The Effects of Bisphenols on Endochondral Ossification in Murine Limb Bud Cultures

By

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A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Master of Science

December 2020

ABSTRACT

Environmental exposure to chemical toxicants and their impact on human health are issues of growing concern in modern society. Bisphenol A (BPA) is the main monomer of polycarbonate plastics and epoxy resins and is widely used in consumer goods. Many alternatives to BPA, such as BPAF, are now emerging in consumer products, despite a lack of safety testing. There is evidence that BPA is an endocrine disruptor and *in utero* exposure to this chemical affects skeletal development in animal models. However, it remains largely unknown whether BPA and its alternatives specifically disrupt endochondral ossification. My goal was to determine the effects of BPA and BPAF on limb development in the mouse by using an *ex vivo* limb bud culture model, and to investigate the effects of these two bisphenols on the expression of *Sox9*, *Runx2*, and *Sp7*, the master regulators of endochondral ossification.

CD1 mice that express COL2A1-eCFP, COL10A1-mCherry, and COL1A1-YFP were used, allowing us to visualize proliferative chondrocytes, hypertrophic chondrocytes, and osteoblasts, respectively. Gestational day 13 embryonic forelimbs were cultured for six days in the presence of vehicle, BPA, or BPAF and then imaged with fluorescence microscopy. To determine effects on gene expression, limb buds were similarly cultured in the presence of BPA or BPAF for 3, 6, 24, or 48 hours. Changes in mRNA levels were determined using qRT-PCR.

BPA and BPAF delayed endochondral ossification and had an adverse effect on the differentiation of the carpals and the phalanges in the limb. Exposure to 10 μM BPA or 1 μM BPAF reduced the differentiation of hypertrophic chondrocytes and osteoblasts, as seen by a reduction in COL10A1-mCherry and COL1A1-YFP fluorescence. At 50 and 100 μM BPA, or 5 and 10 μM BPAF, cartilage template development was arrested and COL10A1-mCherry and COL1A1-YFP fluorescence were strongly suppressed. Exposure to 50 μM BPA downregulated

the expression of *Runx2* at all time points and suppressed the upregulation of *Runx2* and *Sp7* that was observed in control limbs. Exposure to 5 μM BPAF downregulated the expression of *Sp7* at the 24h time point while exposure to 1 μM BPAF decreased transcript levels at 48h.

These data indicate that BPAF, a replacement bisphenol, may be more detrimental to endochondral ossification than BPA. Further studies are needed to determine the effects of other BPA replacements on endochondral ossification and to elucidate the signalling pathways underlying these effects, with the goal of identifying responsible replacements for BPA in consumer goods.

RÉSUMÉ

L'exposition environnementale aux substances toxiques et leurs effets sur la santé humaine sont des sujets de plus en plus préoccupants dans la société moderne. Le bisphénol A (BPA) est le monomère principal trouvé dans les plastiques en polycarbonate et dans les résines époxydes, et est largement utilisé pour produire des biens de consommation. De nombreux alternatifs au BPA, tel que le BPAF, commencent maintenant à être utilisés dans les produits en plastique malgré un manque de tests prouvant l'innocuité de ces produits. Le BPA est un perturbateur endocrinien et l'exposition intra-utérine au BPA affecte le développement squelettique chez les modèles animaux. Cependant, il est inconnu si le BPA et les autres bisphénols perturbent spécifiquement l'ossification endochondrale. Mon objectif était de déterminer les effets du BPA et du BPAF sur le développement des membres chez la souris à l'aide d'un système de culture de membres *ex vivo*. J'ai également caractérisé les effets de ces deux bisphénols sur l'expression de *Sox9*, *Runx2* et de *Sp7,* les principaux régulateurs de l'ossification endochondrale.

Des souris CD1 transgéniques qui expriment le COL2A1-eCFP, COL10A1-mCherry et COL1A1-YFP ont été utilisées, ce qui nous permet de visualiser les chondrocytes prolifératifs, les chondrocytes hypertrophiques et les ostéoblastes, respectivement. Les membres antérieurs des embryons ont été mis en culture pendant six jours en présence de DMSO, BPA ou BPAF, puis imagés à l'aide d'un microscope à fluorescence afin de visualiser l'expression des trois marqueurs. Afin de déterminer l'expression de l'ARNm, les membres murins ont été placés en culture de la même manière, en présence de BPA ou BPAF pendant 3, 6, 24 ou 48 heures. Les niveaux d'expression de l'ARNm ont été déterminés grâce à la PCR quantitative (Réaction en Chaîne par Polymérase ou *qRT-PCR*).

Le BPA et le BPAF ont retardé le processus d'ossification et ont eu un effet négatif sur la différenciation des os carpiens et des phalanges dans les membres. L'exposition des membres à 10 μM BPA ou 1 μM BPAF a réduit la différenciation des chondrocytes hypertrophiques et des ostéoblastes, tel qu'indiqué par la réduction de la fluorescence du COL10A1-mCherry et COL1A1-YFP. Chez les membres exposés à 50 et 100 μM BPA, ou 5 et 10 μM BPAF, la différenciation du cartilage était dramatiquement réduite et la fluorescence du COL10A1-mCherry and COL1A1-YFP était fortement inhibée. Le traitement des membres avec 50 μM BPA a réprimé l'expression de *Runx2* à chacune des périodes d'incubation testées; ce traitement a également réprimé l'augmentation de l'expression de *Runx2* et de *Sp7* qui a été observée chez les membres non-traités. Le traitement des membres avec 5 μM BPAF a réprimé l'expression de *Sp7* à 24h alors que l'exposition à 1 μM BPAF a réduit son expression à 48h.

Ces résultats démontrent que le BPAF, le bisphénol utilisé pour remplacer le BPA, peut en fait être plus nuisible à l'ossification endochondrale que le BPA. Des études supplémentaires seront nécessaires afin de déterminer les effets que pourront avoir d'autres bisphénols sur l'ossification endochondrale. Il sera également important d'élucider les voies de signalisation responsables pour ces effets néfastes, permettant ainsi d'identifier des remplacements non toxiques pour le BPA dans les biens de consommation.

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ABBREVIATIONS

AER	Apical ectodermal ridge
AHR	Aryl hydrocarbon receptor
ALP	Alkaline phosphatase
BFR	Brominated flame retardant
BMD	Bone mineral density
BPA	Bisphenol A
BPAF	Bisphenol AF
BPDP	Tert-butylphenyl diphenyl phosphate
CD	Campomelic dysplasia
CDD	Cleidocranial dysplasia
COL2A1	Collagen Type II Alpha 1
COL10A1	Collagen Type X Alpha 1
COL1A1	Collagen Type I Alpha 1
DDT	Dichlorodiphenyltrichloroethane
DES	Diethylstilbestrol
E2	17β-estradiol
EDC	Endocrine disrupting chemical
ER	Estrogen receptor
ERE	Estrogen response element
ERRγ	Estrogen-related receptor gamma
FR	Flame retardant
GD	Gestational day
GPER	G protein-coupled estrogen receptor
GR	Glucocorticoid receptor
HMG	High mobility group

IPPP	Isopropylated triphenyl phosphate
LBD	Ligand binding domain
OPE	Organophosphate ester
PBDE	Polybrominated diphenyl ether
PCB	Polychlorinated biphenyl
PND	Postnatal day
PPAR	Peroxisome proliferator-activated receptor
PZ	Progress zone
RANKL	Receptor activator of nuclear factor kappa-B ligand
RHD	Runt homology domain
Runx2	Runt-related transcription factor 2
SHH	Sonic hedgehog
Sox9	Sex determining region Y-related high mobility group box 9
SULT1A1	Sulfotransferase family 1A member 1, phenol-sulfating phenol sulfotransferase
TBT	Tributyltin
TMPP	Tris(methylphenyl) phosphate
TPHP	Triphenyl phosphate
TPT	Triphenyltin
TR-alpha	Thyroid hormone receptor alpha
TRAP	Tartrate-resistant acid phosphatase
UGT	Uridine 5'-diphospho-glucuronosyltransferase
ZPA	Zone of polarizing activity

ACKNOWLEDGMENTS

First and foremost, I would like to thank Dr. Barbara Hales and Dr. Bernard Robaire. Your knowledge in your respective fields is unparalleled and I was honoured to have you both as supervisors and as mentors. Thank you for always being available, in person or remotely, to discuss science, to provide feedback on my work, and for hosting weekly journal clubs where I learned more than I expected. On a more personal note, I am very grateful for your hospitality, for your pride in my accomplishments, and for lending a kind ear when I was homesick or frustrated with my experiments. Your patience and guidance led me to where I am today.

Thank you to the Department of Pharmacology and Therapeutics for all of their help. I must also thank Tina Tremblay for her kindness, patience, and support. You were there when I needed you most, and your open door policy showed how much you truly care for the students.

Thank you to the members of my committee, Dr. Jacquetta Trasler, Dr. Koren Mann, and Dr. Niladri Basu for their feedback and advice during my committee meetings.

My deepest appreciation is extended to Trang Luu for keeping the lab together and for all of her help with my experiments. Thank you for never judging me when I had difficulty doing dilutions! Without you Trang, the lab would catch on fire…too soon?

A huge thank you to all of the members of the Hales and Robaire labs, both past and present: Heather Fice, Aimee Katen, Zixuan Li, Abishankari Rajkumar, Seline Vancolen, Vicky Wang, and Oscar Yu. You were all paramount in making my experience memorable. Thank you also to Elyssa Frohlich and Paulina Nguyen, whose friendship means so much to me. You both made me believe that I could handle anything the world threw at me! Lastly, a heartfelt thank you to Elise Boivin-Ford. I appreciate all the hard work you do in and out of the lab.

To Aileen Yan, thank you is not enough. You started off as a mentor and colleague and eventually became one of my most cherished friends. I am indebted to you for all of the knowledge you have passed on to me; everything I know about the limb bud culture is thanks to you. I am grateful for everything you have done to help me achieve my goals.

To my dearest friend, Emilie Fogal, my honey buns, I owe you the world. I truly am not sure if I would have gotten through my toughest days without you. You are like a sister to me and you have always known just what to say to put a smile back on my face. Although we talk almost every single day, you never seem to tire of hearing me complain about the same things over and over again. I will forever be grateful that I sat next to that weird girl on that weird day in 2014. I also could not have completed this thesis without Nadia Landry's friendship. We went through graduate school together, but cities apart, and I am so grateful to have you on my team. Thank you for always listening to me, and for making me feel at home when I am with you.

Last but not least, thank you to my loving family. To my mom and dad, Mayssam El-Halwani and Khairallah Iskandarani, thank you for your unconditional love, your unwavering patience, and for being my biggest supporters. I truly don't know what I would do without you. To my amazing brother, Mohamad Iskandarani, thank you for your tough love and your no-nonsense approach to life; without you, I would still be worried about sending in my graduate school application to McGill. Mama, Baba, and Hammoudi: I love you to the moon and back. This accomplishment is as much yours as it is mine.

PREFACE

Format of the Thesis

This thesis is comprised of three chapters and conforms to the guidelines for thesis preparation, as outlined by Graduate and Postdoctoral Studies at McGill University. Chapter One is an introduction, and provides an overview of the use, exposure, and toxicity of bisphenols A and AF. The first chapter also highlights the process of limb formation and development, ending with the hypothesis and objectives of the project.

Chapter Two is a data chapter, and includes an introduction, detailed methodology, results of the thesis project as well as a short discussion. This chapter is written in a manuscript format to be submitted for publication.

Chapter Three provides a summary and a more detailed discussion of the results, suggestions for future studies, and a final conclusion. A full reference list is provided at the end of the thesis, in alphabetical order.

Contribution of the Authors

All experiments and analyses presented in this thesis were performed by the candidate under the supervision of both Dr. Barbara Hales and Dr. Bernard Robaire.

Chapter One: Introduction

1.1 Endocrine Disrupting Chemicals

An endocrine disrupting chemical (EDC) is an exogenous compound that can affect the action of hormones (Gore et al. 2015). Hormones are made by numerous endocrine glands (Figure 1.1), and they act on target tissues that are found throughout the body; therefore, disruption of this system can interfere with homeostasis and has been associated with obesity, cancer, as well as with adverse effects on the thyroid, adrenal, and on male and female reproduction, both in animals and in humans (Diamanti-Kandarakis et al. 2009).

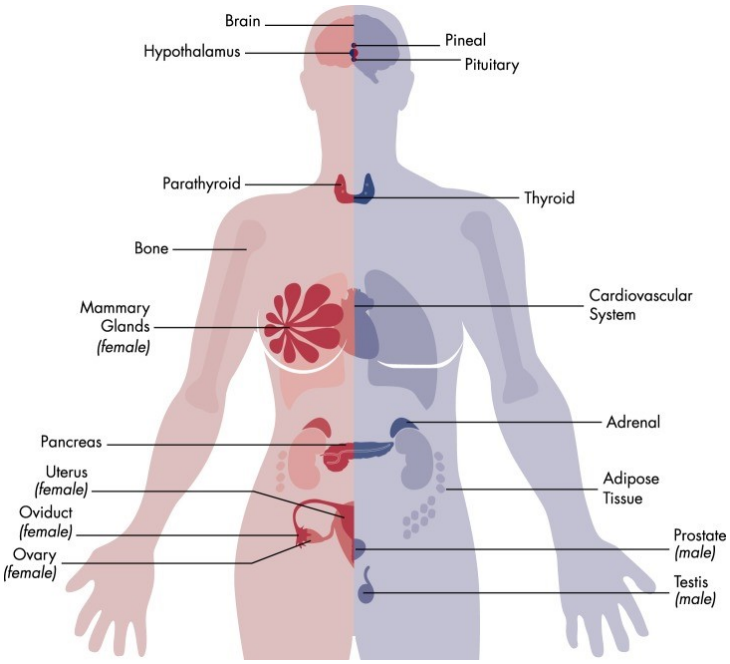


Figure 1.1: Illustration of the human body's endocrine glands. The endocrine glands in females (left) and in males (right) produce hormones, which are then released into the circulatory system and act as signalling molecules (Gore et al. 2015).

Although EDCs act primarily through nuclear hormone receptors, such as the estrogen receptors (ERs), they also exert their actions through nonnuclear hormone receptors, nonsteroid receptors, and modulation of hormone biosynthesis and metabolism, among other mechanisms of

action (Gore et al. 2015). Some of the most extensively studied EDCs include plasticizers (phthalates), pesticides (dichlorodiphenyltrichloroethane, DDT), polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs), and plastics (bisphenols) (Gore et al. 2015).

1.1.1 Bisphenol A

Bisphenol A (BPA, CAS Number: 80-05-7) was synthesized in 1891 by Aleksandr Dianin (Ribeiro et al. 2017).

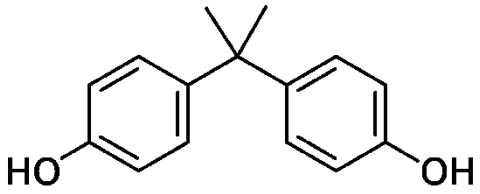


Figure 1.2: The chemical structure of BPA

It was not until the 1930s that the estrogenic properties of BPA were discovered by Edward Charles Dodds (Vogel 2009). In his search for a synthetic estrogen, Dobbs also discovered diethystilbestrol (DES), which was later used as a drug for the prevention of miscarriages (Veurink et al. 2005; Vogel 2009). However, DES was banned in 1971 due its association with an increased risk of rare cancers and reproductive anomalies in children exposed to the drug *in utero* (Herbst et al. 1971; Veurink et al. 2005). In contrast, BPA was never prescribed as a drug; instead, it found its use in the manufacture of plastics (Vogel 2009).

1.1.1.1 Common uses of BPA

BPA is produced in high volumes around the world; it is estimated that around one million tonnes were produced in the United States in 2019. BPA is most commonly polymerized to produce polycarbonates and epoxy resins (Geens et al. 2011). Although less common, BPA can also be used as an additive (Geens et al. 2011). The polymer applications of BPA are numerous; polycarbonate plastics are used to produce food and beverage containers, electronics, leisure and safety equipment, as well as medical equipment (Vogel 2009; Geens et al. 2011). As polymers of BPA, epoxy resins are usually found in the lining of food and beverage cans, while polysulfones are used to produce membranes and plumbing equipment (Geens et al. 2011). Finally, BPA can also be used as an additive in the thermal paper used as cash register receipts or plane and train tickets, among others (Geens et al. 2011; Bernier and Vandenberg 2017).

1.1.1.2 Routes of exposure to BPA

Human exposure to BPA can happen through many routes, but can only occur once this chemical leaches from the products containing it. Exposure may occur due to the fact that a small amount of free BPA is left unpolymerized during the production of polycarbonates and epoxy resins (Geens et al. 2011). Although unlikely, the polymers themselves can also breakdown, causing low amounts of BPA to leach; this occurs when the plastic is heated at high temperatures, or washed in alkaline detergents (Brede et al. 2003; Biedermann-Brem et al. 2008; Geens et al. 2011). Exposure is highest when BPA is used as an additive, such as in thermal receipts, because it is present in an unpolymerized or free form (Geens et al. 2011; Bernier and Vandenberg 2017).

Environmental exposure to BPA often occurs through the diet, especially due to the consumption of canned foods (Geens et al. 2012; Huang et al. 2017; Lehmler et al. 2018). Exposure to BPA can also occur through inhalation or ingestion of household dust, and dermal exposure to personal care products or thermal receipt paper (Biedermann et al. 2010; Liao and Kannan 2014; Wang et al. 2015; Bernier and Vandenberg 2017). Due to the various routes of exposure, environmental contact with BPA is ubiquitous. According to the Canadian Health Measures Survey (2012 and 2013), 90 % of Canadians aged 3 to 79 had detectable levels of BPA in their urine, the average concentration being 1.1 μg/L.

1.1.1.3 Mechanisms of action of BPA

The exact mechanisms of action of BPA are continuously debated by scientists; however, the scientific community agrees that BPA acts through different hormone receptors in the body, thus disrupting the endocrine system (Nagel and Bromfield 2013). BPA is considered a xenoestrogen due to its similarity to estradiol as well as its ability to bind the estrogen receptors ERα and ERβ (Rubin 2011; Nagel and Bromfield 2013; Acconcia et al. 2015). Although BPA has been shown to have 1,000 to 10,000-fold less affinity for the ERs compared to 17β-estradiol (E2), it is still capable of driving a cellular response at low concentrations. ERα and ERβ are nuclear receptors known as ligand-activated transcription factors. Under normal circumstances, E2 will bind, causing a change in conformation in the receptor and allowing it to migrate to the nucleus (Acconcia et al. 2015). Once in the nucleus, the ERs can interact with coactivators or corepressors, as well as with estrogen response elements (EREs) in order to modify the activation of target genes of estradiol (Ascenzi et al. 2006; Acconcia et al. 2015). However, when BPA (or an exogenous ligand) binds to the ERs, the conformational change in the ligand-binding domain (LBD) of the receptor is different because of the steric hindrance BPA causes;

this changes the transcriptional response of the ER (Pike et al. 1999; Acconcia et al. 2015). Furthermore, LBD displacement can differ between the two ERs, causing some exogenous ligands to act as agonists to one receptor while antagonizing the other (Ascenzi et al. 2006). In fact, it seems that BPA acts as an ERα agonist (similar to E2) and as an antagonist to ERβ (Acconcia et al. 2015).

Bisphenol A has been found to bind even more strongly and to be disruptive to estrogen-related receptor gamma (ERRɣ) (Liu et al. 2019). In fact, the binding of BPA to ERRɣ is now considered a primary mechanism of action. Although estradiol does not bind to ERRɣ, the latter is present in the developing embryo and in the placenta, which may explain some of the detrimental effects of BPA on early development (Takeda et al. 2009; Rubin 2011). It was discovered that a member of the G protein-coupled receptor family, GPR30, was capable of binding to estrogen, thus mediating downstream cellular signaling (Prossnitz et al. 2008; Acconcia et al. 2015). Although BPA was found to bind with strong affinity to this receptor, the identity of GPR30 as an estrogen receptor continues to be debated (Thomas and Dong 2006; Levin 2009).

Additional actions of BPA, although not extensively studied, include binding to the androgen receptor and causing antiandrogenic effects (Rubin 2011; Acconcia et al. 2015). BPA may also interfere with thyroid hormone pathways, and cause epigenetic modifications (Rubin 2011; Acconcia et al. 2015). In a study of neonatal rats exposed to BPA, it was found that developmental exposure to BPA can lead to altered DNA methylation, which is an epigenetic change; this was considered the first insult (Prins et al. 2007). Prostatic dysplasia, and eventually, cancer, were observed when this first insult was followed by a second insult, such as high levels of estradiol (Prins et al. 2007).

1.1.1.4 Metabolism of BPA

Once in the body, BPA is metabolized by glucuronidation and sulfation, glucuronidation being the main detoxification pathway (Yokota et al. 1999; Nachman et al. 2014). Once ingested, BPA is rapidly transformed into BPA glucuronide by uridine diphosphate glucuronosyltransferases (UGTs) in the liver; this metabolite is then excreted in the urine (Völkel et al. 2002; Nachman et al. 2014). In rats, this is completed by a UGT isoform known as UGT2B1 (Yokota et al. 1999). It is of note that the levels and enzymatic activity of UGT in human fetal liver are lower compared to the human adult liver (Gail McCarver and Hines 2002; Nachman et al. 2014; Jalal et al. 2018). The parent compound is therefore not immediately metabolized; this may contribute to the detrimental effects of BPA early in development.

The secondary metabolic pathway for BPA is completed through sulfation, transforming BPA into BPA sulfate (Jalal et al. 2018). This is accomplished by sulfotransferases, such as the simple phenol (P)-form phenol sulfotransferase (SULT1A1) (Suiko et al. 2000; Jalal et al. 2018). Glucuronidation and sulfation quickly inactivate BPA and transform it into biologically inert metabolites which are able to be excreted in urine (Suiko et al. 2000; Völkel et al. 2002). Unfortunately, BPA glucuronide can be deconjugated back into the biologically active BPA, which is why it remains a cause for concern despite its rapid metabolism (Nachman et al. 2014; Corbel et al. 2015).

1.2 The Toxic Effects of BPA

Since its discovery, BPA has been the subject of over 9,800 research articles (PubMed, June 18, 2020) outlining its presence and adverse effects on cells, animals, and humans. The effects of BPA on male and female reproduction in humans have been determined through

different association studies. In men, BPA exposure is associated with increased prolactin and estradiol levels (Liu et al. 2015; Ma et al. 2019). BPA is also associated with decreased sexual function and desire, an increased risk of subfertility as well as a decrease in sperm count and quality (Li et al. 2010; Den Hond et al. 2015). With respect to female reproduction, BPA exposure is associated with an increased risk of infertility, as well as adverse pregnancy outcomes (Shen et al. 2015; Wang et al. 2018). In children, BPA exposure is correlated with decreased anogenital distance in newborn boys, decreased height in boys, delayed puberty in girls, and earlier puberty in boys (Berger et al. 2018; Mammadov et al. 2018; Wang et al. 2019).

Metabolic disorders, such as obesity and type 2 diabetes, have also been shown to be associated with exposure to BPA (Ma et al. 2019). Urinary BPA levels are positively associated with the risk of obesity, and the detection of higher levels of BPA in the urine is correlated with an increase in abdominal obesity (Carwile and Michels 2011). In regards to the respiratory system, postnatal urinary BPA concentrations are associated with child wheezing as well as with asthma (Spanier et al. 2012). In postmenopausal women, serum BPA levels are positively associated with elevated mammographic breast density (Sprague et al. 2013). Although this study did not evaluate breast cancer risk directly, breast tissue density is associated with an increased risk for breast cancer (Boyd et al. 1995; Boyd et al. 2007; Duffy et al. 2018). In female mice, BPA accelerates the development of type 1 diabetes (Xu et al. 2019). Prenatal exposure to BPA in rats, in combination with a high-fat diet, increases breast cancer risk in the offspring (Leung et al. 2017). Finally, a recent study exposed mice to BPA and phthalates at concentrations equivalent to those measured in the urine of patients before and after cardiac surgery. It was determined that male mice exposed to BPA and phthalates had decreased survival and delayed recovery after a surgery-induced myocardial infarction (Shang et al. 2019).

The adverse effects of BPA have been analyzed in studies with endpoints ranging from male and female reproduction to cancer. However, the association between BPA and bone has been rarely studied. The main mechanism of action of BPA involves binding to the estrogen receptors and then affecting downstream signalling, which changes the transcriptional response of the ERs/ERRs. Considering that estrogens play a crucial role in the development of bone and its homeostasis, it becomes essential to study the effects of a xenoestrogen such as BPA (Jalal et al. 2018). Bone metabolism involves the bone forming osteoblasts as well as the bone resorbing osteoclasts, which are coupled and which also possess estrogen receptors (Väänänen and Härkönen 1996; Jalal et al. 2018). One of the ways estrogens are able to regulate bone metabolism is by inhibiting osteoclasts and activating osteoblasts, thereby decreasing bone resorption and maintaining bone formation (Väänänen and Härkönen 1996; Cauley 2015). Unfortunately, very little is known about how BPA directly affects these processes. The following sections outline and summarize what is currently known about the effects of BPA on bone and bone cells in *in vitro*, animal, and human studies.

1.2.1 *In vitro* studies

The effects of BPA on osteoclast formation and osteoblast differentiation from bone marrow-derived macrophages were analyzed in RAW 264.7 and MC3T3-E1 cells, respectively; BPA (0.5 to 12.5 μM) concentration-dependently inhibited the differentiation of osteoblasts and induced the apoptosis of both osteoclasts and osteoblasts (Hwang et al. 2013; Chin et al. 2018). Similar results were obtained in cultured goldfish scales, where it was shown that BPA suppressed the activities of osteoclasts and osteoblasts as indicated by their markers, tartrate-resistant acid phosphatase (TRAP) and alkaline phosphatase (ALP), respectively (Suzuki and Hattori 2003). Thus, BPA exposure is associated with adverse effects on bone cells *in vitro*.

1.2.2 Animal studies

In mice exposed to BPA (10 μg/kg/day) via a mini-osmotic pump from gestational day (GD) 11 to postnatal day (PND) 12, BPA increased adult femur length but did not have an effect on tensile strength (Pelch et al. 2012). However, BPA did decrease the torsional strength and energy to failure ("the total amount of torsional energy the bone can withstand prior to fracture") in female mice (Pelch et al. 2012). In a similar study, pregnant rats were gavaged with 25 to 50,000 μg BPA/kg/day from GD 7 until weaning (Lejonklou et al. 2016). In the offspring of these exposed dams, the femurs were elongated (in females), the total bone mineral content of the diaphysis was lower (in females), the diaphyseal cortex of the femur was thicker (in males), and there were no differences in bone strength (males and females) (Lejonklou et al. 2016).

In aromatase knockout mice exposed to 0.116 g/kg or 1.16 g/kg body weight of BPA, there was a dose-dependent prevention of bone loss (Toda et al. 2002). Aromatase knockout mice lack Cyp19, the aromatase gene; they are therefore incapable of producing estrogen and thus act as a model of estrogen deficiency (Fisher et al. 1998). In these mice, there was an increase in bone resorption, resulting in bone loss that could be reversed with 17β-estradiol (Miyaura et al. 2001). The reduction of bone density in the femur of these knockout mice was also reversed by treatment with either dose of BPA (Toda et al. 2002). However, it is important to note that BPA did not affect bone density in wild-type mice; the protective effects of BPA are due to the lack of estrogens in the knockout mice, and the estrogenicity of BPA is not sufficient to have an effect in wild-type mice. Taken together, these studies indicate that developmental exposure to BPA may decrease bone strength and increase fractures, but may also have protective effects in estrogen deficiency models. Furthermore, these responses are often sex-dependant.

1.2.2.1 CLARITY-BPA

In 2012, the National Institute of Environmental Health Sciences (NIEHS), the National Toxicology Program (NTP), and the U.S. Food and Drug Administration (FDA) initiated the Consortium Linking Academic and Regulatory Insights on BPA Toxicity (CLARITY-BPA) program. This program was created in order to investigate the health effects resulting from exposure to BPA. CLARITY-BPA is made up of two components: the core study, conducted at the National Center for Toxicological Research, and the Grantee Studies, which were conducted by NIEHS-funded university researchers. The core study evaluated the effects of perinatal and chronic exposure to a wide range of BPA doses, while the grantee studies tested a variety of additional endpoints.

The final CLARITY-BPA Core Study (NTP RR-09 2018; Camacho et al. 2019) reported the effects of exposure to 2.5–25,000 μg/kg bw/day of BPA on litter parameters, preweaning and postweaning survival, bodyweights, vaginal opening, vaginal cytology, hematology, organ weights, sperm, and the histopathology of various organs. BPA did not seem to cause any adverse effects on the endpoints analyzed. The grantee studies focused on a wide range of endpoints, such as behaviour, diabetes, obesity, the immune system, and the male and female reproductive systems, among others. In most of the grantee studies, BPA caused detrimental effects at the lower doses tested (Heindel et al. 2020). Interestingly, an in-depth analysis on the effects of BPA on bone or bone cells was not done in either the core study or the grantee studies.

1.2.3 Human studies

There are very few human studies that have reported on the effects of BPA on bone. The goal of one of these studies was to analyze the association between serum BPA concentrations

and bone mineral density (BMD) and bone markers (Kim et al. 2012). In the 51 postmenopausal women tested, it was found that serum BPA concentrations did not correlate with BMD or with C-terminal telopeptide (CTX, a marker of bone resorption) and osteocalcin (a marker of bone formation) (Kim et al. 2012). However, the sample size of this study was quite small and included only postmenopausal women. In a larger study of 246 premenopausal women, very similar results were observed: there was no correlation between urinary BPA concentrations and BMD, N-terminal telopeptide (NTX, also a marker of bone resorption) and osteocalcin (Zhao et al. 2012). Finally, a longitudinal study was conducted on 754 children aged 9 to 18 to determine the association between BPA and height (Wang et al. 2019). The results indicated an inverse association between urinary BPA concentrations and height growth in boys, but not in girls (Wang et al. 2019). Since longitudinal bone growth during puberty accounts for 20% of the final adult height, choosing pubertal (as opposed to prepubertal) children is important (Perry et al. 2008; Wang et al. 2019). Although the sample size of this study was larger than the other studies mentioned, not including prepubertal children and collecting spot urine samples are limitations of the study design.

1.3 BPA Regulatory Policies

The multitude of studies exposing BPA as a potential toxic chemical raised concerns among government agencies as well as the public. In 2008, Canada was the first country to label BPA as a dangerous substance; at the time, however, the government had not yet imposed any restrictions on this chemical (Mittelstaedt; Vogel 2009). In 2010, BPA was officially declared toxic by Health Canada, and in the same year, its use in plastic baby bottles was banned (Resnik and Elliott 2015). The FDA followed suit, and in 2012, banned BPA in plastic baby bottles and children's cups (Resnik and Elliott 2015). Interestingly, manufacturers and retailers had already

begun pulling plastic baby bottles containing BPA off shelves, not because of the imminent regulatory decisions or because of its potential toxicity, but due to the fact that the consumers themselves no longer purchased products that contained BPA (Vogel 2009; Resnik and Elliott 2015). Public pressure, and the banning of BPA from baby products, forced manufacturers to find alternatives to this substance, leading to the emergence of other lesser studied bisphenols.

1.4 BPA Replacements

The bisphenols are a very large family of chemicals that are similar in structure and function. Unfortunately, with the exception of BPA, very little is known about their potential toxicity. With the phasing out of BPA, many alternatives are now emerging in consumer products, such as bisphenol AF (BPAF), bisphenol S (BPS), and bisphenol F (BPF). These chemicals are promoted as "alternatives" to BPA, but are rarely studied and analyzed before being used in consumer products.

1.4.1 Bisphenol AF

In bisphenol AF (BPAF, CAS Number: 1478-61-1), the two methyl groups found in BPA are replaced with trifluoromethyl groups (Figure 1.3).

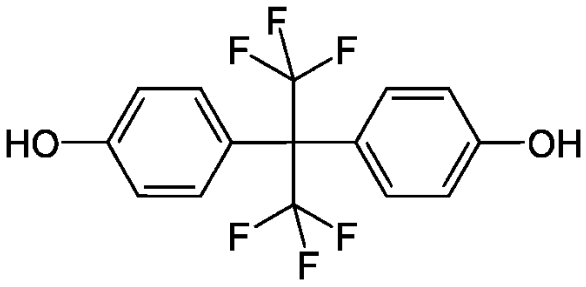


Figure 1.3: The chemical structure of BPAF

Although less extensively studied than BPA, the few studies on BPAF have shown that this chemical was detected in indoor dust samples as well as in foods and beverages, although at lower concentrations than BPA (Liao et al. 2012; Liao and Kannan 2013). It was also found that BPAF possesses endocrine disrupting activities through its binding to the ERα as well as to the G protein-coupled estrogen receptor (GPER) (Li et al. 2014). Furthermore, BPAF has a higher binding affinity to GPR30 compared to BPA, which could lead to stronger estrogenic effects (Cao et al. 2017).

Exposure of human osteosarcoma cells to BPAF for 3 months, resulted in an altered profile of gene expression for genes involved in different processes, including down-regulation of the expression of some bone-related genes (Fic et al. 2015). There are currently no published articles reporting on the effects of BPAF on bone or on ossification in *in vivo* animal or human epidemiological studies. Thus, very little is known about emerging BPA replacements and their potential adverse effects on humans. Similarly, there are very few studies examining the effects of BPA and its replacements on bone and bone cells.

1.5 Limb Formation and Development

Bone formation is a dynamic process that can be influenced by environmental factors, yet, it is rarely studied in the context of the effects of chemical pollutants such as BPA (McGowan 1996). The murine limb bud is a model that can address this large knowledge gap because it captures the process of endochondral ossification as a whole. Furthermore, the cells and pathways involved in murine long bone formation are very similar to those in humans.

1.5.1 The limb bud

In vertebrates, limb buds form at specific positions along the anterior-posterior axis, and these positions correlate with Hox gene expression levels (Iimura and Pourquié 2007). Furthermore, the identity of the limb (forelimb versus hindlimb) is determined by the expression of a specific T-box transcription factor (TBX) protein (Rodriguez-Esteban et al. 1999). TBX4 is specifically expressed in the hindlimb while TBX5 is expressed in the forelimb (Rodriguez-Esteban et al. 1999). In the early stages, the limb bud consists of mesoderm cells covered in ectoderm (Tickle 2015). When mesenchymal cells from the lateral plate mesoderm and from the somites begin proliferating, they accumulate and create a bulge under the overlying ectodermal cells (Tickle 2015). This bulge is what is known as the "limb bud" (Figure 1.4).

The signal that activates the formation of limb buds comes from the lateral plate mesoderm cells; these cells secrete FGF10, the protein that initiates the interactions between the mesoderm and the ectoderm (Tickle 2015). The binding of FGF10 to its receptor in the ectoderm leads to the formation of a structure called the apical ectodermal ridge (AER), which itself secretes FGF8 (Ohuchi et al. 1997; Xu et al. 1998; Tickle 2015). The AER, through secretion of FGF8, helps maintain the mesenchymal cells in a proliferating phase, thereby regulating the proximal-distal growth of the limb. FGF8, when binding to its receptor in the mesoderm (in an area called the progress zone, PZ), leads to the release of FGF10 and thus, the maintenance of the AER. This creates a positive feedback loop between the proliferating cells from the PZ and the AER, allowing the limb bud to grow (Tickle and Eichele 1994; Tickle 2015). The AER is the first signalling center of the limb bud and, eventually, a second signalling center called the zone of polarizing activity (ZPA) appears in the posterior area of the limb (Tickle and Eichele 1994;

Tickle 2015). The ZPA secretes sonic hedgehog protein (SHH), which will define patterning along the anterior-posterior axis (Litingtung et al. 2002; Rodrigues et al. 2017).

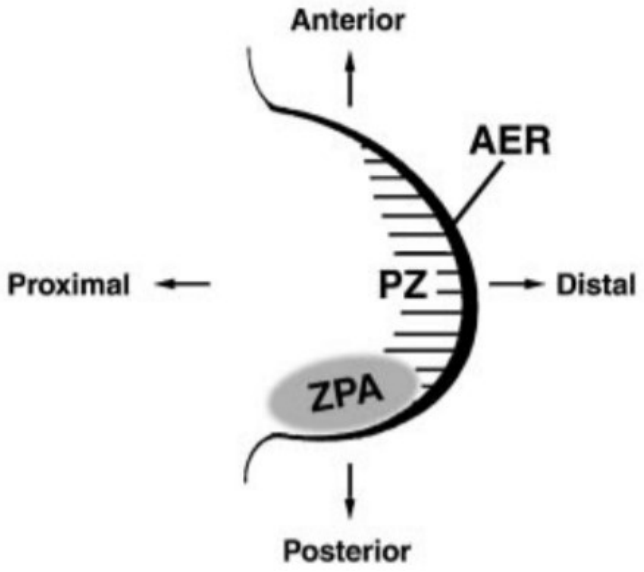


Figure 1.4: Overview of the zones present during limb bud induction and outgrowth. AER: apical ectodermal ridge, PZ: progress zone, ZPA: zone of polarizing activity. Adapted from (Capdevila and Belmonte 2001).

1.5.2 Endochondral ossification

In vertebrates, skeletogenesis begins once mesenchymal cells migrate and aggregate to form mesenchymal condensations (Mackie et al. 2008; Lefebvre and Bhattaram 2010). They then differentiate into chondrocytes (cartilage cells) and osteoblasts (bone cells) (Lefebvre and Bhattaram 2010). The skeleton begins as a cartilaginous entity but with time, bone gradually begins replacing the cartilage template to form the mature skeleton in a process called endochondral ossification (Figure 1.5) (Lefebvre and Bhattaram 2010). The long bones of the body, such as the limbs, develop through this process. The flat bones of the skull, the scapula, and the clavicles are all formed by intramembranous ossification, a process in which bones

develop directly from mesenchymal tissue without the need for a cartilage template precursor (Franz-Odendaal 2011).

The first step of endochondral ossification begins when the mesenchymal cells condense and differentiate into reserve and proliferative chondrocytes, which form the cartilage template of the bone (Gilbert 2000; Mackie et al. 2008). This resting hyaline cartilage is surrounded by a membrane called the perichondrium (Gilbert 2000; Franz-Odendaal 2011). The reserve chondrocytes that make up this cartilage help align the growth plate and inhibit the premature differentiation of proliferative chondrocytes into hypertrophic chondrocytes (Abad et al. 2002). Proliferative chondrocytes, on the other hand, are flatter, more tightly packed, and divide rapidly (Mackie et al. 2008). Furthermore, these chondrocytes secrete collagen type II alpha 1 (COL2A1) (Zhao et al. 1997). Eventually, proliferative chondrocytes cease to divide and undergo hypertrophy to become the much larger hypertrophic chondrocytes (Mackie et al. 2008). The contents of the matrix they secrete is altered and they begin to express collagen type X alpha 1 (COL10A1) and other extracellular matrix proteins such as alkaline phosphatase (ALP) (Gilbert 2000; Ballock and O'Keefe 2003). Both alkaline phosphatase and COL10A1 are essential for the calcification and mineralization of the cartilage matrix (Ballock and O'Keefe 2003; Mackie et al. 2008). Once the matrix has been mineralized, hypertrophic chondrocytes die and blood vessels carrying osteogenic cells invade the cartilage template (Mackie et al. 2008). Osteoblast precursors then differentiate into mature osteoblasts and begin secreting a collagen type I alpha 1 (COL1A1)-rich bone matrix, using the cartilage matrix as a template (Gilbert 2000; Mackie et al. 2008). Thus, cartilage is gradually replaced by bone.

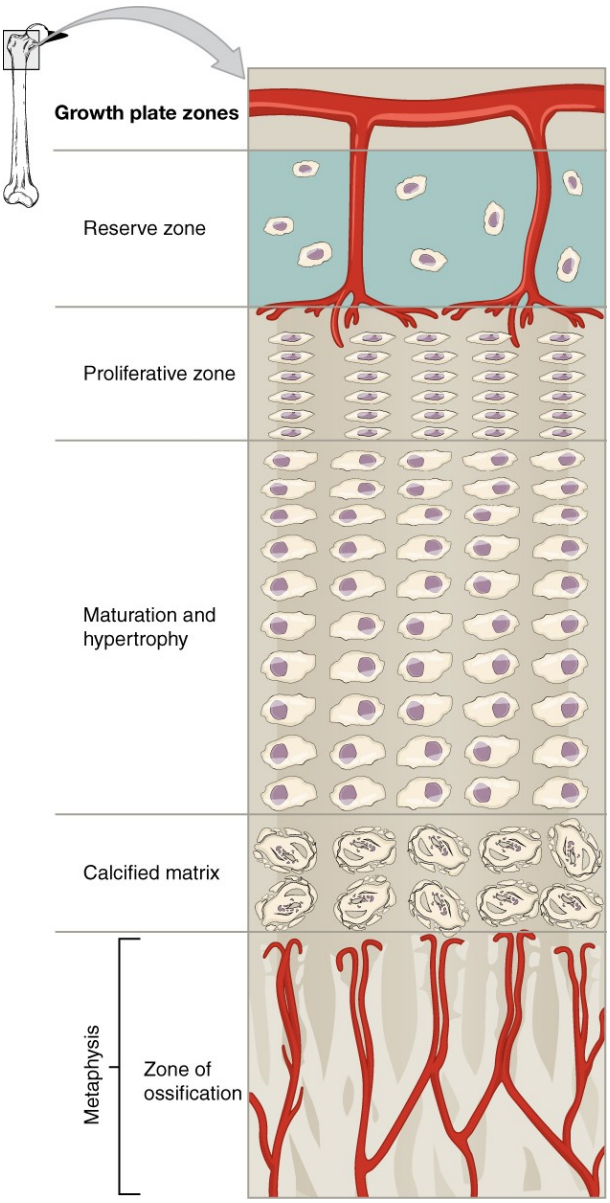


Figure 1.5: Organization of the cells present in the growth plate during endochondral ossification. Adapted from (Kannian and Ryan 2018).

Steps in the progression from resting cartilage all the way to invasion by blood vessels allow the perichondrium to become the periosteum, which forms the periosteal bone collar (Figure 1.6) (Mackie et al. 2008). This creates the primary ossification centre. As this is occurring in the centre of the cartilage model, chondrocytes are continuing to divide, to differentiate, and to be replaced at the ends of the bone, thereby forming the secondary ossification centres near the epiphyses (Mackie et al. 2008). As the skeleton matures, cartilage will be present both at the joints and at the growth plate (area between the primary and secondary ossification centres). To allow elongation of the long bones, the same events that occurred in the primary ossification centre (proliferation of chondrocytes, hypertrophy of chondrocytes and their death, invasion by blood vessels and deposition of bone by osteoblasts) will occur again thanks to the growth plate (Mackie et al. 2008). In adults, the two centres of ossification gradually replace all cartilage as they continue to grow, causing the epiphyseal and metaphyseal bones to fuse. Thus, the growth plate is completely replaced by bone and longitudinal growth is no longer possible. The only cartilage that remains is the permanent articular cartilage present at the joints (Mackie et al. 2008).

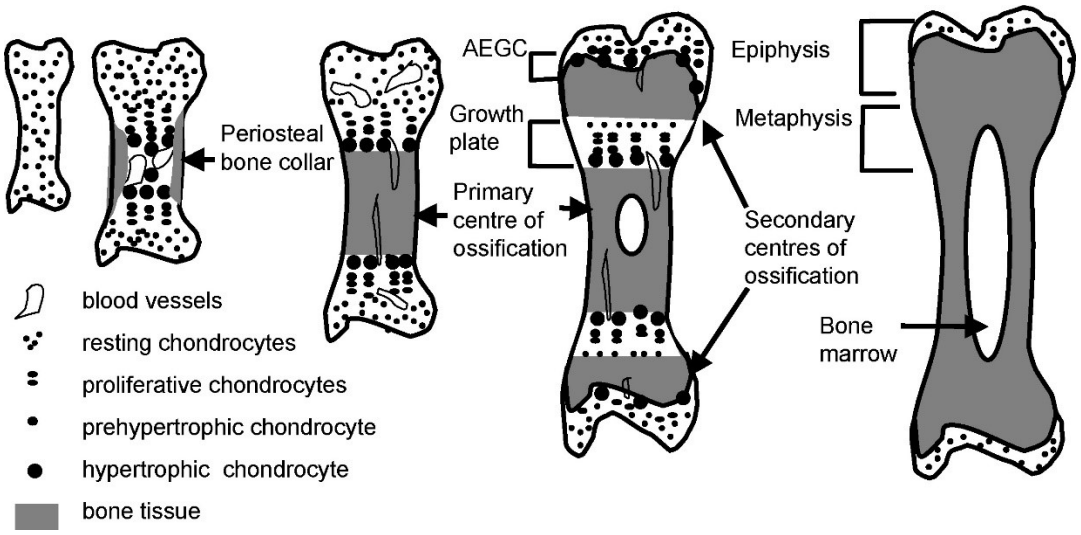


Figure 1.6: The process of endochondral ossification, from a cartilage template to a fully ossified adult bone. AEGC: Articular-epiphyseal growth cartilage.
Adapted from (Mackie et al. 2008).

1.5.3 Master regulators of endochondral ossification

The process of endochondral ossification is tightly regulated by three transcription factors: SOX9, RUNX2, and SP7.

1.5.3.1 SOX9 signalling

SOX9, or sex determining region Y (SRY)-related high mobility group (HMG) box 9, is a transcription factor from the Sox family, encoded by the *Sox9* gene. The proteins from this family are highly conserved and share a HMG domain, through which these proteins recognize and bind to specific sequences in DNA (Kamachi and Kondoh 2013). *Sox9* is expressed in many tissues, including in mesenchymal cells and chondrocytes (Jo et al. 2014; Lefebvre and Dvir-Ginzberg 2017). SOX9 is vital to chondrogenesis because it maintains the survival of proliferative chondrocytes and prevents premature hypertrophy (Figure 1.7) (Ikeda et al. 2004; Jo et al. 2014). By binding directly to specific sequences, Sox9 regulates and activates the gene encoding COL2A1, which is expressed in proliferative chondrocytes and forms the bulk of the cartilage matrix (Bell et al. 1997; Jo et al. 2014).

The presence of a mutation on one allele of the *Sox9* gene causes campomelic dysplasia (CD) in humans (Foster et al. 1994; Wagner et al. 1994). CD is characterized by sex reversal, defects in cartilage formation, and skeletal abnormalities such as bowing of the long bones (Foster et al. 1994). In cells that lack both alleles of *Sox9*, COL2A1 is no longer expressed, thereby inhibiting cartilage development (Bi et al. 1999). Sox9 is therefore essential for chondrocyte differentiation and is the master regulator of chondrogenesis.

1.5.3.2 RUNX2 and SP7 signalling

RUNX2, or runt-related transcription factor 2, is a protein encoded by the *Runx2* gene. *Runx2* is mainly expressed in osteoblasts but has also been detected in chondrocytes (Komori et al. 1997). Similarly to SOX9, RUNX2 possesses a highly conserved region called *runt* homology domain (RHD) which serves as its DNA-binding domain (Inada et al. 1999). RUNX2, together with the co-transcription factor Cbfb/Pebp2β, can form heterodimers and bind to specific sequences (Inada et al. 1999). During bone formation, RUNX2 has two main roles: the first is to promote the maturation and differentiation of proliferative chondrocytes into hypertrophic chondrocytes and the second is to promote the differentiation of mesenchymal stem cells into osteoblasts (Figure 1.7) (Komori et al. 1997; Inada et al. 1999).

In mice lacking *Runx2*, COL10A1 is no longer expressed in the humerus and femur, thereby indicating that the differentiation of hypertrophic chondrocytes is blocked (Inada et al. 1999). Thus, osteoblast progenitors fail to differentiate into mature osteoblasts and both intramembranous and endochondral ossification are completely arrested in *Runx2* null mice (Komori et al. 1997). The mice die shortly after birth since the absence of bone formation in the ribs makes breathing impossible (Komori et al. 1997). In contrast, mice with a heterozygous mutation in *Runx2* show a milder phenotype, with delayed ossification and hypoplastic clavicles and nasal bones (Komori et al. 1997). In humans, cleidocranial dysplasia (CDD) is caused by mutations in *RUNX2*, leading to hypoplastic clavicles, extra teeth, and short stature, among other skeletal anomalies (Inada et al. 1999; Jarvis and Keats).

RUNX2 acts in concert with SP7 to promote osteoblastogenesis (Figure 1.7) (Nakashima et al. 2002). SP7, or Osterix, is the third major transcription factor involved in endochondral ossification, and is encoded by the *Sp7* gene. Mice lacking *Sp7* also show no ossification because

the osteoblasts fail to differentiate; this occurs despite the presence of RUNX2, indicating that SP7 acts downstream of RUNX2 to promote bone formation (Nakashima et al. 2002). Levels of COL1 (the collagen that makes up the bone matrix) were also dramatically reduced (Nakashima et al. 2002). Thus, RUNX2 is essential for endochondral ossification, allowing chondrocytes to mature and begin producing a matrix that is predominantly COL10A1, and through SP7, RUNX2 is indispensable for osteoblastogenesis, a process vital for the laying down of bone (Komori et al. 1997; Inada et al. 1999; Nakashima et al. 2002).

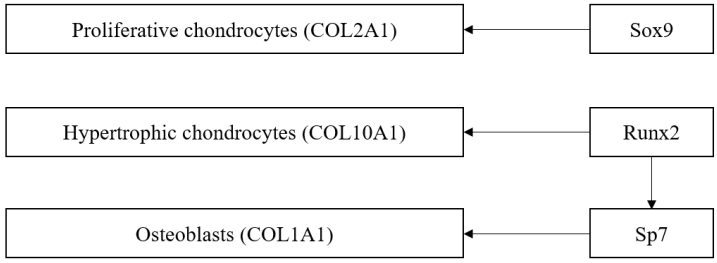


Figure 1.7: Organization and roles of the master regulators of endochondral ossification.

1.6 Bone as a Target for Other Endocrine Disrupting Chemicals

Bisphenols are far from being the only endocrine disruptors capable of causing adverse effects on bone metabolism and ossification. In fact, there is a wide variety of chemicals that are associated with altered skeletal development.

1.6.1 Organotins and diethylstilbestrol

Organotin compounds, like tributyltin (TBT) and triphenyltin (TPT), are created from the combination of tin and hydrocarbon compounds, and are mostly used as biocides and wood preservatives (Agas et al. 2013). *In utero* exposure to 10 mg/kg and 20 mg/kg TBT from GD 0 to GD 19 resulted in delayed and reduced ossification of the fetus (Adeeko et al. 2003).

Furthermore, stem cells isolated from mice exposed to TBT *in utero* were predisposed to become adipocytes, at the expense of osteogenesis and the osteogenic lineage (Kirchner et al. 2010). Diethylstilbestrol (DES), another EDC known to cause adverse effects on bone, is a potent estrogen agonist (Agas et al. 2013). Mice exposed to 0.1 μg/kg/day DES from GD 11 to PND 12 had increased femur length and decreased tensile strength, the combination of which decreased torsional ultimate strength (Pelch et al. 2012).

1.6.2 Flame retardants

Flame retardants (FRs) are industrial chemicals added to a variety of materials in order to reduce flammability and slow the growth of fire (Birnbaum and Staskal 2004). Brominated flame retardants (BFRs), such as polybrominated diphenyl ethers (PBDEs), are some of the most widely used FRs (Birnbaum and Staskal 2004). Due to their widespread use, human exposure to PBDEs became common, and studies regarding their toxic effects began emerging in the literature. Pups of Sprague Dawley rats exposed to an environmentally relevant mixture of PBDEs showed an increase in digit anomalies as well as decreased skeletal ossification; skeletal variations were prevalent at relatively low exposure levels (Berger et al. 2014; Tung et al. 2016). PBDEs were eventually phased out due to their toxicity, and replaced with orgonaphosphate esters (OPEs). Unfortunately, new evidence is now emerging concerning the safety of these alternative FRs. Using the same model outlined in this thesis, our lab has previously determined the effects of four different OPEs [triphenyl phosphate (TPHP), tert-butylphenyl diphenyl phosphate (BPDP), tris(methylphenyl) phosphate (TMPP), or isopropylated triphenyl phosphate (IPPP)] on endochondral ossification (Yan and Hales 2019). The effects of OPEs were compared to those caused by BDE-47, the legacy PBDE congener, and it was determined that OPEs may not be safer alternatives than their brominated predecessors (Yan and Hales 2019). In fact,

concentrations as low as 1 μM of the four OPEs had an adverse effect on chondrogenesis, while concentrations equal to or greater than 3 μM dramatically supressed osteogenesis (compared to 50 μM BDE-47). Furthermore, TPHP and BPDP downregulated the expression of *Runx2* and *Sp7*, while BDE-47 had a relatively minor impact on the same transcription factors (Yan and Hales 2019). These effects are very similar to those observed in the present thesis.

Organotins, alkylphenols, diethylstilbestrol, dioxins, phthalates, PBDEs, and OPEs are all EDCs that have been associated with effects on bone (Agas et al. 2013; Yan and Hales 2019). BPA and BPAF are clearly not unique in their ability to adversely affect bone and the results obtained in this thesis are one more piece of the larger puzzle of endocrine disrupting chemicals.

1.7 The Triple Transgenic Mouse Model

Our laboratory uses a triple transgenic CD1 mouse model that expresses fluorescently tagged markers of chondrocyte and osteoblast differentiation (Figure 1.8) in order to determine the effects of various chemicals on endochondral ossification (Maye et al. 2011). These mice were a gift from David L. Butler (University of Cincinnati) and David Rowe (University of Connecticut Heath Center), and were generated using bacterial recombination with a BAC clone (Maye et al. 2011). The proliferative chondrocytes in these transgenic mice express COL2A1 tagged with enhanced cyan fluorescent protein (COL2A1-eCFP). Their hypertrophic chondrocytes express COL10A1 tagged with mCherry fluorescent protein (COL10A1-mCherry), and their osteoblasts express COL1A1 tagged with yellow fluorescent protein (COL1A1-YFP). Because each type of collagen that is mentioned is highly expressed in a specific cell population, they can be used as markers for those populations, allowing us to track and visualize the process of endochondral ossification over time. Thus, using a fluorescence microscope, we are able to

visualize the differentiation status of proliferative chondrocytes, hypertrophic chondrocytes, and osteoblasts.

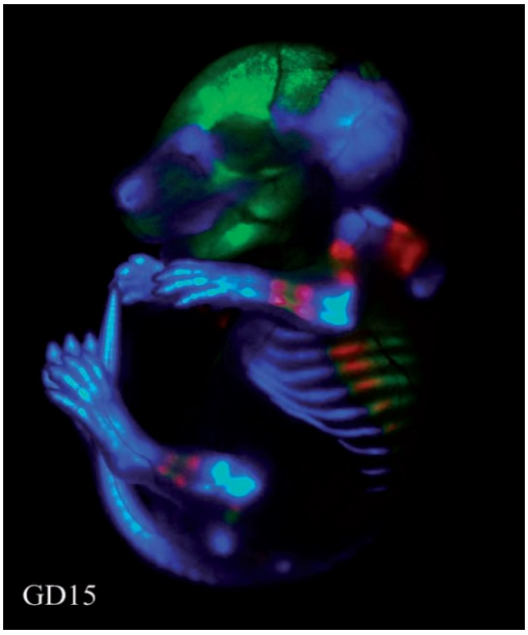


Figure 1.8: Gestational day (GD) 15 triple transgenic mouse embryo. These triple transgenic reporter mice allow for the visualization of skeletal development thanks to fluorescently tagged markers of chondrocyte and osteoblast differentiation. In blue are proliferative chondrocytes that express COL2A1-eCFP (cartilage), in red are hypertrophic chondrocytes that express COL10A1-mCherry, and in green are osteoblasts that express COL1A1-YFP (ossified bone) (Paradis and Hales 2015).

1.8 Hypothesis and Objectives

A number of studies have shown that exposure to BPA is linked to alterations in skeletal development and possible detrimental effects on bone cells. However, the mechanisms by which BPA disrupts limb development are largely unknown, and no currently published article addresses the effects of BPA on endochondral ossification in mammals. Even less is known about the possible effects of BPA replacements, such as BPAF. As BPA and its replacements continue to be produced in large quantities around the world, making human exposure

widespread, it is important to study the effects of bisphenols on skeletal development.
I hypothesize that both BPA and BPAF will adversely affect and delay endochondral ossification in the murine limb. My specific goals are:

Objective 1: To determine the effects of BPA and BPAF on endochondral ossification, by visualizing the expression of proliferative chondrocytes, hypertrophic chondrocytes, and osteoblasts in *ex vivo* limb buds.

Objective 2: To investigate the effects of BPA and BPAF on the expression of *Sox9*, *Runx2*, and *Sp7*, the master regulators of endochondral ossification.

With the results of objectives 1 and 2, we are able to compare the toxicity of BPAF with BPA in the context of endochondral ossification.

Chapter Two: The Effects of Bisphenol A and Bisphenol AF on Endochondral Ossification in Ex Vivo Murine Limb Bud Cultures

Abstract

The bisphenols are a family of chemicals commonly used to produce polycarbonate plastics and epoxy resins. *In utero* exposure to bisphenol A (BPA) was found to affect skeletal development in animal models. However, it remains unknown whether BPA and its alternatives specifically disrupt endochondral ossification, the process by which bone is formed. Using an *ex vivo* murine limb bud culture system, we determined the effects of BPA and BPAF on endochondral ossification. The major stages of this process were visualized using triple transgenic mice that express fluorescent markers of collagen: COL2A1-eCFP (proliferative chondrocytes), COL10A1-mCherry (hypertrophic chondrocytes), and COL1A1-YFP (osteoblasts). The forelimbs of gestation day 13 embryos were cultured in the presence of vehicle, BPA, or BPAF. BPA (≥10 μM) and BPAF (≥1 μM) reduced the differentiation of hypertrophic chondrocytes and osteoblasts, as seen by a reduction in COL10A1-mCherry and COL1A1-YFP fluorescence. Concentrations of ≥50 μM BPA and ≥5 μM BPAF suppressed chondrogenesis; osteogenesis was almost completely arrested at 100 μM BPA and 10 μM BPAF. Both BPA and BPAF affected the expression of *Sox9*, *Runx2*, and *Sp7*, the master regulators of endochondral ossification. The expression of *Sox9* was altered after exposure to BPA. Exposure to 50 μM BPA downregulated the expression of *Runx2* at all time points and suppressed the overall upregulation of *Runx2* and *Sp7* that was observed in control limbs. BPAF had a less pronounced effect on these transcription factors. Our data suggest that BPAF, the replacement bisphenol, may be more detrimental to endochondral ossification than BPA.

Introduction

The bisphenols are a family of chemicals used to produce polycarbonate plastics and epoxy resins (Geens et al. 2011). Bisphenol A (BPA), the most commonly studied bisphenol, has been used extensively in the production of reusable plastic water bottles and food storage containers, and has been detected in thermal paper, medical equipment, and in the lining of metal cans (Geens et al. 2011; Bernier and Vandenberg 2017). Human exposure normally occurs through the diet after consumption of food from canned products containing BPA (Jalal et al. 2018). It can also occur through dermal contact with thermal receipt paper as well as through dust inhalation (Wang et al. 2015; Bernier and Vandenberg 2017; Jalal et al. 2018). Due to its widespread use, exposure to BPA has become ubiquitous. It is associated with adverse effects such as infertility, obesity, and cancer in humans and in animal models (Shen et al. 2015; Leung et al. 2017; Wang et al. 2018; Ma et al. 2019). Because of concerns regarding its toxicity, BPA was banned from plastic baby bottles in Canada in 2010, and later by the FDA (Resnik and Elliott 2015).

With the global phase-out of BPA, many alternative bisphenols are now emerging in consumer products, such as bisphenol AF (BPAF). BPAF is one of the most widely used BPA replacements, with increasing human exposure; BPAF is also associated with adverse health effects (Andújar et al. 2019). It is therefore essential to identify whether BPAF is more or less toxic than the legacy bisphenol it has replaced.

Endocrine disruptors such as BPA have many possible toxicological targets within the body. One such target that is often overlooked is the skeleton, which begins as a cartilaginous entity. With time, bone gradually replaces this cartilage template to form the mature skeleton in a

process called endochondral ossification (Lefebvre and Bhattaram 2010). The epiphyseal plate is essential for the process of ossification. Within this plate, the proliferative chondrocytes that make up the cartilage template are continuously dividing, and will eventually mature into hypertrophic chondrocytes, that are ultimately replaced by the osteoblasts that lay down the bone matrix (Mackie et al. 2008). These events are regulated by three transcription factors known as the master regulators of endochondral ossification: sex determining region Y (SRY)-related high mobility group (HMG) box 9 (SOX9), runt-related transcription factor 2 (RUNX2), and SP7 (Kronenberg 2003; Long and Ornitz 2013).

SOX9 maintains the survival of proliferative chondrocytes and prevents premature hypertrophy (Ikeda et al. 2004; Jo et al. 2014). SOX9 also regulates and activates the gene encoding COL2A1, which is expressed in proliferative chondrocytes and forms the bulk of the cartilage matrix (Bell et al. 1997; Jo et al. 2014). In cells lacking *Sox9*, COL2A1 is no longer expressed, thereby inhibiting cartilage development (Bi et al. 1999). SOX9 is therefore essential for chondrocyte differentiation. RUNX2 promotes the maturation and differentiation of proliferative chondrocytes into hypertrophic chondrocytes as well as the differentiation of mesenchymal stem cells into osteoblasts (Komori et al. 1997; Inada et al. 1999). In mice lacking *Runx2*, COL10A1 is no longer expressed, thereby indicating that the differentiation of hypertrophic chondrocytes has been blocked (Inada et al. 1999). RUNX2 acts in concert with SP7 to promote osteoblastogenesis (Nakashima et al. 2002). Mice that lack *Sp7* show no ossification and a dramatic reduction in COL1 (type I collagen secreted by osteoblasts); this occurs despite the presence of RUNX2, indicating that SP7 acts downstream of RUNX2 to promote bone formation (Nakashima et al. 2002)

Exposure to bisphenols, such as BPA and BPAF, has been associated with a variety of effects on bone and bone cells. In cultured goldfish scales, exposure to BPA suppressed the activities of tartrate-resistant acid phosphatase (TRAP) and alkaline phosphatase (ALP), markers of osteoclasts and osteoblasts, respectively (Suzuki and Hattori 2003). Similarly, in RAW 264.7 and MC3T3-E1 cells, BPA inhibited the differentiation of both osteoblasts and osteoclasts, while stimulating their apoptosis (Hwang et al. 2013). Hwang et al. also reported that the expression of *Sp7* (also known as Osterix) and *Runx2* were downregulated by BPA. The adverse effects of BPA were not limited to *in vitro* studies. Developmental exposure to BPA in C57BL/6 mice led to increased femur length and decreased torsional strength (in females) (Pelch et al. 2012). In Wistar rats, developmental exposure to BPA was associated with altered femoral bone geometry in both males and females (Lejonklou et al. 2016). Finally, adult rare minnows (*Gobiocypris rarus*) were exposed to BPA, and it was found that maternal BPA exposure delayed the ossification of craniofacial bone, reduced bone size, and downregulated the expression of *Sox9*, *Runx2*, and *Col2a1* (Fan et al. 2018).

There are very few studies investigating the effects of BPA on bone formation in animals. To our knowledge, there are no currently published articles outlining the *in vivo* or *ex vivo* effects of BPAF on bone or on ossification. Therefore, the aim of the current study is to elucidate the effects of BPA and BPAF on endochondral ossification in the murine *ex vivo* limb bud culture model. This model system that allows us to visualize the expression of proliferative chondrocytes, hypertrophic chondrocytes, and osteoblasts (Paradis et al. 2019). Using this limb bud culture system, we investigated the effects of BPA and BPAF on the expression of *Sox9*, *Runx2*, and *Sp7*, the master regulators of endochondral ossification.

Materials and Methods

Animals

CD1 reporter mice expressing Collagen Type II alpha 1-enhanced cyan fluorescent protein (COL2A1-eCFP), Collagen Type X Alpha 1-mCherry (COL10A1-mCherry), and Collagen Type I Alpha 1-yellow fluorescent protein (COL1A1-YFP) were a gift from David L. Butler (University of Cincinnati, Cincinnati, Ohio) and David Rowe (University of Connecticut Health Center, Farmington, Connecticut) (Maye et al. 2011). These mice were maintained on a 12h light/12h dark cycle and housed at the McIntyre Animal Resource Centre (McGill University, Montreal, QC, Canada). Animals were provided with food and water ad libitum. Female virgin mice were allowed to mate overnight; the presence of a vaginal plug the next morning was considered gestation day (GD) 0. Pregnant mice were euthanized on GD 13 by CO_2 asphyxiation followed by cervical dislocation, after which the uteri and embryos were explanted in Hanks' balanced salt solution (Sigma-Aldrich Canada Co., Oakville, ON, Canada). All experiments were approved by the Animal Care Committee of McGill University (protocol #7892) and complied with the guidelines outlined in the *Guide to the Care and Use of Experimental Animals*, prepared by the Canadian Council on Animal Care.

Limb bud cultures and treatments

GD 13 embryos were cultured as previously described (Paradis et al. 2019). Briefly, the forelimbs of each embryo were excised, pooled, and placed in glass bottles containing 6 mL of a culture medium consisting of 75% BGJb medium (Gibco, ThermoFisher Scientific), 25% salt solution, supplemented with ascorbic acid (0.16 mg/mL) and gentamycin (50 mg/mL, Wisent Bioproducts, QC, Canada). Hindlimb development is delayed by 0.5 days compared to the forelimb; for this reason, only the forelimbs of each embryo were used, and limbs from male and

female embryos were cultured together. Culture bottles were then gassed for two minutes with 5% CO_2, 50% O_2 balanced with N_2. Each bottle subsequently received one of three treatments: dimethyl sulfoxide (DMSO, vehicle control), bisphenol A (BPA: 1, 10, 50, or 100 μM) [CAS No. 80-05-7], or bisphenol AF (BPAF: 0.1, 1, 5, or 10 μM) [CAS No. 1478-61-1] (gifts from Environmental Health Science and Research Bureau, Health Canada, Ottawa, ON, Canada). Initial experiments revealed that BPAF concentrations equivalent to those of BPA caused an almost complete disintegration of the limbs; thus, the concentrations of BPAF used in subsequent experiments were lower than those of BPA. The limbs were cultured at 37°C for up to 6 days; the medium was changed and reoxygenated on day 3, with no addition of vehicle or bisphenols. Exposure was not continuous to allow for possible recovery of ossification by day 6.

Limb Morphology and Scoring

Limbs (n = 5–14 culture bottles, 5–8 limbs per bottle) were imaged using a Leica DFC450C digital camera (Leica Microsystems, Wetzlar, Germany) connected to a Leica M165 Fluorescent Stereo Microscope (Leica Microsystems) on day 1 (24 hours after initial culture), day 3, and day 6 in order to visualize the expression of the three fluorescent markers. Following the 6-day culturing period, the photographs of the COL2A1-eCFP-positive cartilage from day 3 and day 6 limbs were quantified using a morphogenetic differentiation scoring system (Paradis et al. 2019). Briefly, each component of the forelimb (radius, ulna, carpals, and all five digits) received a score from 0 (unrecognizable) to 30 (excellent differentiation for a cultured limb). The assigned scores allowed us to assess the extent of cartilage template development.

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

Limbs were cultured as previously described, collected after 3, 6, 24, or 48h, and placed in RNAlater until extraction. Limb buds (n = 5 culture bottles, 3–4 limbs per bottle) were

homogenized, and total RNA was extracted using the RNeasy Mini kit (Qiagen, Mississauga, ON, Canada). The concentration and purity of the isolated RNA were assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts). Samples were then diluted to a concentration of 2 ng/µL, and transcripts were quantified using Power SYBR Green RNA-to-C_T 1-Step Kit (Applied Biosystems, Foster City, California) and the Viia7 Real-Time PCR System (Applied Biosystems). One mixture was created for each primer and consisted of 170 µL SYBR Green Master Mix, 48.3 µL Rnase-Dnase-free water, 34 µL of the specific primer, and 2.72 µL Reverse Transcriptase Mix. Therefore, each 20 µL reaction consisted of 10 µL SYBR Green Master Mix, 2.84 µL Rnase-Dnase-free water, 2 µL of primer, 0.16 µL Reverse Transcriptase Mix, and 5 µL of the RNA sample. The following conditions were used for PCR: 48°C for 30 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 55°C for 30 s, and 72°C for 30 s. The primers were obtained from QuantiTect Primer Assays (Qiagen): *Sox9* (QT00163765), *Runx2* (QT00102193), *Sp7* (QT00293181), and ribosomal protein L8 (*Rpl8*, QT00253379). Reactions were done in triplicate, averaged, and normalized to the amount of *Rpl8* transcripts. Data were analyzed using QuantStudio™ Real-Time PCR Software (Version 1.3).

Statistical Analysis

Data were analyzed using GraphPad Prism 8 (Version 8.3.0, GraphPad Software, La Jolla, California). Limb differentiation scores were compared using Bonferroni-corrected Mann-Whitney U tests; $p < 0.05$ was considered statistically significant. mRNA transcript levels were compared using 2-way ANOVA followed by Dunnett's test; $p < 0.01$ was considered statistically significant.

Results

BPA and BPAF adversely affect chondrogenesis and osteogenesis

To assess the effect of BPA and BPAF on endochondral ossification, triple transgenic embryonic forelimbs were cultured for six days in the presence of different concentrations of these bisphenols. After 1, 3, and 6 days in culture, images of the limbs were captured using fluorescence microscopy (Figure 2.1, Figure 2.3). Control limbs developed as expected; over the course of the 6-day period, there was elongation of the radius and the ulna of these limbs. We also observed the development of the metacarpals as well as the differentiation of the carpals and the proximal and medial phalanges; usually, the distal phalanges also appeared (data not shown). The control forelimbs consistently enter into the later stages of endochondral ossification, as shown by the differentiation of the hypertrophic chondrocytes and of the osteoblasts. This differentiation is indicated by the appearance of COL10A1-mCherry and COL1A1-YFP fluorescence on day 3; these zones of hypertrophy and ossification lengthened by day 6.

After exposure to 1 μM BPA, there was no observable effect on cartilage template development; the radius and ulna elongated normally and the metacarpals, carpals and phalanges differentiated (Figure 2.1). There was also no effect on the differentiation of the hypertrophic chondrocytes; COL10A1-mCherry fluorescence remained identical to control. However, by day 6, osteoblast differentiation was affected, as observed by a decrease in COL1A1-YFP fluorescence. Exposure to 10 μM BPA decreased the elongation of the radius and of the ulna, but had no effect on the development of the carpals and the phalanges. However, this concentration of BPA did adversely affect the progression of the limbs into the later stages of endochondral ossification. There was reduction in the differentiation of hypertrophic chondrocytes and

osteoblasts, as seen by a reduction in COL10A1-mCherry and COL1A1-YFP fluorescence, as early as day 3 that was then maintained to day 6.

After exposure to 50 μM BPA, the cartilage template was strongly affected (Figure 2.1). The distal phalanges never developed, and the medial phalanges as well as the carpals were poorly defined. 50 μM BPA also inhibited the differentiation of the hypertrophic chondrocytes and osteoblasts, as seen by a dramatic reduction in COL10A1-mCherry and COL1A1-YFP fluorescence (Figure 2.1). At 100 μM BPA, cartilage template development was arrested and the limb remained primitive throughout the 6 days in culture. COL10A1-mCherry and COL1A1-YFP fluorescence were completely suppressed on day 3, and there was only a slight recovery of COL10A1-mCherry fluorescence by day 6; osteoblast differentiation was never observed.

After exposure to 1 μM BPA, the limb differentiation scores were not significantly different from control (Figure 2.2). The limb morphogenetic differentiation score at 10 μM was lower compared to control but this did not reach statistical significance. Exposure to 50 μM BPA significantly decreased the limb differentiation score. Finally, the limb morphogenetic differentiation score at 100 μM BPA was dramatically reduced compared to control.

Exposure to BPAF had observable effects at lower concentrations than BPA (Figure 2.3). There was no effect on cartilage template development or on the progression of endochondral ossification when the limbs were exposed to 0.1 μM BPAF. There was also no effect on cartilage when the limbs were exposed to 1 μM BPAF. However, at a concentration as low as 1 μM BPAF, there was a reduction in COL10A1-mCherry and COL1A1-YFP fluorescence at day 6, indicating a suppression of chondrocyte hypertrophy and osteoblast differentiation. At 5 μM BPAF, there are fewer cartilage condensations in the phalanges as well as a dramatic decrease in COL10A1-mCherry and COL1A1-YFP fluorescence at days 3 and 6. Similarly to the effects

seen after exposure to 100 μM BPA, exposure to 10 μM BPAF caused strong adverse effects on the cartilage template as well as a dramatic suppression in the progression of endochondral ossification.

There was no change in the limb differentiation scores at the 0.1 and 1 μM BPAF exposure levels (Figure 2.4), which quantitatively supports the fluorescence images. At 5 μM BPAF, we observed a significant decrease in the limb morphogenetic differentiation score due to the fact that there are fewer cartilage condensations in the phalanges. Finally, the limb morphogenetic differentiation score at 10 μM BPAF was dramatically reduced compared to control.

In summary, the cartilage template is adversely affected at higher concentrations of the bisphenols; these affects become apparent and significant after exposure to 50 μM BPA or 5 μM BPAF. Growth is completely halted at the highest concentrations tested: 100 μM BPA and 10 μM BPAF. Starting at 10 μM BPA and 1 μM BPAF, there is dose-dependent decrease in COL10A1-mCherry and COL1A1-YFP fluorescence, indicating a suppression of chondrocyte hypertrophy and osteoblast differentiation.

BPA and BPAF affect the master regulators of endochondral ossification

To determine the effects that BPA and BPAF will have on the expression of *Sox9*, *Runx2*, and *Sp7*, limb buds were cultured in the presence of BPA or BPAF for 3, 6, 24, or 48h. Changes in mRNA levels were determined using quantitative RT-PCR. The expression of *Sox9* in control limbs only increased slightly, whereas *Runx2* and *Sp7* expression was highly upregulated (Figure 2.5). As the limb bud progresses from the cartilage template into chondrocyte maturation and

hypertrophy, and finally into osteoblast differentiation, there is an upregulation of *Runx2*, and to a greater extent, *Sp7* expression.

Sox9 was slightly downregulated following exposure to 1 μM BPA after 24 and 48h. This is consistent with chondrocytes exiting the proliferation stage and entering the hypertrophy stage of endochondral ossification. *Sox9* transcript levels decreased after exposure to 10 or 50 μM BPA at 3h (Figure 2.5). 50 μM BPA upregulated *Sox9* at 6h but this effect was not maintained. 50 μM BPA downregulated *Runx2* expression at all time points, consistent with the observation that COL10A1-mCherry fluorescence was lower at this concentration. None of the other concentrations of BPA tested had any effect on *Runx2* expression. Exposure to 1 μM BPA had no effect on *Sp7* expression. 10 μM BPA caused a consistent upregulation of *Sp7* starting at the 6h time point. This is interesting because 10 μM BPA decreased COL1A1-YFP fluorescence on days 3 and 6 of the cultures. Compared to control, 50 μM BPA slightly but significantly increased *Sp7* transcript levels after 3 and 6h, but they then declined after 24 and 48h. In summary, consistent with the appearance of COL10A1-mCherry and COL1A1-YFP fluorescence at day 3 of the 6-day culture, there was an increase in *Runx2* transcript levels after 24 and 48h, independently of the concentration tested. This increase was much more dramatic in the case of *Sp7*. However, 50 μM BPA suppressed this upregulation of *Runx2* and *Sp7* that was seen in control limbs.

We observed a downregulation of *Sox9* at the 3h time point in limbs exposed to 1 or 5 μM BPAF, while only exposure to 1 μM BPAF caused a downregulation of *Sox9* at 48h (Figure 2.6). Exposure to 0.1 μM BPAF downregulated *Sox9* at the 6h and 24h time points. Exposure to 0.1, 1, and 5 μM BPAF significantly upregulated *Runx2* transcript levels at the 3h, 6h, and 48h time points, respectively. Finally, exposure to 1 μM BPAF downregulated the expression of *Sp7*

at the 48h time point, while exposure to 5 μM BPAF decreased transcript levels at the 24h time point. Both of these observations are consistent with the decrease observed in COL1A1-YFP fluorescence when compared to control.

Discussion

Both BPA and BPAF were detrimental to endochondral ossification, as witnessed in the six-day cultures; BPA's effects on COL10A1-mCherry and COL1A1-YFP fluorescence became apparent at 10 μM of exposure while BPAF's effects were observable at a concentration as low as 1 μM. Cartilage template differentiation was supressed starting at 50 μM BPA and 5 μM BPAF, while osteogenesis was dramatically supressed at the highest concentrations tested. In terms of mRNA expression, 50 μM BPA suppressed the upregulation of *Runx2* and *Sp7* that was seen in control limbs, consistent with the observation that COL10A1-mCherry and COL1A1-YFP fluorescence was lower at this concentration. The effects seen after exposure to BPAF are comparable to those of BPA, but are observable at concentrations that are one order of magnitude lower. For example, 10 μM BPA can be considered equivalent to 1 μM BPAF. Despite this comparison, BPAF had a less pronounced effect on these transcription factors. It is possible that BPAF has an entirely different mechanism of action when compared to BPA, considering that it possesses stronger agonistic activity against ERα and ERβ (Kojima et al. 2019). This is the first study to demonstrate that BPA and BPAF can disrupt endochondral ossification in murine embryonic limb buds. Importantly, the adverse effects of BPAF are occurring at concentrations that are ten times lower than those of BPA, the legacy bisphenol.

Although both BPA and BPAF altered the progression of endochondral ossification, these effects did not always translate into observable changes in the expressions of *Sox9*, *Runx2*, and *Sp7*. For example, 10 μM BPA caused a consistent upregulation of *Sp7* starting at the 6h time point, whereas this exposure decreased COL1A1-YFP fluorescence on days three and six of the cultures. First, it is important to consider that the detrimental effects on ossification in our culture system only become apparent after six days of incubation, whereas the transcript levels are

measured only up to 48 hours. This raises the possibility that the transcript downregulation that we would expect to see will occur after the 48-hour mark. Increases in transcript levels when there is a decrease in fluorescence may also hint at an unknown compensatory mechanism, leading to an upregulation in the transcription factors in order to offset the detrimental effects caused by the bisphenol. In fact, upregulation of the expression of one of the *Runx2* isoforms has been known to occur when the other isoform is knocked out in mice (Xiao et al. 2004). Although this did not lead to completely normal function in these mice, this compensatory mechanism could provide a hint as to why some transcripts are at or near normal control levels even though fluorescence is dramatically reduced. Finally, it is also possible that the effects observed are not mediated by mRNA expression but by the post-translational modifications of the proteins themselves. For example, RUNX2 has phosphorylation sites that can be targeted by different kinases, which can then lead to reduced protein stability due to ubiquitin-proteasome degradation; this negatively regulates osteoblast differentiation (Wee et al. 2002; Kugimiya et al. 2007; Huang et al. 2012). SP7 can also be targeted for ubiquitin-proteasome mediated degradation, causing detrimental effects on osteogenesis (Choi et al. 2015).

The exact mechanism by which BPA and BPAF affect endochondral ossification is currently unknown. However, a few studies have explored the possible ways that BPA might be affecting bone. It is now known that BPA binds strongly to and has antagonistic effects on the estrogen-related receptor gamma (ERRϒ) (Liu et al. 2019). Interestingly, studies show that inhibition of ERRϒ negatively regulates osteoblast differentiation, which may explain why BPA disrupts bone formation (Kim et al. 2015). Other studies show that BPA suppresses the expression of RANK, *Runx2*, *Sp7*, and β-catenin in RAW 264.7 and MC3T3-E1 cells (Hwang et al. 2013). Receptor activator of nuclear factor kappa-B ligand (RANKL) is the ligand that binds

to its receptor, RANK, and regulates bone resorption by activating osteoclast differentiation (Thent et al. 2018). By downregulating RANK, BPA reduces the differentiation of osteoclasts (Hwang et al. 2013). The Wnt/β-catenin signaling pathway is essential for the differentiation of mesenchymal stem cells into osteoblasts (Zuo et al. 2012). By downregulating β-catenin, BPA reduces the differentiation of osteoblasts, thereby inhibiting bone formation (Hwang et al. 2013). As seen in previous studies, BPA downregulates *Sox9*, *Runx2*, and *Sp7*, inhibits the formation of osteoblasts, and is therefore detrimental to ossification. These observations are consistent with the effects described in the present study. However, it will require further investigation to determine whether the effects on the estrogen receptors, RANK, and the Wnt/β-catenin signaling pathway are the mechanisms at play in our model system.

BPAF caused detrimental effects on endochondral ossification, but at concentrations that were ten times lower than those tested in the case of BPA. This raises the concern that the common BPA replacements currently being used in consumer products may not be as harmless as originally thought. This study highlights the importance of research on the toxicity of bisphenols, so that we may eventually identify responsible replacements for BPA.

Figures and Legends

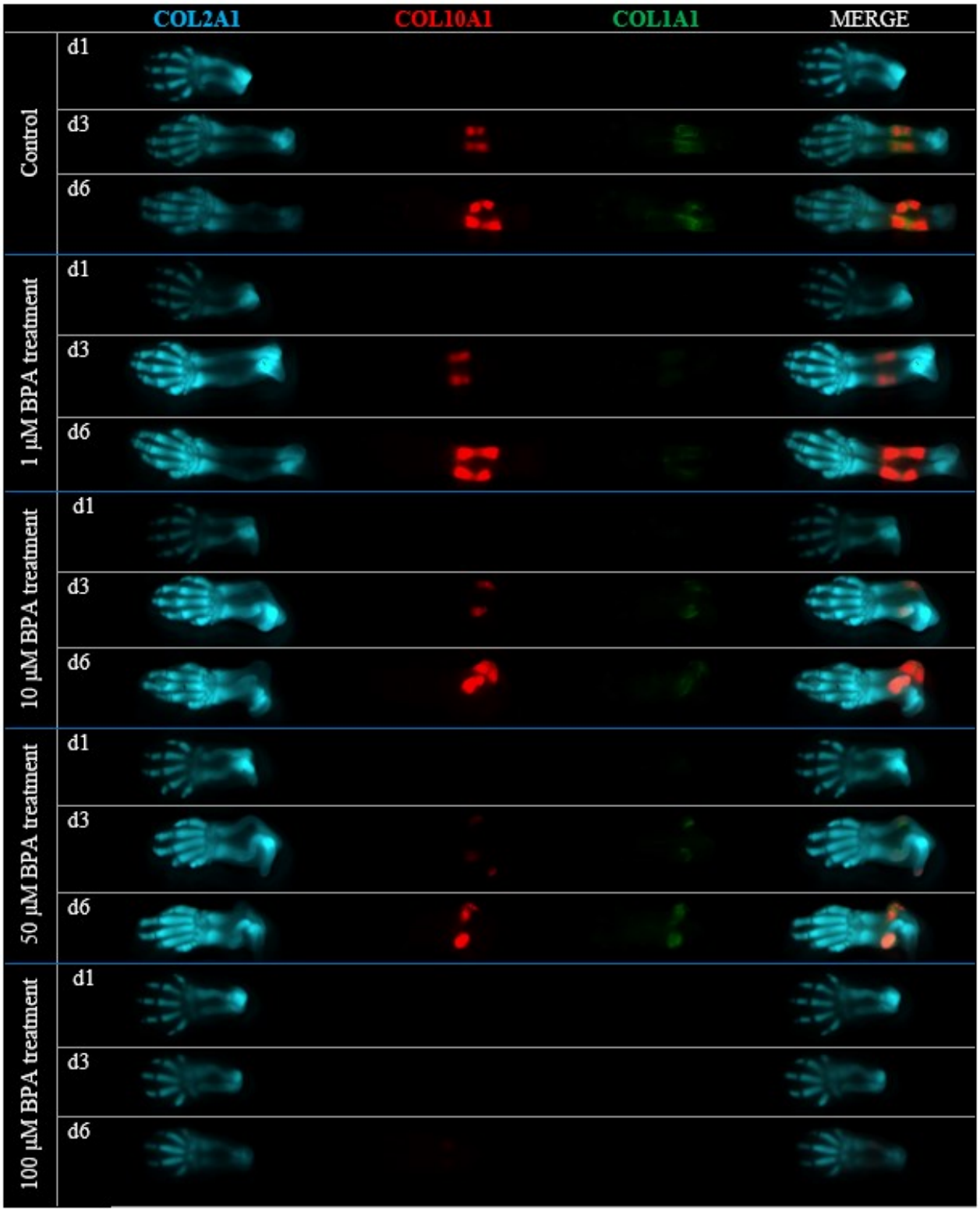


Figure 2.1: Representative fluorescence microscopy images of triple transgenic murine embryonic limb buds. GD 13 forelimbs were cultured in the presence of vehicle (control), 1, 10, 50, or 100 μM BPA, then photographed on days 1, 3, and 6 of the 6-day culture period. COL2A1-eCFP: proliferative chondrocytes (cartilage), COL10A1-mCherry: hypertrophic chondrocytes, COL1A1-eYFP: osteoblasts (osteogenesis); n = 9–14.

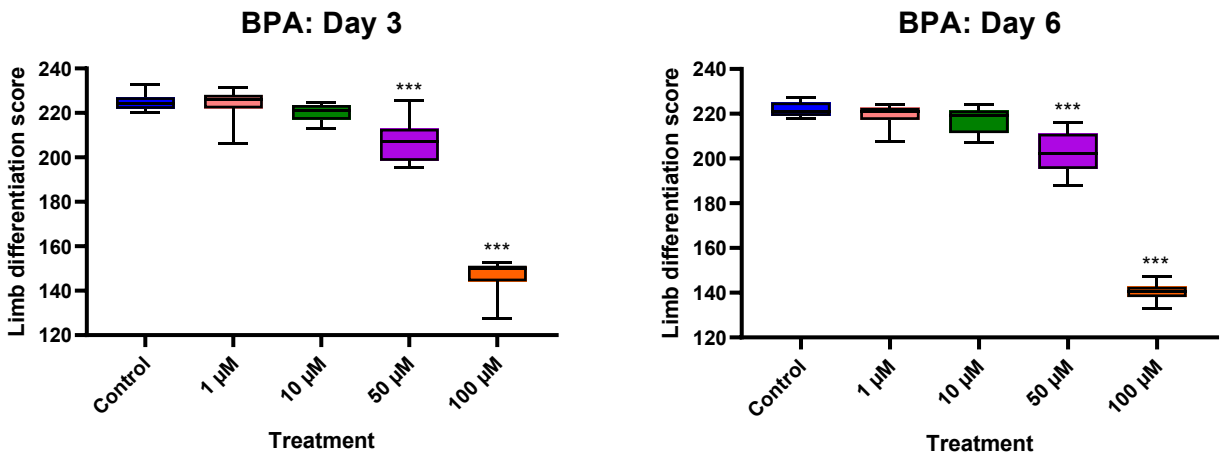


Figure 2.2: Limb differentiation scores are assigned based on the extent of cartilage template development (COL2A1-CFP). Each component of the forelimb receives a score from 0 (unrecognizable) to 30 (excellent differentiation for a cultured limb); a high score indicates good morphological development. *$p < 0.05$, ***$p < 0.001$, n = 9–14 (BPA).

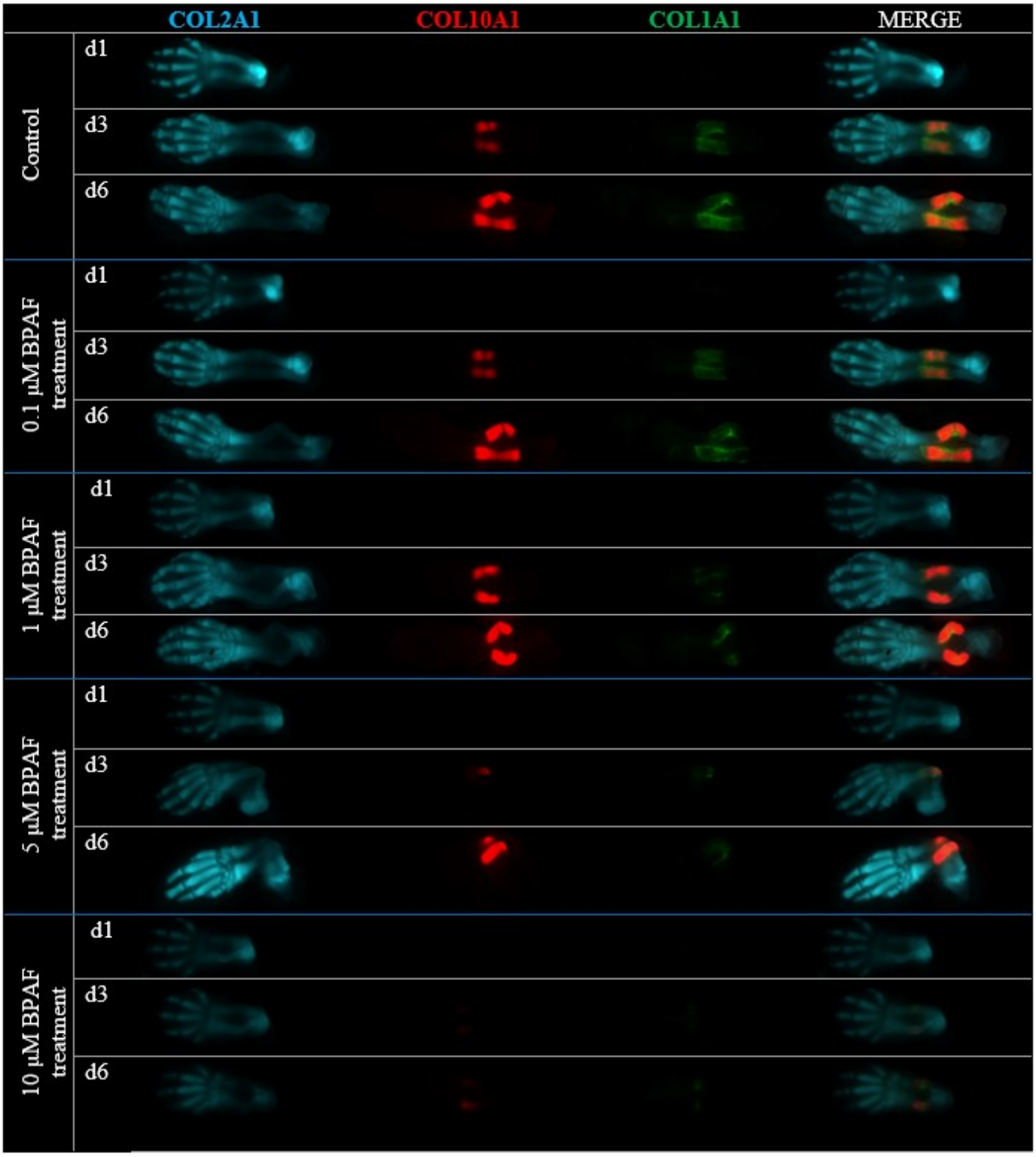

COL2A1
COL10A1
COL1A1
MERGE
Control
0.1 μM BPAF treatment
1 μM BPAF treatment
5 μM BPAF treatment
10 μM BPAF treatment
d1
d3
d6

Figure 2.3: Representative fluorescence microscopy images of triple transgenic murine embryonic limb buds. GD 13 forelimbs were cultured in the presence of vehicle (control), 0.1, 1, 5, or 10 μM BPAF, then photographed on days 1, 3, and 6 of the 6-day culture period. COL2A1-eCFP: proliferative chondrocytes (cartilage), COL10A1-mCherry: hypertrophic chondrocytes, COL1A1-eYFP: osteoblasts (osteogenesis); n = 5–7.

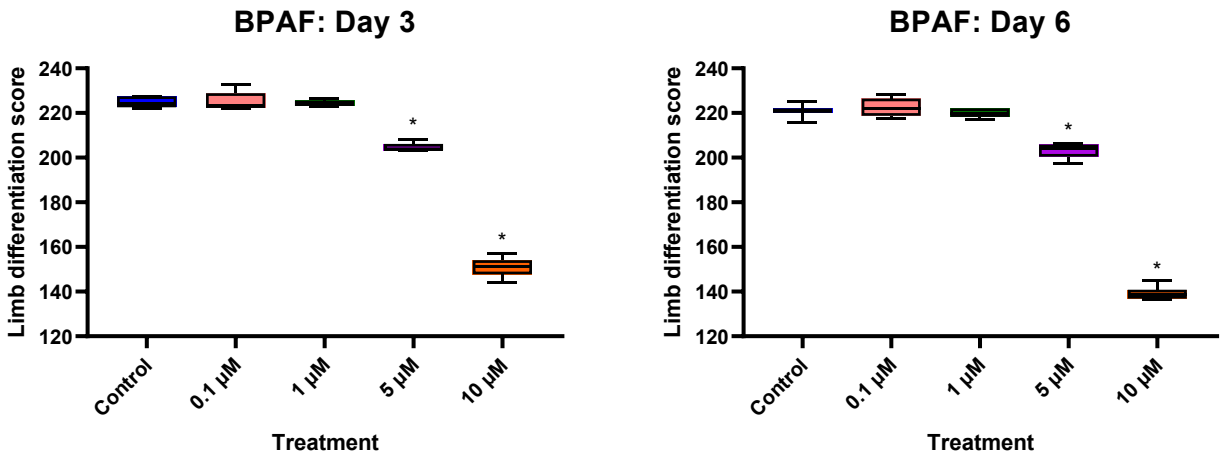
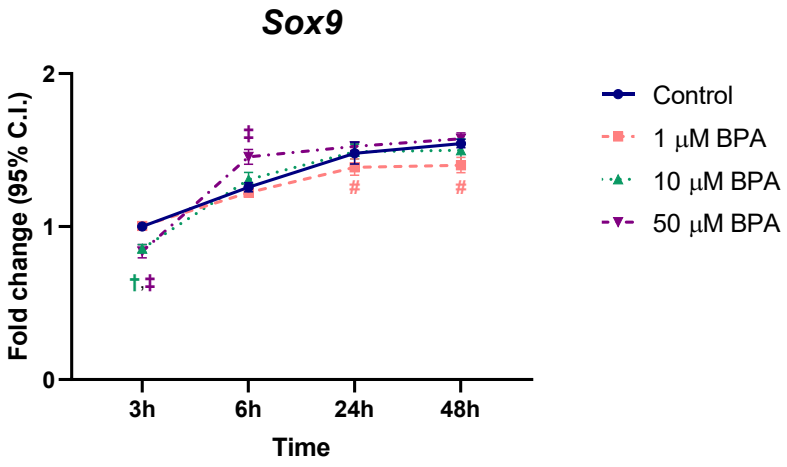
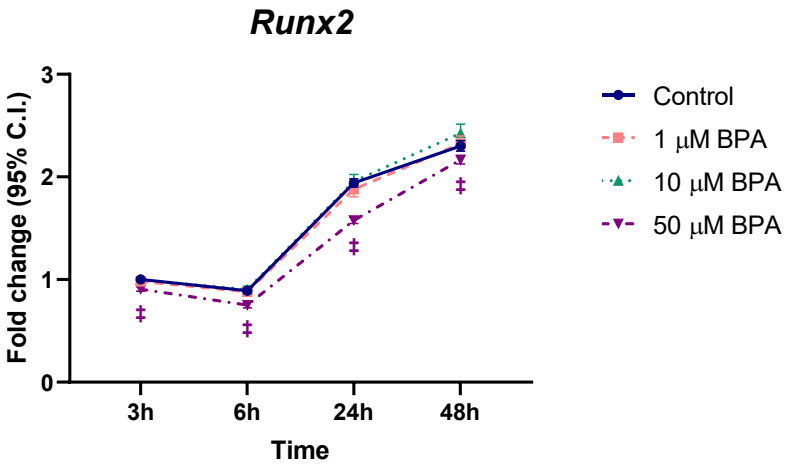


Figure 2.4: Limb differentiation scores are assigned based on the extent of cartilage template development (COL2A1-CFP). Each component of the forelimb receives a score from 0 (unrecognizable) to 30 (excellent differentiation for a cultured limb); a high score indicates good morphological development. *$p < 0.05$, ***$p < 0.001$, n = 5–7 (BPAF).


Sox9
Fold change (95% C.I.)
2
1
0
3h
6h
24h
48h
Time
Control
1 μM BPA
10 μM BPA
50 μM BPA


Runx2
Fold change (95% C.I.)
3
2
1
0
3h
6h
24h
48h
Time
Control
1 μM BPA
10 μM BPA
50 μM BPA

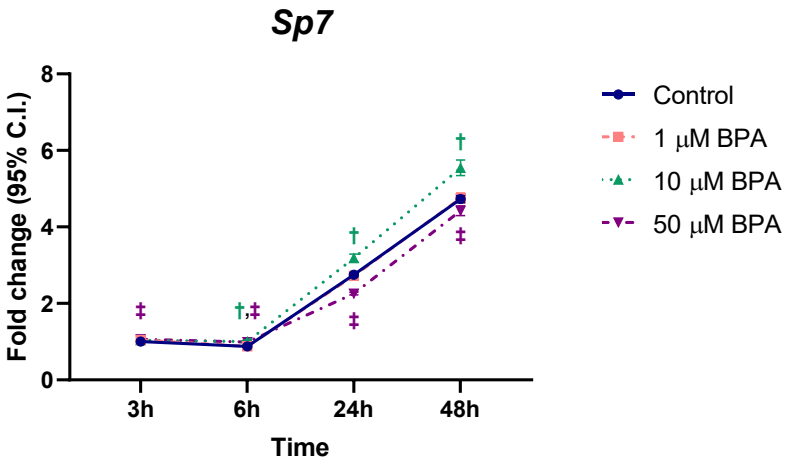
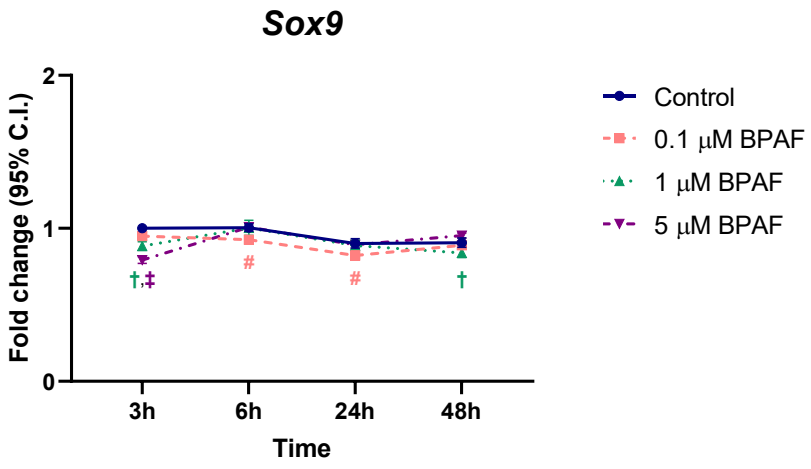
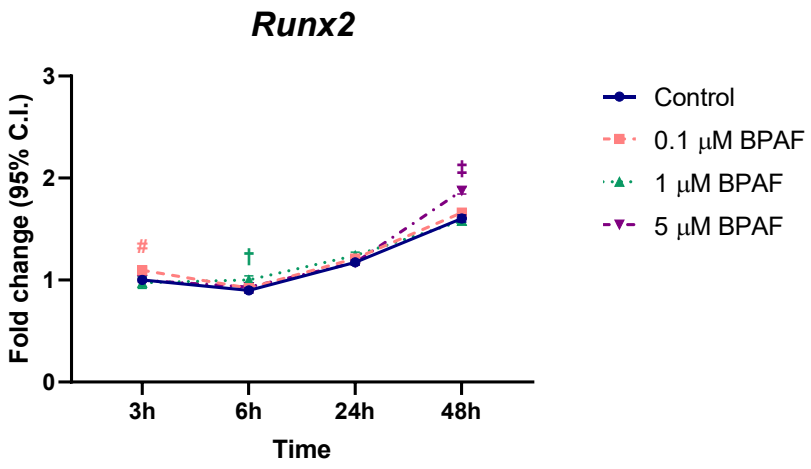

Sp7
Fold change (95% C.I.)
8
6
4
2
0
3h
6h
24h
48h
Time
Control
1 μM BPA
10 μM BPA
50 μM BPA

Figure 2.5: mRNA expression levels measured by quantitative RT-PCR. Limb buds were exposed to vehicle (control), 1, 10, or 50 μM BPA and collected at four different time points. Expression was normalized to *Rpl8* transcript levels. #$p < 0.01$ control versus 1 μM BPA, †$p < 0.01$ control versus 10 μM BPA, ‡$p < 0.01$ control versus 50 μM BPA, n = 5.


Sox9
Fold change (95% C.I.)
0
1
2
3h
6h
24h
48h
Time
Control
0.1 μM BPAF
1 μM BPAF
5 μM BPAF


Runx2
Fold change (95% C.I.)
0
1
2
3
3h
6h
24h
48h
Time
Control
0.1 μM BPAF
1 μM BPAF
5 μM BPAF

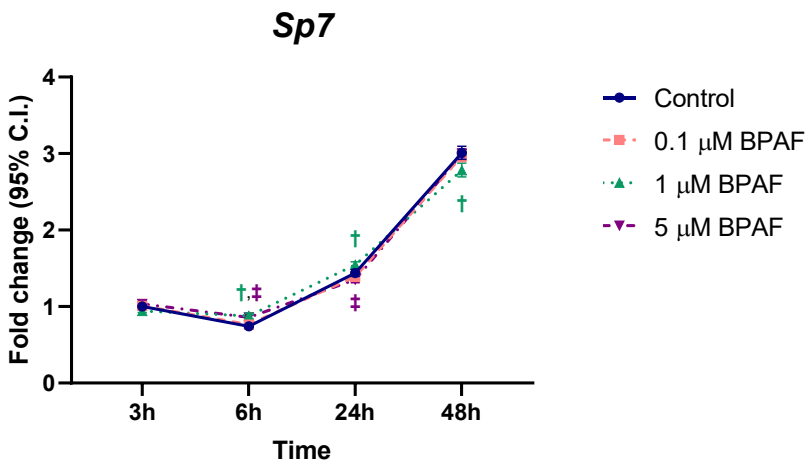

Sp7
Fold change (95% C.I.)
0
1
2
3
4
3h
6h
24h
48h
Time
Control
0.1 μM BPAF
1 μM BPAF
5 μM BPAF

Figure 2.6: mRNA expression levels measured by quantitative RT-PCR. Limb buds were exposed to vehicle (control), 0.1, 1, or 5 μM BPAF and collected at four different time points. Expression was normalized to *Rpl8* transcript levels. #$p < 0.01$ control versus 0.1 μM BPAF, †$p < 0.01$ control versus 1 μM BPAF, ‡$p < 0.01$ control versus 5 μM BPAF, n = 5.

Chapter Three: Discussion and Conclusions

3.1 Summary

The goal of my thesis project was to determine the effects of BPA and BPAF on the process of endochondral ossification as well as on the major transcription factors responsible for this process. Triple transgenic embryonic murine limb buds were exposed to different concentrations of these bisphenols in culture, then incubated for up to six days. Proliferative chondrocytes, hypertrophic chondrocytes, and osteoblasts were then visualized using fluorescence microscopy.

We determined that both BPA and BPAF had detrimental effects on endochondral ossification. BPA reduced the differentiation of hypertrophic chondrocytes and osteoblasts, as seen by a reduction in COL10A1-mCherry and COL1A1-YFP fluorescence starting at 10 μM. Chondrogenesis was supressed beginning at 50 μM, and osteogenesis was almost completely arrested at 100 μM BPA. BPAF had similar adverse effects on the differentiation of hypertrophic chondrocytes and osteoblasts, but these effects were observed after exposure to 1 μM. At concentrations equal to or above 5 μM, chondrogenesis was suppressed, while osteogenesis was almost completely arrested at 10 μM BPAF. Both bisphenols also affected the expression of *Sox9*, *Runx2*, and *Sp7*, the master regulators of endochondral ossification. Exposure to 50 μM BPA downregulated the expression of *Runx2* at all time points and suppressed the overall upregulation of *Runx2* and *Sp7* that was observed in control limbs. Although BPAF also had significant effects on these transcription factors, the extent of the effects was less pronounced despite its toxicity in the six-day cultures.

3.2 BPA's Potential Mechanisms of Action Against Bone

Based on the results obtained in this study, it is not currently possible to determine the exact mechanism by which BPA and BPAF have affected endochondral ossification. However, their pleiotropic effects indicate that their mechanism of action may not be limited to a single pathway or target. In fact, the adverse effects are likely the result of many different pathways combining to create the observed phenotype.

It is now clear that BPA mediates many of its effects by binding to the estrogen receptors ERα and ERβ, due to its similarity to estradiol (Rubin 2011; Nagel and Bromfield 2013; Acconcia et al. 2015). Depending on the specific ER subtype, BPA can act either as an agonist or an antagonist (Acconcia et al. 2015). When it comes to the skeleton, estrogens are crucial for the process of bone growth and bone turnover (Väänänen and Härkönen 1996). Estrogens promote bone formation and decrease bone resorption through direct effects on osteoblasts and osteoclasts, respectively (Khosla et al. 2012). Considering that BPA is able to cause antagonistic effects on the ERs, it is possible that BPA's adverse effects on the limb buds stem from its similarity to estradiol. This is plausible because the estrogen receptors are found on a variety of cells, including chondrocytes, osteoblasts, and osteoclasts (Manolagas et al. 2013). Furthermore, both ERα and ERβ are expressed throughout the fetal development of the mouse as well as in the murine fetus itself (Bondesson et al. 2015).

More recently, it has been determined that BPA binds strongly to and has antagonistic effects on the estrogen-related receptor gamma (ERRɣ) (Liu et al. 2019). Studies show that antagonizing ERRɣ negatively regulates osteoblast differentiation by downregulating osteogenic genes such as *Bmp2* (bone morphogenetic protein) and *Runx2* (Kim et al. 2015). Considering that

BPA is a strong antagonist to ERRϒ, this may be a possible mechanism that explains why BPA is able to disrupt bone formation in our study.

Other studies have found that BPA suppresses the expression of RANK (Hwang et al. 2013). Receptor activator of nuclear factor kappa-B ligand (RANKL) is a ligand that binds to its receptor, RANK, and is crucial for bone resorption through the activation, differentiation, and survival of osteoclasts (Streicher et al. 2017). By downregulating RANK expression, BPA reduces the differentiation of osteoclasts (Hwang et al. 2013). A recent study has shown that bone resorption (through osteoclasts) is coupled to bone formation (through osteoblasts) (Ikebuchi et al. 2018). The accepted signalling pathway, as mentioned above, involved RANKL being released by osteoblasts and osteocytes, where it can then bind to the RANK receptor on hematopoietic stem cells to activate their differentiation into osteoclasts (Ikebuchi et al. 2018). More recently it was discovered that osteoclasts themselves can release vesicular RANK, which can then bind to RANKL found on the surface of osteoblasts; this then activates RUNX2, thereby promoting bone formation (Ikebuchi et al. 2018). By suppressing the differentiation of osteoclasts, BPA can indirectly supress bone formation through this coupling process.

The Wnt/β-catenin pathway, which is essential for the differentiation of mesenchymal stem cells into osteoblasts, has also been known to be affected by BPA (Zuo et al. 2012; Hwang et al. 2013). β-catenin is essential for osteoblastogenesis, and any interruption in Wnt/β-catenin signalling will affect skeletal development (Zuo et al. 2012). High concentrations of BPA downregulate the expression of β-catenin, which reduces the differentiation of osteoblasts, thereby inhibiting bone formation (Hwang et al. 2013). In the same study, BPA caused an upregulation of caspases 3, 8, and 9, which stimulate the apoptosis of osteoclasts and osteoblasts (Hwang et al. 2013).

As mentioned previously, Fan et al. exposed adult rare minnows (*Gobiocypris rarus*) to BPA, following which their offspring were assessed for bone development and the expression of ossification related genes (Fan et al. 2018). It was found that maternal BPA exposure delayed the endochondral ossification of craniofacial bone, reduced bone size, and downregulated the expression of *Sox9*, *Runx2*, and *Col2a1* (Fan et al. 2018). These results are all consistent with those obtained in the present study.

3.3 BPAF's Estrogenicity as a "Safer Alternative"

Both BPA and BPAF are known to bind to the estrogen receptors. Recently, a study has found that BPAF had the strongest agonistic activity against ERα and ERβ, compared to BPA, BPAP, BPB, BPE, BPF, BPP, BPS, and BPZ (Kojima et al. 2019). This might explain why BPAF is a more potent endocrine disruptor, and why its effects on endochondral ossification were more severe. Furthermore, BPAF had a higher binding affinity to GPR30 compared to BPA, which could also lead to stronger estrogenic effects (Cao et al. 2017). By binding more strongly, BPAF can more effectively compete with estradiol and lead to the activation of different signalling pathways (Cao et al. 2017)

3.4 Future Directions

The findings in this thesis were the first demonstration of the effects of BPA and BPAF on endochondral ossification in embryonic murine limb buds, and on the transcription factors involved in this process. However, more research will be required to elucidate the exact mechanism by which bisphenols cause adverse effects in bone. Performing RNAseq on limb bud tissue samples that have been exposed to bisphenols will be critical to this understanding. RNA sequencing is a high-throughput sequencing technology that allows us to analyze the

transcriptome by determining the levels of transcripts (and their isoforms) that are present at a specific time or under a specific physiological condition (Wang et al. 2009).

We have previously shown that many nuclear receptors are expressed in limb buds at the developmental stage that we are interested in; these include the thyroid hormone receptor alpha (TR-alpha), the glucocorticoid receptor (GR), the peroxisome proliferator-activated receptors (PPARs), the estrogen receptors (ERs), and the aryl hydrocarbon receptor (AHR) (Yan and Hales 2020) [Gene Expression Omnibus (GSE155435)]. These receptors, and many others, could be the downstream targets through which the bisphenols are acting to cause their detrimental effects. Based on the relative toxicity of BPA and BPAF at different time points and concentrations, which are outlined in this thesis, RNAseq will be performed for three time points (3h, 24h, and 48h), three treatment groups per bisphenol (control, 10 μM, and 50 μM for BPA and control, 1 μM, and 5 μM for BPAF), and an N of 5. Therefore, through RNAseq data analysis, we aim to determine the genetic mechanisms of bisphenols.

In this thesis, we have established the effects of two bisphenols, the legacy BPA and one of the most widely used replacements, BPAF. However, the number of bisphenols that are on the market is ever growing; in fact, there are now over a dozen different bisphenols that have been synthesized, and concerns over their toxicity are beginning to emerge. BPA analogues may not be safer alternatives, as they also exhibit endocrine disrupting activity (Moon 2019). A new study could utilize the *ex vivo* limb bud culture model to assess the effects of BPS and BPF, as these are some of the most commonly used substitutes (Chen et al. 2016). Eventually, it would be interesting to determine the effects of all BPA analogues, including but not limited to BPAP, BPB, BPE, BPM, BP-TMC, and BPZ.

Lastly, it is important to consider that the present study determine the effects of individual bisphenols on the process of ossification. In reality, humans are exposed to variety of bisphenols in their day-to-day life and many different bisphenols are detected in urine (Lehmler et al. 2018; Wang et al. 2020). Just like with BFRs and OPEs, studying the effects of an environmentally relevant mixture of bisphenols is critical to our understanding of real world exposure. This will also allow us to assess whether bisphenol mixtures are more detrimental to health than the individual BPs.

3.5 Significance of Findings and Final Conclusions

To our knowledge, this is the first report of the effects of BPA and BPAF on endochondral ossification in the embryonic murine limb bud, as well as on the effects of these two bisphenols on the master regulators of endochondral ossification, *Sox9*, *Runx2*, and *Sp7*.

Research on bisphenols is essential as their use keeps increasing and research on their toxic effects becomes clearer. In fact, BPA continues to be detected in human urine years after its ban from polycarbonate baby bottles and its phase out in consumer goods (Lehmler et al. 2018; Wang et al. 2020). BPAF, a now commonly used BPA substitute, may be just as toxic as its predecessor. In conclusion, exposure to BPA and BPAF leads to adverse effects on bone by delaying endochondral ossification and causing detrimental effects on the differentiation of the carpals and the phalanges in the limb.

1) Exposure to 10 μM BPA and 1 μM BPAF reduced the differentiation of hypertrophic chondrocytes and osteoblasts, as seen by a reduction in COL10A1-mCherry and COL1A1-YFP fluorescence. At 50 and 100 μM BPA, and at 5 and 10 μM BPAF, cartilage template development was arrested, leading to significantly decreased limb

differentiation scores. COL10A1-mCherry and COL1A1-YFP fluorescence were dramatically suppressed at these high concentrations.

2) The expression of *Sox9* was altered after exposure to BPA. Exposure to 50 μM BPA downregulated the expression of *Runx2* at all time points and suppressed the upregulation of *Runx2* and *Sp7* that was observed in control limbs. Exposure to 5 μM BPAF downregulated the expression of *Sp7* at the 24h time point while exposure to 1 μM BPAF decreased *Sp7* transcript levels at 48h. In general, BPAF had a less pronounced effect on the three transcription factors.

Thus, in the context of endochondral ossification, our data suggest that BPAF may be more detrimental than BPA. More research will be required to identify responsible replacements for BPA.

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