of the Signaling Pathway Identified *in vitro* can be Observed in BPA-exposed Fetal Lungs

The identification of the molecular mechanisms triggered upon exposure to BPA highlighted a potential pathway by which BPA may exert its adverse effects on the fetal lungs. In Chapter Four, a novel molecular pathway was identified where BPA acts on ERβ to activate the NF-κB signaling pathway, which in turn leads to diminished GR signaling in lung epithelial cells. These findings suggest that BPA induces NF-κB activation through binding to ERβ, which in turn inhibits GR activity. Future studies will be required to determine whether this molecular pathway operates *in vivo* in fetal lungs following prenatal exposure to BPA. These include: ***(1)*** determine whether exposure to BPA results in decreased GR nuclear translocation in fetal lungs. ***(2)*** Measurement of p65 expression and activation in fetal lungs at various time points (i.e. E15.5-E18.5). ***(3)*** Measurement of IκB-α in fetal lungs at various time points (i.e. E15.5-E18.5). ***(4)*** Measurement of ERβ-target gene expression in BPA-exposed fetal lungs, which will indicate whether the ERβ signaling pathway is activated in fetal lungs following BPA exposure. The proposed experiments will demonstrate whether the observed molecular pathway of BPA is evident in fetal lungs following prenatal exposure to BPA. This will provide a potential pathway by which BPA acts to disrupt fetal lung maturation, as well as provide critical insights into the adverse effects of developmental exposure to BPA on fetal organ development.

5.2.3 The Effects of Prenatal Exposure to BPA on Fetal Lung Maturation in ERβ-null Mice

To determine whether ERβ mediates the effects of BPA on fetal lung maturation.

Several studies have demonstrated that estrogen is required for the formation and complete development of alveoli, for the maintenance of existing alveoli, for lung homoeostasis, and for alveolar regeneration in adult mice[65–67]. Interestingly, ERβ-null mice do not have a lung immaturity phenotype at birth[68]. However, later in life, female mice begin to show signs of decreased numbers of alveoli, suggesting deficient alveolar formation as well as evidence of surfactant accumulation in their lungs[65,69]. The data presented in this thesis indicates that BPA inhibits glucocorticoid signaling likely via ERβ-mediated induction of NF-κB. Since ERβ-null mice do not have a lung immaturity phenotype at birth, these mice provide a suitable model to examine whether ERβ is mediating the disruptive effects of BPA on fetal lung maturation. I hypothesize that offspring of ERβ knockout mice will exhibit normal lung development following prenatal exposure to BPA. If confirmed, this will provide definitive evidence for a critical role of ERβ in mediating the effects of BPA on fetal lung maturation.

5.2.4 To Determine the Short- and Long-term Consequences of Prenatal Exposure to BPA

Considering that BPA-exposed newborn mice survive into adulthood, the immediate (neonatal) and long-term (adulthood) consequences of BPA exposure during pregnancy should be determined, particularly since chronic lung diseases such as asthma and COPD have at least part of their origins in early life[23,70,71]. Based on the findings presented in this thesis (i.e., fetal mice exposed to BPA via maternal diet display severe lung immaturity), and given that epidemiological as well as animal studies suggest that early life exposure to BPA is associated with impaired lung function and diseases later in life, it is important to determine whether the severe lung immaturity observed in our BPA-exposed fetal mice increases susceptibility to lung dysfunction and disease in both the neonatal period and during adulthood. It is very likely that these developmental defects persist into adulthood, leading to impaired lung function and diseases later in life. Moreover, the link between early life exposure

to BPA and the susceptibility for airway disease in adult life has never been explored. Examining the effects of prenatal BPA exposure on the adult lung and combining these findings with our previous data documenting the disruptive effects of BPA on the fetal lung will establish a direct link between fetal and adult lung disruptions resulting from prenatal exposure to BPA.

5.3 Conclusions

Adverse events during early life can severely impact an individual's long-term health. Often, adverse events are unrecognized or only noted when developmental processes have already been affected. Early life exposure to BPA is of concern for several reasons. First, it affects the development of various fetal organs[2,72–80]. Second, it acts on multiple signaling pathways involved in organ development[81,82]. Third, this disrupting chemical is highly pervasive in our environment[83,84]. While the ban of BPA in baby bottles reduces postnatal exposure, fetal exposure to BPA via maternal exposure remains. Both epidemiological and animal studies clearly indicate that exposure to BPA, especially during fetal life, results in lung dysfunction and diseases later in life. Thus, it is imperative to examine the adverse effects of early life exposure to BPA on fetal lung development. The focus of this thesis was to examine the hypothesis that the previously reported lung dysfunction and diseases following BPA exposure may have a fetal origin. The first set of experiments described in this thesis demonstrate that exposure to environmentally relevant doses of BPA during the critical period of fetal development significantly impairs fetal lung maturation in mice. The second set of experiments provides evidence that BPA inhibits GR activity via ERβ-induced activation of the NF-κB signaling pathway in lung epithelial cells. A schematic diagram depicting the precise molecular pathway through which BPA represses ENaCγ expression in lung epithelial cells is shown in Figure 5.1.

This model postulates unconjugated (free) BPA readily crossing the cell membrane, binding to and activating the nuclear receptor ERβ. Once activated, ERβ translocates to the nucleus where it inhibits IκB-α expression. With less IκB-α to sequester NF-κB in the cytosol, more NF-κB is activated and translocates to the nucleus. As NF-κB is known to antagonize GR in both the cytoplasm and the nucleus, it is postulated that GR nuclear

translocation will significantly decrease, followed by decreased activation of GR-target genes, such as ENaCγ. As a result, ENaCγ mRNA and protein expression, following treatment with BPA, is significantly reduced. My present findings provide evidence for a novel molecular pathway where BPA acts on ERβ to activate the NF-κB signaling pathway, which in turn leads to reduced GR activity and consequent repression of ENaC**γ** expression in lung cells. Clearly, this model is speculative and much work is required to provide definitive evidence for this signaling pathway in mediating the effects of BPA on fetal lung development. Nevertheless, the findings presented in this dissertation raise concerns about the potentially deleterious effects of BPA on lung development in humans, especially given that the BPA doses used in this study were within the range of exposure in humans. Our hope is that this data, alongside epidemiological evidence of the increasing incidence of lung diseases in human populations exposed to BPA during various developmental stages, encourages regulatory agencies to ban this disruptive chemical from use in consumer products.

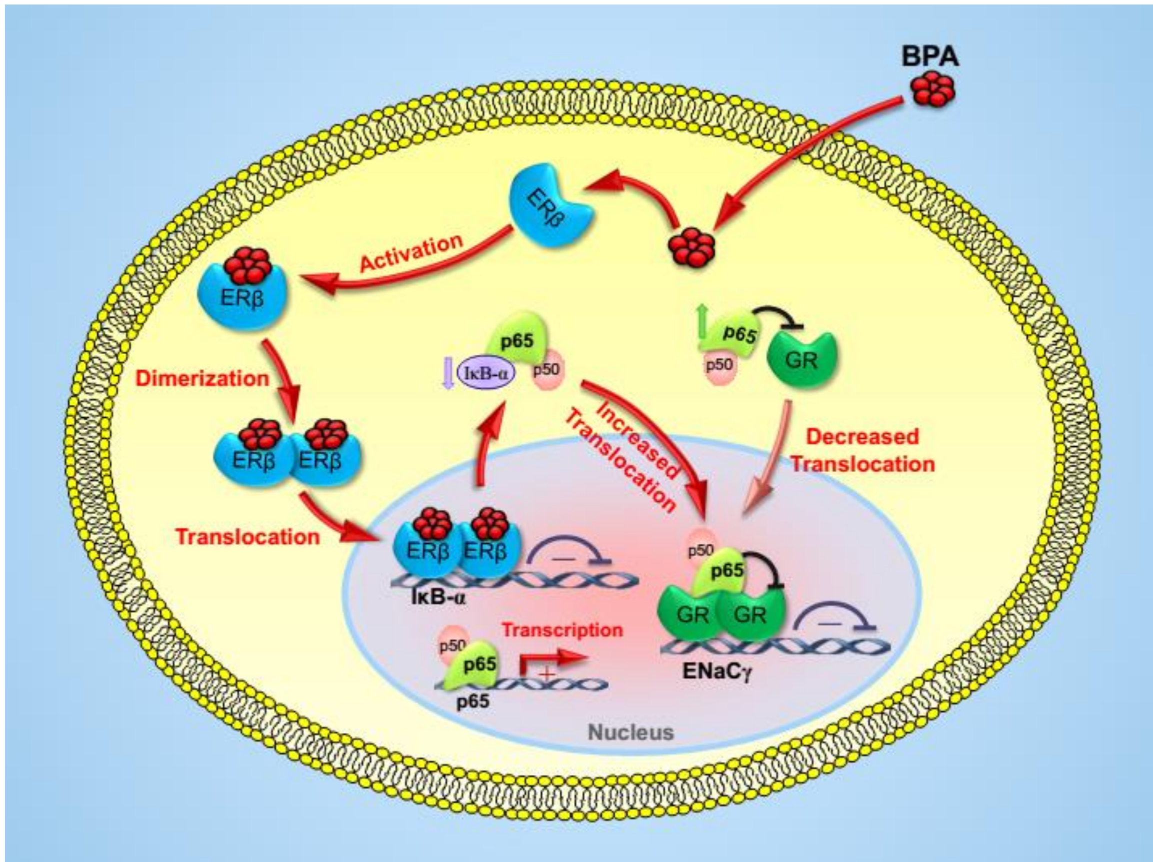

Figure 5.1: A schematic representation of the postulated molecular pathway by which BPA represses ENaCγ expression in lung epithelial cells. In this model, BPA enters the cell where it binds to ERβ, which in turn disassociates with its chaperone proteins, and acquires the ability to translocate to the nucleus. Once inside the nucleus, ERβ binds to the promoter of IκB-α and inhibits its transcription. With less IκB-α to sequester NF-κB in the cytosol, more NF-κB is activated and translocates to the nucleus. As NF-κB is known to antagonize GR in both the cytoplasm and the nucleus, it is postulated that GR nuclear translocation will significantly decrease, followed by reduced expression of GR-target genes, such as ENaCγ.

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6 Appendix

6.1 Appendix 1

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To whom it may concern,

My name is Ayten Hijazi, I am writing to ask for copyright permission to include my publication from the article (Hijazi A, Guan H, Cernea M, Yang K. Prenatal exposure to bisphenol A disrupts mouse fetal lung development. FASEB J. 2015;

6.2 Appendix 2

2012-004::3:
AUP Number: 2012-004
AUP Title: Molecular Mechanisms of Fetal Growth Restriction

Yearly Renewal Date: 06/01/2015
The YEARLY RENEWAL to Animal Use Protocol (AUP) 2012-004 has been approved, and will be approved for one year following the above review date.

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.
The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.
Submitted by: Kinchlea, Will D
on behalf of the Animal Use Subcommittee

7 Curriculum Vitae

Ayten Hijazi

EDUCATIONAL BACKGROUND

PhD | **September 2011 – June 2016**

Western University, London, Ontario, Canada
Department of Pharmacology and Toxicology
Collaborative Program in Developmental Biology
THESIS: The Effects of Prenatal Exposure to Environmental Toxins on Fetal Development
GPA 4.0/4.0 – all Graduate level courses

Hon. BSc | **September 2007 – June 2011**

Western University, London, Ontario, Canada
Department of Biology

SCHOLARSHIPS

Schulich Graduate Scholarship 2011- 2016

PUBLICATIONS

1. **Hijazi A**, Guan H, Cernea M, and Yang K. Prenatal exposure to bisphenol A disrupts mouse fetal lung development. *FASEB J* 2015; 29(12):4968-77.
2. **Hijazi A**, Guan H, and Yang K. Bisphenol A suppresses glucocorticoid target gene expression via a novel ERβ-NF-κB-GR signaling pathway in lung epithelial cells. *Arch Toxicol* 2016, Accepted with minor revisions.

ABSTRACTS

1. **Hijazi A**, Guan H, and Yang K. Bisphenol A suppresses ENaCγ expression via a novel ERβ-NF-kB-GR signaling pathway in lung epithelial cells. The 14th Annual Paul Harding Research Day in London, ON. Abstract and oral presentation (*First Prize for Graduate Student oral presentation*).

2. **Hijazi A**, Guan H, and Yang K. Bisphenol A disrupts fetal lung maturation via the glucocorticoid signaling pathway. The 13th Annual Paul Harding Research Day in London, ON. Abstract and Poster presentation *(Honorable Mention).*

3. **Hijazi A**, Guan H, and Yang K. Bisphenol A disrupts fetal lung maturation via the glucocorticoid signaling pathway. The 7th Annual Developmental Biology Research Day in London, ON. Abstract and Poster presentation.

4. **Hijazi A**, Guan H, and Yang K. Prenatal exposure to Bisphenol A impairs fetal lung maturation in the mouse. The 11th Annual Paul Harding Research Day in London, ON. Abstract and Oral presentation.

5. **Hijazi A**, Guan H, and Yang K. Prenatal exposure to Bisphenol A impairs fetal lung maturation in the mouse. The 5th Annual Developmental Biology Research Day in London, ON. Abstract and Oral presentation.

RELATED WORK EXPERIENCE

Western University, London, Ontario, Canada

Teaching Assistant - Pharmacology 3580y Sept 2014 – April 2016

- Set-up for class (preparing materials for lab sessions, inputting information in e-learning platform)
- In-class instruction (supervising a lab and leading a tutorial)
- Reading electronic communication from students and corresponding with students relevant to the
  assigned course (email and e-learning)
- Marking essays/reports/assignments/exams

Western University, London, Ontario, Canada

Teaching Assistant - Pharmacology 2060b Jan 2012- July 2013

- Instructed and prepared online tutorials
- Reading electronic communication from students and meeting with students to clarify course material

- Proctoring and marking exams

CERTIFICATES

Certificate in Business and Consulting – Mini MBA Jan 2016 – March 2016

Top 4 in Case Analysis Presentation

VOLUNTEER

Western University, London, Ontario, Canada Oct 2010-April 2011

Volunteer

- Lab Assistant at Dr. Gardner's lab
- Prepared specimens for analysis using Electron Microscopes
- Analyzed specimens from fungal tissue