ARYL HYDROCARBON RECEPTOR ACTIVATION AND ITS ROLE IN SKIN PHYSIOLOGY AND TOXICOLOGY

Jyoti Bhuju

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ARYL HYDROCARBON RECEPTOR ACTIVATION AND ITS ROLE IN SKIN PHYSIOLOGY AND TOXICOLOGY

by

Jyoti Bhuju

A Dissertation
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Major: Biology

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ABSTRACT

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is a persistent organic pollutant that causes toxicity by activating the aryl hydrocarbon receptor (AHR). Activation of the AHR has been linked to an increased risk of developing skin diseases including chloracne and atopic dermatitis (AD). We investigated the effects of perinatal exposure to TCDD on the development of the epidermal barrier and the susceptibility to skin diseases in C57BL/6J mice. Mice were exposed in utero to 5 µg/ kg bw TCDD on embryonic day 12 and the cutaneous effects were studied from postnatal day 1 (P1) through adult life. TCDD-exposed pups exhibited diffuse epidermal hyperplasia at birth; however, this effect did not persist. TCDD-exposed animals did not develop AD-related pathologies including skin lesions, increased serum IgE and Th2 immune responses between birth to P135. At P21, TCDD-exposed skin exhibited sebaceous gland hypoplasia, reminiscent of chloracne, that was reversible by P35. Analysis of the skin microbiome identified a change in bacterial community structure between control and treated mice at P21, that was no longer evident by P35. Similarly, CYP1A1 and CYP1B1 RNA and protein expression increased transiently in TCDD-exposed skin at P13-21. Both CYP1A1 and CYP1B1 protein expression co-localized with leucine-rich repeats and immunoglobulin-like domains protein 1 (LRIG1)-expressing progenitor cells at the infundibulum. CYP1B1 also co-localized with leucine rich repeat containing G protein-coupled receptor 6 (LGR6)-expressing progenitor cells at the junctional zone. Parallel studies with human keratinocytes showed that activation of the AHR with TCDD increased terminal differentiation. Surprisingly, knockdown of the AHR resulted in increased keratinocyte differentiation. We determined that levels of cyclin-dependent kinase inhibitor 1B
(CDKN1B) were increased in these AHR knockdown cells. Further studies are required to explore the relationship between AHR, CDKN1B, cell cycle, and cell differentiation.

In conclusion, perinatal exposure of mice to TCDD resulted in a chloracne-like phenotype in the skin, without evidence of atopy. The identity of specific skin cells affected by TCDD will aid ongoing research into the mechanisms of chloracne which include sebaceous gland dysmorphogenesis and epidermal hyperplasia.
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<td>AD</td>
<td>atopic dermatitis</td>
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<tr>
<td>AHR</td>
<td>aryl hydrocarbon receptor</td>
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<tr>
<td>AHR-CA</td>
<td>constitutively active AHR</td>
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<td>AHRR</td>
<td>AHR repressor</td>
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<tr>
<td>ARNT</td>
<td>AHR nuclear translocator</td>
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<tr>
<td>BaP</td>
<td>benzo(a)pyrene</td>
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<tr>
<td>bHLH</td>
<td>basic helix-loop-helix</td>
</tr>
<tr>
<td>Blimp1</td>
<td>B lymphocyte-induced maturation protein1</td>
</tr>
<tr>
<td>BNF</td>
<td>β-napthoflavone</td>
</tr>
<tr>
<td>CDKN1B</td>
<td>cyclin-dependent kinase inhibitor 1B</td>
</tr>
<tr>
<td>CYP</td>
<td>cytochrome P450</td>
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<tr>
<td>DLC</td>
<td>dioxin-like halogenated compounds</td>
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<tr>
<td>DMBA</td>
<td>7,12-dimethyl-benz(a)anthracene</td>
</tr>
<tr>
<td>DOHaD</td>
<td>developmental origins of health and disease</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme linked immunosorbent assay</td>
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<tr>
<td>EPB</td>
<td>epidermal permeability barrier</td>
</tr>
<tr>
<td>FICZ</td>
<td>6-formylindolo [3,2-b] carbazole</td>
</tr>
<tr>
<td>FLG</td>
<td>filaggrin</td>
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<tr>
<td>HAHs</td>
<td>halogenated aromatic hydrocarbons</td>
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<tr>
<td>Hprt</td>
<td>hypoxanthine-guanine phosphoribosyltransferase</td>
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<tr>
<td>HRNR</td>
<td>hornerin</td>
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<tr>
<td>IgE</td>
<td>immunoglobulin E</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IVL</td>
<td>involucrin</td>
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<tr>
<td>LGR6</td>
<td>leucine rich repeat containing G protein-coupled receptor 6</td>
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<td>LRIG1</td>
<td>leucine-rich repeats and immunoglobulin-like domains protein 1</td>
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<td>NF-κB</td>
<td>nuclear factor-kappa beta</td>
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<tr>
<td>NLS</td>
<td>nuclear localization signal</td>
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<tr>
<td>NRF2</td>
<td>nuclear factor-erythroid 2-related factor-2</td>
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<td>OTU</td>
<td>operational taxonomic unit</td>
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<td>PAHs</td>
<td>polycyclic aromatic hydrocarbons</td>
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<td>PAS</td>
<td>period- AHR nuclear translocator (ARNT)- single-minded</td>
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<tr>
<td>PCB</td>
<td>polychlorinated biphenyls</td>
</tr>
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<td>PCDD</td>
<td>polychlorinated dibenzo-p-dioxins</td>
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<tr>
<td>PCDF</td>
<td>polychlorinated dibenzo-p-furans</td>
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<tr>
<td>POP</td>
<td>persistent organic pollutant</td>
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<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
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<td>SC</td>
<td>stratum corneum</td>
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<tr>
<td>TCDD</td>
<td>2,3,7,8-tetrachlorodibenzo-p-dioxin</td>
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<tr>
<td>TERT</td>
<td>telomerase reverse transcriptase</td>
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<tr>
<td>TEWL</td>
<td>trans-epidermal water loss</td>
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<tr>
<td>TGM1</td>
<td>transglutaminase1</td>
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<tr>
<td>XRE</td>
<td>xenobiotic responsive elements</td>
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Aryl Hydrocarbon Receptor (AHR)

The AHR is a ligand activated transcription factor of the basic helix-loop-helix / Period- AHR nuclear translocator (ARNT)- single-minded (Sim) [bHLH/ PAS] family (Hankinson, 1995). All bHLH/ PAS family members function by forming hetero- or homodimer protein complexes. The AHR dimerizes with ARNT to form a heterodimer that interacts with downstream genes. Both the AHR and ARNT consist of an N-terminal bHLH domain, an internal PAS domain, and a C-terminal transcription activation domain (Figure 1). The PAS domain is a highly conserved region consisting of two adjacent repeats of about 130 amino acids, PAS-A and PAS-B. The PAS-A domain along with bHLH domain is involved in AHR-ARNT dimerization. The PAS-B domain of the AHR is involved in ligand binding (Gu et al., 2000).

**Figure 1.** Schematic of the functional domains of the AHR protein. The N-terminal basic helix-loop-helix region is involved in DNA binding and protein-protein interactions. The two PAS domains, PAS-A and PAS-B are required for protein-protein interactions and for ligand binding. The transcriptional activation domain is located in the C-terminal residues of the protein.

The AHR is highly conserved through evolution. It is expressed in multiple tissues and is particularly known for its function as a sensor of xenobiotics. Activation of the AHR results in induction of xenobiotic metabolizing enzymes such as the cytochrome
P450s (CYPs) (Nguyen & Bradfield, 2008). Its discovery followed soon after the finding that the extent of CYPs induction in response to xenobiotics was different in different strains of mice (Poland et al., 1976). 2,3,7,8-tetrachlorodibenzo-\(p\)-dioxin (TCDD), an environmental contaminant is one of the most extensively studied AHR ligand. The extent of CYP1A1 induction in response to TCDD was found to be less in DBA/2 strain as compared to C57BL/6 and this difference was attributed to different Ahr alleles that these mice express. C57BL/6 strains express the more responsive Ahrb allele with 10-fold higher ligand binding affinity as compared to Ahrd allele in DBA/2 (Nebert et al., 1975; Poland et al., 1994). In models of genetic knockout of the receptor, the AHR-null mice exhibit many phenotypic abnormalities such as hepatic defects, decreased body weight and a defective immune system indicating a developmental role for the AHR (P. Fernandez-Salguero et al., 1995; Gonzalez & Fernandez-Salguero, 1998; Schmidt et al., 1996). And so, initially identified as regulator of xenobiotic metabolism, several studies now suggest regulatory roles of the AHR in physiological processes, development, and homeostasis (Abel & Haarmann-Stemmann, 2010; Puga et al., 2005).

In its basal state, the AHR is primarily located in the cytoplasm in a protein complex consisting of two molecules of the 90-kDa heat shock protein (hsp90), the co-chaperone p23, tyrosine kinase c-SRC and the immunophilin-like XAP2 protein, also known as AHR interacting protein (AIP) (Denis et al., 1988; Dong et al., 2011; Ma & Whitlock, 1997; Perdew, 1988). Once bound by a ligand, the AHR-protein complex undergoes structural reorganization exposing a nuclear localization signal (NLS). The AHR is then translocated into the nucleus where it dissociates from the protein complex and dimerizes with ARNT. The AHR-ARNT dimer binds to xenobiotic responsive
elements (XREs) in the promoter regions of responsive genes to regulate their transcription (Gonzalez & Fernandez-Salguero, 1998; Hankinson, 1995). Subsequently, upon ligand binding, the AHR undergoes ubiquitination which leads to its degradation by 26S proteasome (Ma & Baldwin, 2000). Additionally, AHR activation also induces the transcription of the AHR repressor (AHRR) that negatively regulates AHR expression by competing with the AHR itself to form heterodimers with ARNT (Mimura et al., 1999). The AHRR-ARNT heterodimer is inactive, but the unavailability of ARNT for the AHR-ARNT complex blocks expression of AHR-responsive target genes.

Besides the canonical AHR-ARNT heterodimer complex (Figure 2), the AHR has also been found to interact and form complexes with other regulatory proteins. The AHR associates with nuclear factor-kappa beta (NF-κB) to activate transcription of c-myc (D. W. Kim et al., 2000) and interleukin-8 (Vogel et al., 2007). Similarly, the AHR has been shown to associate with Kruppel-like factor 6 (Klf6) and regulate gene expression of targets such as plasminogen activator inhibitor-1 (PAI-1) and the cyclin-dependent kinase inhibitor, p21Cip1 (Huang & Elferink, 2012; Jackson et al., 2014). These studies show non-canonical AHR activity that is independent of the ARNT and based on non-XRE, novel DNA motifs.

Besides being a transcription factor, AHR has also been identified as an E3 ubiquitin ligase that mediates ubiquitination of target proteins for proteasomal degradation (Ohtake et al., 2007). This non-transcriptional role of AHR is facilitated by the association of AHR with an E3 ubiquitin ligase, cullin 4B, to form a complex, CUL4B<sup>AhR</sup>. Some of the most prominent targets of this complex include the steroid hormone receptors, including the estrogen and androgen receptor (Ohtake et al., 2009).
Moreover, this complex has been found to promote ubiquitination leading to proteasomal degradation of β-catenin in colon tumor cell line (Kawajiri et al., 2009).

**Figure 2.** Canonical and non-canonical AHR signaling pathways. In a canonical pathway, ligand binding of the AHR results in nuclear localization and dimerization with ARNT. Together the proteins bind to regulatory regions (XRE) on target genes to regulate transcription. In a non-canonical pathway, the AHR is activated by the ligands and translocated to the nucleus, but it associates with novel partners such as NF-κB to bind to non-XRE motifs on target DNA. In a non-transcriptional role, the AHR functions as an E3 ubiquitin ligase marking proteins for proteasomal degradation.

**AHR ligands**

In general, ligands of the AHR are classified into two groups- i) xenobiotic compounds that are environmental pollutants and products of industrial processes, and ii) naturally occurring dietary compounds. Ligands of the first group include the most extensively studied polycyclic aromatic hydrocarbons (PAHs) and halogenated aromatic hydrocarbons (HAHs) (Abel & Haarmann-Stemmann, 2010). HAHs include polychlorinated biphenyls (PCBs), dibenzo-p-furans (PCDF) and dibenzo-p-dioxins (PCDD) that are high-affinity AHR ligands with binding affinities in the pM to nM
range. Comparatively, PAHs including benzo(a)pyrene (BaP), 3-methylcholanthrene (3-MC) and 7,12-dimethyl-benz(a)anthracene (DMBA) have lower binding affinities in the nM to µM range (Denison & Nagy, 2003). Moreover, HAHs are resistant to metabolism allowing for a persistent AHR activation and toxicity. PAHs on the other hand, are labile to metabolism and fail to produce AHR-dependent toxicity (Denison et al., 2011).

TCDD, commonly referred to as dioxin, is a high affinity AHR ligand of the HAH family (Schecter et al., 2006). Dioxins are produced during various industrial processes including manufacturing of herbicides and pesticides, incineration and bleaching of pulp and papers. Being extremely resistant to degradation, these are persistent in the environment for long periods of time with bioaccumulation in food sources like meat, fish and dairy. The basic chemical structure of all dioxins consists of two benzene rings connected by two oxygen atoms and substituted with four to eight chlorine atoms. There are 75 congeners. TCDD contains chlorine atoms at positions 2, 3, 7, and 8 on the benzene rings (Figure 3) (Schecter et al., 2006).

![Chemical structure of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD).](image)

**Figure 3.** Chemical structure of 2,3,7,8–tetrachlorodibenzo-p-dioxin (TCDD).

Toxicity of TCDD and similar HAHs is mediated by the AHR. Studies with genetically modified mice that are null for the receptor or carrying a mutation in the nuclear localization sequence of the receptor failed to develop responses in absence of nuclear localization and binding of AHR to regulatory elements (Bunger et al., 2003; P.
M. Fernandez-Salgueiro et al., 1996). While a number of structurally diverse ligands can bind to and activate the AHR, each interaction is unique and elicit diverse downstream effects (Denison et al., 2011). Studies have shown different ligands interact with different residues in the ligand binding domain of the AHR resulting in differences in the conformation of AHR. This ligand-dependent changes in AHR conformation then alters the downstream signaling and responses (Denison et al., 2011; Soshilov & Denison, 2014).

TCDD-mediated AHR activation induces a number of xenobiotic metabolizing enzymes. These include Phase I metabolizing enzymes of the cytochrome P450 family, CYP1A1, CYP1A2, CYP1B1, and Phase II enzymes such as aldehyde dehydrogenase, UDP glucuronosyltransferase, NAD(P)H: menadione oxidoreductase, and glutathione transferase (Nebert et al., 2000). Most xenobiotics undergo two phases of transformation to be eliminated from the body. During phase I, xenobiotics undergo oxidation, reduction, hydrolysis, and hydration reactions to form intermediate metabolites that are further converted into hydrophilic substrates in phase II reaction, making products that are easily excreted in urine or bile (Ramadoss et al., 2005; Xu et al., 2005). Although TCDD is the most potent inducer of CYP1A1, the presence of the chlorine atoms on TCDD inhibits its oxidation. Therefore, TCDD is not metabolized, contributing to its delayed clearance from the body and its deposition in organs like liver and adipose tissue (Sorg et al., 2009). Studies report an approximate half-life of TCDD in rodents to be about 11 days (Birnbaum, 1986; Gasiewicz et al., 1983). In humans, the half-life of TCDD has been estimated to be 7–11 years, the exact time being dependent on the dose and body composition of an individual (Kerger et al., 2006; Pirkle et al., 1989).
TCDD induces a spectrum of toxic effects involving multiple systems across multiple species. In animals, toxic doses of TCDD results in wasting syndrome where they can lose as much as half of the total body weight (Birnbaum, 1994). Immunotoxicity has been observed in multiple species, where even low doses of TCDD results in involution of the thymus gland (Poland and Knustson, 1982). Other adverse effects of TCDD include hepatomegaly with altered liver functions, cardiotoxicity, reproductive toxicity, teratogenesis, and carcinogenesis (White & Birnbaum, 2009). However, the extent of these TCDD effects are species and tissue dependent. The LD50 of TCDD is approximately 1 μg/ kg body weight for guinea pigs, 22 μg/ kg for rats, 114 μg/ kg for C57BL/6J mice, but about 1000 μg/ kg for hamsters (Poland & Knutson, 1982; Schecter et al., 2006; Vos et al., 1974). In humans, the most significant indicator of TCDD toxicity is the skin condition called chloracne, which is characterized by hyperkeratinization of the epidermis and metaplastic changes of sebocytes (Panteleyev & Bickers, 2006; Suskind, 1985). Chloracne lesions consist of hyperplasia and hyperkeratosis of the interfollicular epidermis, hyperkeratosis of the hair follicle, especially at the infundibulum, and squamous metaplasia of the sebaceous glands which form keratinaceous comedones and cysts.

Naturally occurring AHR-ligands include substances of dietary plant origin such as flavonoids, polyphenols, alkaloids, and indole derivates. These are found ubiquitously in fruits and vegetables. Flavonoids, including flavones, flavanols, flavanones, and isoflavones, form the largest group of dietary AHR ligands (Nguyen & Bradfield, 2008). As compared to the synthetic ligands, naturally occurring compounds are weak modulators of the AHR with majority of flavonoids being AHR antagonists/ inhibitors.
Agonists include compounds such as quercetin, diosmin, tangeritin, and tamarixetin. Besides the plant derived compounds, natural ligands include the endogenous compounds that are found in the body. Many tryptophan metabolites such as ITE [2-(1’H-indole- 3’-carbonyl)-thiazole-4-carboxylic acid methyl ester], kynurenines or FICZ [6-formylindolo [3,2-b] carbazole] fall into this group (Denison & Nagy, 2003; Nguyen & Bradfield, 2008). ITE is an AHR agonist isolated from porcine lung tissue and its ability to activate AHR suggests a physiological role (Song et al., 2002). Kynurenines are another potent AHR ligands that are generated from the breakdown of tryptophan via the kynurenine pathway. FICZ on the other hand is ultraviolet photoproduct of tryptophan. FICZ has high affinity to AHR similar to dioxin but is rapidly metabolized. Additionally, two tryptophan metabolites, indigo and indirubin, were isolated from human urine and shown to activate the AHR in a yeast system (Adachi et al., 2001). However, the ability of these naturally occurring compounds to activate the AHR in vivo remains to be established.

**AHR ligands in skin diseases**

Skin is the largest organ of the body and forms an interface between the external and internal environment. The outermost layer, epidermis is a stratified squamous epithelial layer that is in the process of continuous renewal. It protects the body against external pathogens, physical and chemical insults. It also serves as a permeability barrier that prevents excessive loss of water from the body. The epidermis is maintained by keratinocytes that proliferate and differentiate to form different layers: basal, spinosum, granulosum, and corneum (Figure 4). The process of terminal differentiation begins when basal cells withdraw from the cell cycle and lose adhesion from basement membrane to
initiate differentiation (J. Segre, 2003). As these cells travel outward in the layers, they undergo morphological and biochemical changes. Approaching the outermost layer, they lose their organelles such as nuclei and mitochondria and these cornified cells are enveloped in lipids to form the epidermal permeability barrier (Proksch et al., 2008). In addition to these layers of dead corneocytes and surrounding lipids, that are traditionally described as “bricks and mortar” structures, the intercellular tight junctions have also been illustrated to contribute to the epidermal barrier (Furuse et al., 2002).

Figure 4. Schematic illustration of epidermal permeability barrier. Adapted from (J. Segre, 2003).

Many skin diseases such as atopic dermatitis (AD), psoriasis, and ichthyosis vulgaris are associated with disrupted differentiation leading to abnormalities in the epidermal barrier function (Proksch et al., 2008; J. A. Segre, 2006). Defective barrier allows the penetration of environmental pollutants/allergens that initiate inflammation. The AHR is highly expressed in lesional skin of patients with skin conditions like AD
and psoriasis (H. O. Kim et al., 2014). Additionally, ligand activation of the AHR increases the expression of pro-inflammatory and inflammatory cytokines that aggravate allergic diseases (Ito et al., 2002; H. O. Kim et al., 2014; Kimata, 2003). Studies have shown that TCDD aggravates atopic lesions in mice that have been sensitized with allergen, by increasing IFN-γ production and infiltration of mast cells (Ito et al., 2008). Recently, Hidaka et al. found that cutaneous application of an AHR ligand, DMBA, induces AD-like conditions in mice (Hidaka et al., 2017). Further, it was shown that FICZ, an endogenous ligand, fails to produce similar effects suggesting that probably a prolonged activation of AHR (as induced by DMBA) is required for the development of disease.

In contrast, several studies report AHR as a potential therapeutic target for skin diseases. Topical application of coal tar is one of the oldest therapies for AD. In lesional AD skin, coal tar was shown to activate the AHR which resulted in an increase in epidermal differentiation and restoration of epidermal differentiation proteins like filaggrin, loricrin, hornerin, and involucrin (van den Bogaard et al., 2013). In the same study, coal tar alleviated IL-4 induced AD pathology in a 3D skin equivalent model. AHR activation by ligands such as soybean tar, FICZ, phytoextracts of *Artemisia princep* upregulates Ovo-like protein 1 (OVOL1) to increase expression of filaggrin, loricrin, and involucrin (Hashimoto-Hachiya et al., 2018; Hirano et al., 2017; Tsuji et al., 2017). Similarly, endogenous AHR ligands have also demonstrated therapeutic potential for skin conditions. In one study, Yu et al. reported the protective effects of a tryptophan metabolite in the skin. This study showed that an indole derivative of tryptophan alleviates MC903-induced AD-like condition in mice and this effect was dependent on
the AHR (Yu et al., 2018). Most importantly, an AHR ligand, tapinarof is currently in Phase III trials of its efficacy as a topical treatment of AD (Bissonnette et al., 2018). This would be a first in class therapeutic agent based on the AHR.

Clearly, the AHR plays a role in inflammatory skin conditions. However, the underlying mechanisms that determine the causative role and therapeutic potential of the AHR are not fully understood. The discrepancies in the outcome of AHR activation in case of inflammatory skin conditions may in part be due to the oxidative and antioxidative properties of the AHR ligands (Furue et al., 2018). The involvement of oxidative stress and altered antioxidant defenses in the pathophysiology of AD and similar inflammatory diseases is well-known (Okayama, 2005; Tsukahara et al., 2003). Ligands such as TCDD and BaP are oxidative and induce formation of reactive oxygen species (ROS) (Kennedy et al., 2013; Tsuji et al., 2011) whereas antioxidative ligands such as phytochemical compounds, coal tar, soybean tar inhibit ROS generation by activating nuclear factor-erythroid 2-related factor-2 (NRF2) (Furue et al., 2018).

**Effects of TCDD on the epidermis and epidermal permeability barrier**

Several studies have demonstrated that TCDD alters keratinocyte differentiation both in *in vitro* and *in vivo* models. Activation of AHR on TCDD treatment increased stratification and terminal differentiation in human keratinocytes *in vitro* (Greenlee et al., 1985) and resulted in aberrant expression of several differentiation markers (Loertscher et al., 2001). TCDD also increased expression of numerous genes in the epidermal differentiation complex and ceramide biosynthesis (Kennedy et al., 2013; Sutter et al., 2011; Sutter et al., 2009). TCDD activation of AHR not only increased differentiation genes, but also increased mitochondrial ROS and nitric oxide and decreased glycolysis
indicating that the AHR regulates metabolic reprogramming in the keratinocytes to promote differentiation (Kennedy et al., 2013; Sutter et al., 2018; Sutter et al., 2020). Inhibiting the AHR with antagonists such as CH223191 and GNF351 significantly reduced expression of terminal differentiation genes in mouse keratinocytes (van den Bogaard et al., 2015). Impairment in differentiation was also demonstrated with AHR deficient primary keratinocytes in the same study. Mice deficient in AHR in the keratinocytes showed high transepidermal water loss (TEWL), in response to tape-stripping, indicating a defective barrier system (Haas et al., 2016). In utero studies showed TCDD exposure increased expression of filaggrin in C57BL/6J mice (Loertscher et al., 2002) and accelerated epidermal barrier formation at embryonic day 15 (E15) by 1 day (Sutter et al., 2011). Further studies showed that in utero exposure to TCDD disrupted tight junction functions resulting in leaky tight junctions (Muenyi et al., 2014). Together, these findings support a role of the AHR in epidermal development and function.

Unlike humans that develop chloracne on TCDD exposure, most lab animals including haired rodents seem to be refractory to TCDD-induced dermatoses. In the case of rodents, only the strains of hairless mice exhibit TCDD-induced chloracne-like pathology such as epidermal hyperplasia, hyperkeratinization of the stratum corneum, absence or involution of sebaceous glands and formation of dermal cysts (Puhvel et al., 1982). In a recent in vivo study with C57BL/6J mice, application of AHR agonists, TCDD and β-napthoflavone (BNF), resulted in atrophy of the sebaceous glands without the development of cutaneous lesions as seen in chloracne (Fontao et al., 2018). Moreover, these effects were reversible. Besides this report, chloracne-like skin lesions
was reported in B6C3F1 mice treated with a dioxin-like compound, 3,3′,4,4′-tetrachlorazobenzene (TCAB) for 2 years (Ramot et al., 2009). These mice received 10 and 30 mg/kg/day TCAB by oral gavage for 5 days a week for 2 years that led these animals to develop follicular dilations and atrophy of the sebaceous glands, consistent with chloracne-like lesions. On the other hand, transgenic mice expressing constitutively active AHR (AHR-CA) in the epidermis revealed that these mice developed an AD-like phenotype in adulthood with skin lesions, pruritus, skin inflammation, and immunological imbalance (Tauchi et al., 2005).

Our hypothesis for this study is that perinatal TCDD exposure results in defects in skin barrier development in mice and increases susceptibility to skin diseases.

Significance

The mechanism of toxicity of environmental pollutants on skin is not completely understood. However, several studies demonstrate that AHR activation is one of the underlying factors, along with disrupted barrier, inflammatory responses, oxidative stress and microbiome alterations (Mancebo & Wang, 2015). Our study investigates the role of AHR activation in skin physiology and toxicology. TCDD appears to disturb epidermal homeostasis leading to abnormal proliferation and differentiation of keratinocytes. Previous studies in our lab showed that in utero exposure to TCDD at a dose of 10 µg/ kg body weight (bw) accelerated the development of the epidermal permeability barrier (EPB) E15 (Sutter et al., 2011; Muenyi et al., 2014). TCDD-exposed pups also exhibited acanthosis, hyperkeratosis and defective barrier with leaky tight junctions at postnatal day 1 (P1) (Muenyi et al., 2014). According to the concept of the ‘Developmental Origins of
Health and Disease (DOHaD), environmental modulations during early life impacts tissue physiology and homeostasis determining long-term health (Barker, 2007). The current study determines the effects of in utero and lactational exposure to TCDD on barrier formation and function, extending these observations from P1 to adult mice. The effects of dioxin exposure on the development and function of epidermis and microbiome assembly is understudied. This study will broaden our understanding of how dioxin-like environmental pollutants influence barrier formation and health, the microbiome, and susceptibility to skin conditions in adults.

**Aim 1: Investigate the cutaneous effects of in utero and lactational exposure to TCDD**

TCDD can cross the placental barrier and effect development of the fetus. Additionally, TCDD being lipophilic, is accumulated in the adipose tissues of mammary glands. Hence, perinatal exposures occur in utero and via lactation (Gasiewicz et al., 1983; Nau et al., 1986). In mice, the development of the epidermis begins at E9 with a single layer of epidermal cells. The stratification of this single layer starts at E13, and a complete barrier is formed around E16-17 (Hardman et al., 1998; O'Shaughnessy & Christiano, 2004). In this study, time mated pregnant C57BL/6J mice were treated with corn-oil or TCDD (5 or 10 µg/ kg bw) by oral gavage on E12. Exposed pups were then sacrificed at various time-points and effects on barrier formation and function were assessed.
1.1 Determine the effects of TCDD on the development and function of EPB and susceptibility to AD

Previously, exposing mouse embryos to 10 µg/kg bw TCDD resulted in accelerated barrier formation (Muenyi et al., 2014; Sutter et al., 2011). Here, we determined if this effect was dependent on the AHR. TEWL was measured to determine epidermal barrier function.

Susceptibility to AD health was determined by observing clinical symptoms such as skin lesions, erythema, xerosis, scaly skin, hair loss and scratching behavior. Tissue sections were stained with Hematoxylin and Eosin (H&E) and evaluated for changes in epidermal thickness. Development of inflammation was assessed by examining transcript levels of inflammatory cytokines in the skin.

1.2 Determine the effects of TCDD on sebaceous gland development and morphogenesis

Although chloracne, the most consistent indicator of TCDD toxicity in humans has not yet been reported in haired rodents, involution of sebaceous glands have been reported in adult (4-8 weeks) haired mice topically exposed to TCDD for 2 weeks (Puhvel & Sakamoto, 1988) and 5 weeks (Fontao et al., 2018). Sebaceous hypoplasia is one of the key features of chloracne. Therefore, the skin of TCDD-exposed animals was examined carefully for any changes in the sebaceous glands over time. Particularly, Oil Red O staining for lipid staining of sebaceous glands was performed. Further, sebaceous stem cells were analyzed as possible targets of TCDD.
1.3 Determine the effects of TCDD on skin microbiome assembly during postnatal development

Skin has a complex microbiome assembly that adds to the protective barrier to protect against invasion by other pathogens. Fetal skin is sterile in utero, with colonization occurring immediately after birth (Grice et al., 2008). As the individual grows, the skin microbiome is established and stabilized. The microbial community is greatly dependent on the maintenance of the skin topography and immune responses (Prescott et al., 2017). There is a huge shift in the microbial community during disease conditions or wounds that results in establishment of many more types of bacteria including some rare ones on the skin. Particularly, shifts of species like Staphylococcus have been associated with abnormal immune responses and impaired wound healing (Grice et al., 2010; Schierle et al., 2009). Mice skin is inhabited largely by Firmicutes, Proteobacteria, Actinobacteria and Bacteroidetes (Belheouane et al., 2020). The effects of environmental pollutants and subsequent AHR activation on skin microbiome is understudied. Ligand activated AHR can modulate the proliferation and differentiation of immune cells (Hanieh, 2014; Quintana et al., 2008). As such, the effects of AHR activation on the assembly and temporal shifts of the skin microbiome during development was studied.

Aim 2: Determine the effects of AHR knockdown in keratinocyte biology

To further study the role of AHR in epidermal biology, AHR knockdown in immortalized keratinocyte cells, N/TERT-1, was developed. Expression of differentiation markers were studied to determine the role of AHR in differentiation of keratinocytes.
Chapter 2: Materials and methods

Mice

Time-mated, presumed-pregnant C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME). The AHR null mice, B6.129-Ahr<sup>tm1Bra</sup>/J (Schmidt et al., 1996) were also purchased from Jackson Laboratory (Stock # 002831) and maintained as a heterozygous colony by breeding with wild type C57BL/6J. Heterozygous animals were crossed and the day after a vaginal plug was observed was considered as embryonic day 1 (E1). Homozygous offspring were examined for barrier development on E15 and E16. All genotypes were confirmed by PCR amplification of tail genomic DNA.

Animals were housed in clear disposable plastic cages and maintained in a 12:12-hr light: dark cycle in a temperature- (24°C ± 1°C) and humidity- (35% ± 4%) controlled room. Dams received Teklad Global 18% Protein Rodent Diet 2018 until E9 after which they were switched to Teklad Global 16% Protein Rodent Diet 2016 (Harlan Teklad, Madison, WI). Pups were harvested at postnatal day 1 (P1), P6, P13, P21, P35 and P70. Pups remained with the mothers until weaning at P21, after which they were housed in a group of up to 5 per cage.

All animal research protocols were approved by the University of Memphis Institutional Animal Care and Use Committee and carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health.
Animal treatment

TCDD gavage

Treatments were administered as described previously (Muenyi et al., 2014). Briefly, dams were weighed on E12 and randomly distributed into groups to receive either corn-oil (vehicle control) or a single dose of 5 or 10 µg/kg body weight TCDD in 100 µL corn-oil by oral gavage. For preparation of gavage, syringes were loaded with corn-oil or TCDD, being careful not to contaminate any surface with TCDD. Animals were restrained gently, grasping them by the loose skin of the neck and back. Once the animal was immobilized, it was held in an upright position and the gavage needle (9923B, Cadence Science, Cranston, RI) was passed through the mouth. As the animal started to swallow the gavage needle, the needle was advanced to the stomach and treatment dispersed. Following gavage, animals were housed singly in their respective cages.

MC903 treatment

To induce an AD-like pathology, animals belonging to corn-oil and TCDD group were sensitized with the vitamin D analogue, MC903 (Calcipotriol hydrate, Sigma-Aldrich, St. Louis, MO). Each animal received 2 nmol of MC903 prepared in 25 µL of absolute ethanol. For application, animals were anesthetized and MC903 was applied in strokes with a micropipette tip on the left ear (12.5 µL on the inside and 12.5 µL on the outside) once a day for 14 days. On the right ear, ethanol was applied as vehicle control in a similar way. Ear thickening was measured with a Digimatic micrometer (293-831-30, Mitutoyo, Aurora, IL) at indicated times.
Anesthesia

All P1 and P6 animals were sedated with SOMNASOL™ Euthanasia-III solution (National Drug Code no. 11695-4829-1; Butler Schein Animal Health, Dublin, OH). To sedate, 10 µL of 1:10^6 SOMNASOL™ Euthanasia-III solution: PBS was injected intraperitoneally. Bigger animals (P13 and above) were anesthetized using isoflurane. To begin the process, isoflurane vaporizer was filled with isoflurane and the oxygen flow meter was set to 1 L/min flow rate. The animal was placed inside the anesthesia chamber and isoflurane flow started. Isoflurane flow was maintained at 2-3%. Animals were euthanized by cervical dislocation or CO2 asphyxiation.

Skin permeability assay

Barrier formation was determined with an assay using the β-galactosidase substrate, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) (Hardman et al., 1998). Embryos were collected as described previously (Muenyi et al., 2014; Sutter et al., 2011). Briefly, pregnant dams were euthanized by asphyxiation with carbon dioxide, and the entire uterus with embryos was removed. The embryos were then carefully removed from the embryonic sacs and rinsed in phosphate buffered saline (PBS), pH 7.4. Tail snips were collected for genotyping and the embryos were then incubated in the X-gal reaction mixture for 18 hr at 30°C. Next, the embryos were fixed in 10% formalin at 4°C for 24 hr and transferred to 70% alcohol. The embryos were photographed and quantified as cited (Muenyi et al., 2014; Sutter et al., 2011).
**Transepidermal water loss (TEWL)**

TEWL was measured on crown of the head of the animals using the Delfin VapoMeter with a 4.5-mm nail adapter attached (Delfin Technologies Ltd, Stamford, CT) as previously described (Muenyi et al., 2014).

**Isolation of genomic DNA and Polymerase Chain Reaction**

Tail snips were heated in DNA extraction buffer (25 mM NaOH/0.2 mM EDTA) for 45 min at 95°C, vortexing and spinning down the reaction mixture every 15 min. The reaction was neutralized with a neutralization buffer (40 mM Tris HCl, pH 5.5) and supernatant collected. Genomic DNA were amplified using GoTaq Master Mix (PRM7123, Thermo Fisher Scientific, Waltham, MA) and primers listed in Table 1, and PCR products were separated on 1% agarose gel in 1X TAE buffer (40 mM Tris, 20 mM Glacial acetic acid, 1 mM EDTA).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BJ common oIMR0444</td>
<td>TCTTGGGCTCGATCTTGTGTCAGGAACAGG</td>
</tr>
<tr>
<td>BJ mutant oIMR8162</td>
<td>TGGATGTGGAATGTGTGCGAG</td>
</tr>
<tr>
<td>BJ WT oIMR0443</td>
<td>GGATTTGACTTAATTCCCTTCAGCGG</td>
</tr>
</tbody>
</table>

**RNA isolation and qRT-PCR**

To isolate RNA from mouse tissue, a piece of skin scalp was collected in RNAlater solution (AM7020, Thermo Fisher Scientific) in a RNase-free microcentrifuge tube. Samples were stored at -20°C until processing. For processing, samples were thawed, and 50-100 mg tissue was homogenized in 1 mL RNA STAT-60 using a Polytron system (PT-DA 1205/2EC, Kinematica, Switzerland). To isolate RNA from cells, RNA STAT-60 was directly added to the cell culture plate. In both cases, RNA was
extracted with chloroform: isoamyl alcohol (24:1) and washed with 75% ethanol following precipitation with equal volume of isopropanol. Extracted RNA was dissolved in water and quantitated by absorbance using a Nanodrop (2000/2000c, Thermo Fisher Scientific). Quantitative PCR (qPCR) was performed as previously described (Sutter et al., 2009). Briefly, 1µg of RNA was reverse transcribed to cDNA and 6 ng of cDNA was amplified using Absolute Blue SYBR Green qPCR master mix (Thermo Fisher Scientific) and the primers listed in Table 2 for the intended targets. Expression levels were normalized to mouse Hprt or human Tubulin alpha 1C. The efficiencies of each primer set was determined and used in quantitation (Pfaffl, 2001).

Table 2. List of qPCR primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (FP) (5’-3’)</th>
<th>Reverse complement primer (RC) (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AHR (human)</td>
<td>GTGCACCTGCTACATTGTGGG</td>
<td>AAAAGCCATGAAAGCATTTACCT</td>
</tr>
<tr>
<td>Cyp1a1 (mice)</td>
<td>GCACCTCTGTCTACCCCTACA</td>
<td>AGACCTGGTTTTACTGCCCA</td>
</tr>
<tr>
<td>Cyp1a1 (human)</td>
<td>GCAGCAGGATAGCCAGGAAGAGAA</td>
<td>CATCCCCACACGACAACAAAGAGA</td>
</tr>
<tr>
<td>Cyp1b1 (mice)</td>
<td>TAGTAAAGGGCTGGGACGGTGA</td>
<td>CATCCGGGTCTGGTTGGTTT</td>
</tr>
<tr>
<td>FLG (human)</td>
<td>GACACCCCCGGATCTCCTCACC</td>
<td>AGCTGCCATGTCTCCAAACCTAAAC</td>
</tr>
<tr>
<td>Hprt (mice)</td>
<td>ACAGGCCAGACTTTGTGGGA</td>
<td>ACTTGCGCTCATCTTGGCT</td>
</tr>
<tr>
<td>HRNR (human)</td>
<td>CCAGCACAAGAGGAACAAGAAGA</td>
<td>GCCCGCGCCCTGAAGACTGATG</td>
</tr>
<tr>
<td>Il-4 (mice)</td>
<td>CCCCCAGCTAGTTGTCATCCT</td>
<td>CAAGTGATTTTTTGTCGCATCCG (Yu et al., 2018)</td>
</tr>
<tr>
<td>LOR (human)</td>
<td>CAGGGGCACCGATGGGCTTAGAG</td>
<td>TGAGGGCACTGGGTTGGAGGTAG</td>
</tr>
<tr>
<td>Tubulin alpha 1C (human)</td>
<td>GTTGCCGTCCCCCTCGCCTCCTT</td>
<td>CTTGCACACCCCCGTTTCCT</td>
</tr>
</tbody>
</table>
Microbiome analysis

Sample collection

For microbiome sample collection, animals were shaved under anesthesia a day before sample collection. To collect microbiome samples, animals were anesthetized. A sterile foam swab (#25-1506 1PF, Puritan, Guilford, ME) was moistened with sterile PBS and vigorously rubbed over a 2 x 2 cm area on the dorsal region of the animals. The swab with microbiome sample was broken off into a sterile tube (2.0 mL Safe-Lock Biopur tubes, #022600044, Eppendorf) and stored at -80°C until DNA extraction.

DNA extraction

Bacterial DNA was extracted as described previously (Meisel et al., 2016) using the PureLink kit (K182002, Invitrogen, Carlsbad, CA) and quantified using the Quant-iT PicoGreen Assay Kit (P7589, Invitrogen).

16S rRNA gene amplicon library construction and sequencing

The 16S ribosomal RNA genes were amplified using bar-coded PCR primers targeted to the V1-V3 hypervariable region (FP 5’-AGAGTTTGATCCTGGCTCAG-3’; RC 5-ATTACCGCGGTGTGCTGG-3’). PCR reactions were carried out in quadruplicate using Accuprime Taq HiFi (12346086, Invitrogen). Each PCR reaction contained 0.2 µM of each primer, 1 U AccuPrime Taq HiFi, 1X Buffer II, and 2 µL DNA in a total volume of 25 µL. Cycling conditions are as follows: 1 cycle of 95°C for 2 min; 32 cycles of 95°C for 20 s, 60°C for 30 s, and 72°C for 60 s; 1 cycle of 72°C for 5 min. The resulting 16S rDNA amplicons were purified using a 1:1 volume of SPRI beads (09-981-123, GE Healthcare, Chicago, IL), quantified using PicoGreen, pooled in equal amounts, and sequenced on the Illumina MiSeq using 2x300 bp chemistry. Extraction blanks and
DNA-free water were subjected to the same amplification and purification procedure to assess potential environmental contamination. Library preparation and sequencing were performed at the CHOP Microbiome Center (University of Pennsylvania, Philadelphia, PA).

**Bioinformatics analysis of 16S amplicon sequencing data**

Raw FASTQ read files called from Illumina MiSeq machine were demultiplexed using the FLEXBAR program (v2.4) (Dodt et al., 2012) and in-house Perl scripts. All PE reads of every sample were phylogenetically assigned to the curated Greengenes 97% OTU reference tree (v13.8) (DeSantis et al., 2006) using our recently developed phylogenetic placement tool HmmUFOtu (Zheng et al., 2018) with default options.

Subsequently phylogeny-based OTUs and the corresponding OTU-tree were summarized and built using HmmUFOtu with a requirement of a minimum of 5 reads. This cut-off was determined by a rarefaction curve of the remaining number of OTUs.

The OTU table and corresponding tree files were loaded and processed using the Phyloseq R package (McMurdie & Holmes, 2013). The taxonomy aggregated summary of microbiome samples as well as the within sample alpha-diversity analyses were also performed using the Phyloseq R package. To find differentially enriched OTUs (DE-OTUs), the Phyloseq object was first converted into a DESeq2 object, then a negative binomial linear model was trained for the normalized OTU counts using the DESeq2 R package (Love et al., 2014), in which both the postnatal age and exposure (TCDD or corn-oil) factors were included. Significant DE-OTUs were called as FDR-adjusted p-value < 0.1.
**Histological and immunohistochemical analyses**

**Tissue preparation for paraffin and cryo-embedding**

Skin tissue from dorsal back was either embedded in paraffin or frozen in optimal cutting temperature (OCT) compound (Tissue-Tek; Sakura Finetek USA, Torrance, CA). As shown in Figure 5a, the left half of dorsal back samples were collected for paraffin embedding and the right half for OCT embedding. To paraffin embed, samples were fixed in 10% neutral buffered formalin for 24 hr at 4°C and then stored in 70% ethanol at 4°C until ready to embed. For preparation of frozen samples, tissues were fixed in 10% formalin for 24 hr and further immersed in 20% sucrose solution for additional 24 hr at 4°C. For embedding in OCT, tissues were placed on OCT compound in a cryomold (4557, Tissue-Tek) and then placed at -20°C until frozen. OCT embedded samples were transferred to -80°C for storage. For histological analyses, all tissues were cut along the sagittal plane (Figure 5B).

![Figure 5](image)

**Figure 5.** Schema of tissue collection for histological analyses (A) and sagittal versus traverse section (B).
**Hematoxylin and Eosin (H&E) staining**

For histological analysis, paraffin embedded samples were cut into 5 µm sections, deparaffinized and stained with H&E. To stain, deparaffinized tissue sections were incubated in Mayer’s hematoxylin (Cancer Diagnostics, Durham, NC) for 4 min, rinsed in tap water for 5 min and immersed in Scott’s tap water substitute (0.02% magnesium sulfate, heptahydrate; 0.002% sodium bicarbonate; 0.05% sodium hydroxide in MilliQ water) for 10 times to intensify blue coloration of hematoxylin. Tissue sections were then counterstained with 1% Eosin (AC611815000, Acros) for 5 min and rinsed in distilled water 3 times. Next, sections were dehydrated by incubation in 95% ethanol followed by three incubations in 100% ethanol for 1 min each. Lastly, sections were incubated twice in xylene for 1 min each before mounting with Permount™ mounting media (Thermo Fisher Scientific). H&E-stained tissue sections were visualized under a Nikon Eclipse E800 microscope (Nikon, Melville, NY). Epidermal thickness was measured on photographs using ImageJ (National Institutes of Health, Bethesda, MD).

**Oil Red O staining**

Oil Red O for lipid staining of sebaceous glands was performed on frozen tissue. To stain, 10 µm frozen sections were rinsed briefly in 60% isopropanol to remove the OCT compound. A working solution of Oil Red O was prepared by mixing 3 parts of Oil Red O stock (0.5% in isopropanol) and 2 parts of distilled water. The Oil Red O solution was filtered, and tissue sections were incubated in it for 15 min. Sections were then rinsed with 60% isopropanol thrice for 2 min each and counterstained by dipping in Mayer’s hematoxylin solution five times. Finally, sections were mounted in Aqua-Mount (13800, Thermo Fisher Scientific). For analysis of sebaceous glands, multiple images of
consecutive sections were acquired. The areas of sebaceous glands stained with Oil Red O were quantitated with ImageJ using the freehand area selection tool.

**Immunohistochemistry**

Immunohistochemistry was performed on paraffin sections with primary antibodies as listed in Table 3. Signals were detected using streptavidin/ biotin system (VECTASTAIN® Universal Quick HRP Kit, PK-8800, Vector laboratories, Burlingame, CA) in combination with diaminobenzidine (DAB substrate kit, SK-4100, Vector laboratories) as per manufacturer’s instructions. Briefly, deparaffined sections were incubated in 0.3% hydrogen peroxide in methanol for 30 min to inhibit endogenous peroxidase activity. Sections were then blocked in 10% normal horse serum for 30 min and incubated with primary antibodies for 1 hr at room temperature. Next, sections were washed and incubated with biotin linked universal secondary antibody followed by incubation in streptavidin coupled peroxidase solution. Signals were developed with 90 s of incubation in DAB substrate. Finally, sections were counterstained with Hematoxylin (#H-3404, Vector laboratories).

**Immunofluorescence staining**

Paraffin sections were deparaffinized and incubated in blocking solution (PBS, 0.05% Tween 20, 10% NHS) for 30 min. Sections were then incubated in primary antibodies (listed in Table 3) for 1 hr, washed in PBS for 5 min three times and incubated with appropriate species-specific secondary antibodies (1:50 dilution) as listed in Table 4 for 30 min. Slides were then washed in PBS for 5 min three times and mounted in ProLong Diamond antifade mountant (P36962, Thermo Fisher) containing DAPI as
nuclear counterstain. All fluorescent sections were analyzed on an A1 confocal microscope (Nikon).

Table 3. List of antibodies for IHC

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Host</th>
<th>Dilution</th>
<th>Antigen retrieval</th>
<th>Application</th>
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<tr>
<td>CYP1A1</td>
<td>SantaCruz</td>
<td>Mouse</td>
<td>1:50</td>
<td>Citrate buffer (10mM, pH 5.5) at 85°C for 6 min</td>
<td>IHC, IF</td>
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<tr>
<td>CYP1B1</td>
<td>Sutter lab (Walker et al., 1998)</td>
<td>Rabbit</td>
<td>1:250</td>
<td>No</td>
<td>IHC</td>
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<tr>
<td>CYP1B1</td>
<td>Sutter lab (Walker et al., 1998)</td>
<td>Rabbit</td>
<td>1:500</td>
<td>*Citrate buffer (10mM, pH 5.5) at 85°C for 6 min</td>
<td>IF</td>
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<tr>
<td>Lrig1</td>
<td>R&amp;D</td>
<td>Goat</td>
<td>1:25</td>
<td>Citrate buffer (10mM, pH 5.5) at 85°C for 6 min</td>
<td>IF</td>
</tr>
<tr>
<td>LGR6</td>
<td>Santacruz</td>
<td>Mouse</td>
<td>1:100</td>
<td>No</td>
<td>IF</td>
</tr>
<tr>
<td>LGR6</td>
<td>Proteintech</td>
<td>Rabbit</td>
<td>1:200</td>
<td>No</td>
<td>IF</td>
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</table>

IF: immunofluorescence staining; IHC: immunohistochemical staining; *epitope retrieval performed only when co-stained with CYP1A1 and Lrig1

Table 4: List of secondary antibodies for IF

<table>
<thead>
<tr>
<th>Secondary antibody</th>
<th>Source</th>
<th>Host</th>
<th>Conjugate</th>
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<tbody>
<tr>
<td>anti-mouse (A32766)</td>
<td>ThermoFisher</td>
<td>donkey</td>
<td>Alexa Fluor 488</td>
</tr>
<tr>
<td>anti-mouse (A10037)</td>
<td>ThermoFisher</td>
<td>donkey</td>
<td>Alexa Fluor 568</td>
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<td>anti-rabbit (A10042)</td>
<td>ThermoFisher</td>
<td>donkey</td>
<td>Alexa Fluor 568</td>
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<tr>
<td>anti-rabbit (A32795)</td>
<td>ThermoFisher</td>
<td>donkey</td>
<td>Alexa Fluor 647</td>
</tr>
<tr>
<td>anti-goat (A32814)</td>
<td>ThermoFisher</td>
<td>donkey</td>
<td>Alexa Fluor 488</td>
</tr>
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</table>
Measurement of grooming duration and scratching frequency

Individual mice were separated into a fresh cage. After allowing a few minutes for the animals to settle down, the animals were observed for grooming behavior and scratching frequency over a 10-min period. The use of the forepaw or mouth was described as grooming, whereas the use of the hind paw as scratching.

Serum IgE detection

Serum was prepared from trunk blood of animals. Blood samples were allowed to clot at room temperature for 15 min and then centrifuged for 10 min at 10,000 rpm. Serum was collected as supernatant and stored at -80°C. Total IgE in the serum was detected by using the Mouse IgE ELISA MAX Deluxe kit (432404, BioLegend, San Diego, CA) according to the manufacturer’s instructions.

Cell culture

The immortalized keratinocyte cell line, N/TERT-1, were kindly provided by James G. Rheinwald, [(Dickson et al., 2000) Cell Culture Core, Harvard University] and were grown in keratinocyte serum-free media (KSFM) (17005042, ThermoFisher) supplemented with bovine pituitary extract and epidermal growth factor as previously described (Sutter et al., 2018; Sutter et al., 2009).

Knockdown of AHR

N/TERT-1 cells were either transduced with AHR shRNA lentiviral particles (SigmaMillipore, TRCN0000245286) or control vectors (SigmaMillipore, SHC201V) in the presence of polybrene (5 µg/ mL). Transduced cells were screened for stable expression of the vectors using 1 µg/ mL puromycin. Resistant cells were expanded and maintained in 0.5 µg/ mL puromycin for experiments.
**Western blotting**

Total proteins from cells were extracted using RIPA lysis buffer (0.1% SDS, 1% NP40, 5 mM EDTA, 0.5% sodium deoxycholate, 150 mM NaCl, 50 mM Tris-HCl pH 8) supplemented with phenylmethylsulfonyl fluoride and protease inhibitor cocktail (P8340, SigmaMillipore). The samples were centrifuged for 10 minutes at 10,000 x g at 4°C and the supernatant was used for immunoblotting. For FLG detection, pellets from the whole cell lysis were boiled for 5 min in lysis buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 1% 2-beta-mercaptoethanol). The samples were centrifuged for 10 min at 10,000 x g at room temperature and supernatant was used to detect FLG. Protein was quantitated using the Pierce Micro BCA kit (23235, ThermoFisher Scientific). Twenty micrograms of protein samples were separated by PAGE (7.5% and 10% gels, 150 Volts) and transferred to polyvinylidene fluoride membranes (100 Volts, 90 min, 4°C). Blocking and antibody incubations were in Tris-Buffered Saline with 0.1% Tween 20 with 5% milk. All primary and secondary antibodies used are listed on Table 5. Following incubation with Clarity Western ECL Substrate (1705061, Bio-Rad) bands were visualized using the ChemiDoc Touch Imaging System (Bio-Rad). Signal density was quantitated using Bio-Rad Laboratories Image Lab (v5.2.1) software (Hercules, CA).
Table 5: List of antibodies for western blots

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Isotype</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>AHR</td>
<td>17840-1-AP, Proteintech</td>
<td>Rabbit polyclonal</td>
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</tr>
<tr>
<td>FLG</td>
<td>905801, BioLegend</td>
<td>Rabbit polyclonal</td>
<td>1:1,000</td>
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<tr>
<td>LOR</td>
<td>PRB 145P, Biolegend</td>
<td>Rabbit polyclonal</td>
<td>1:1000</td>
</tr>
<tr>
<td>IVL</td>
<td>19018, Sigma</td>
<td>Mouse monoclonal</td>
<td>1:1000</td>
</tr>
<tr>
<td>TGM1</td>
<td>12912-3AP, Proteintech</td>
<td>Rabbit polyclonal</td>
<td>1:1000</td>
</tr>
<tr>
<td>CDKN1B (p27)</td>
<td>3698, Cell signaling</td>
<td>Mouse monoclonal</td>
<td>1:2000</td>
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<td>Beta-actin</td>
<td>A5441, Sigma Aldrich</td>
<td>Mouse monoclonal</td>
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<tr>
<td>goat anti-rabbit secondary antibody</td>
<td>111035144, Jackson ImmunoLabs Research</td>
<td>Goat polyclonal</td>
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<tr>
<td>goat anti-mouse secondary antibody</td>
<td>115035003, Jackson ImmunoLabs Research</td>
<td>Goat polyclonal</td>
<td>1:10,000</td>
</tr>
</tbody>
</table>

_Cornified envelope (CE) assay_

Confluent cells were pretreated in basal KSFM overnight and grown with or without TCDD (10nM) for 5 days in basal KSFM plus 1.8 mM calcium chloride. The formation of CEs were quantitated as described in (Banks-Schlegel & Green, 1980). Cells were counted before they were spun and resuspended in 10 mM Tris-HCl pH 7.5, 1% SDS and 1% 2-mercaptoethanol. CEs were counted following an incubation at 90°C for 10 min. The number of CEs were determined per number of cells and expressed as a percentage.
Statistical analysis

Data are presented as mean ± SD. A two-tailed unpaired Student’s t-test was used for single comparisons. A two-way analysis of variance (ANOVA) followed by Tukey post-hoc test was used to compare differences between multiple independent variables. For non-parametric analyses, Mann Whitney U test was performed. The specific statistical test used is indicated in the legend of each figure. Differences were considered significant when p-values were < 0.05. All data were analyzed with GraphPad Prism software (version 7.03, La Jolla, CA).
Chapter 3: Cutaneous effects of in utero and lactational exposure of C57Bl/6J mice to 2,3,7,8-tetrachlorodibenzo-p-dioxin

Introduction

Early-life exposures to persistent organic pollutants impact development and alter neurological, cardiovascular and reproductive functions in later-life (Boekelheide et al., 2012). A transgenic mice expressing constitutively active AHR (AHR-CA) in the epidermis develop an AD-like phenotype with severe skin lesions, frequent itching, and increased skin inflammation in adulthood (Hidaka et al., 2017; Tauchi et al., 2005). These transgenic mice expressed the constitutively active form of the AHR under the control of the promoter of keratin 14, that is expressed predominantly in the keratinocytes. Similarly, exposing C57BL/6J mice embryos to TCDD, a potent AHR ligand, accelerates epidermal permeability barrier (EPB) formation in mice (Muenyi et al., 2014; Sutter et al., 2011). On birth, exposed pups demonstrate epidermal acanthosis, hyperkeratosis and defective barrier with leaky tight junctions (Muenyi et al., 2014). In contrast, topical application of AHR agonists, TCDD and β-napthoflavone (BNF), on C57BL/6J mice results in atrophy of the sebaceous glands without the development of cutaneous lesions as seen in chloracne (Fontao et al., 2018). Although chloracne is a common skin toxicity in humans exposed to TCDD, it is not the case in animals. With the exception of few animals like rabbits, monkeys and hairless mice, most animals including haired rodents fail to develop chloracne lesions in response to TCDD (Horton & Yeary, 1985; Poland et al., 1984).

The mechanism of toxicity of environmental pollutants on skin is not completely understood. However, several studies demonstrate that AHR activation is one of the
underlying factors, along with disrupted barrier, inflammatory response, oxidative stress and microbiome alteration (Mancebo & Wang, 2015). The aim of this study is to determine the effects of developmental exposure to TCDD on cutaneous health of mice, extending these observations from P1 to adult mice.

Results

AHR-dependent effects of TCDD on the development of the EPB

Previously, we reported that in utero exposure to TCDD accelerated epidermal barrier formation in mice resulting in epidermal acanthosis, hyperkeratosis, and defective barrier with leaky tight junctions at birth (Muenyi et al., 2014; Sutter et al., 2011). To determine whether this effect was dependent on the AHR, AHR null mice, B6.129-Ahr<sup>tm1Bra/J</sup> (Schmidt et al., 1996) were studied. Time-mated wild type and AHR null mice were treated by gavage with TCDD at E12 or E13 and sacrificed at E15 or E16. An X-gal dye exclusion assay was performed on the fetuses to evaluate the development of EPB formation. As previously observed, TCDD accelerated barrier formation of wild type embryos at E16 compared to corn-oil-treated control pups as shown by the increased exclusion of the dye (Figure 6A, B). This effect was absent in AHR null embryos, demonstrating that TCDD-mediated acceleration of barrier formation is dependent on the AHR. Further, TCDD treatment induced thickening of the epidermis at P1, indicating acanthosis. This effect was also dependent on the AHR (Figure 6C, D).
A

<table>
<thead>
<tr>
<th>TCDD</th>
<th>WT</th>
<th>AHR null</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>+</td>
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</tbody>
</table>

B

E15

WT

AHR null

E16

WT

AHR null

C

WT

AHR null

Corn-oil

TCDD

D

Epidemal thickness (μm)

Corn-oil

TCDD (10 μg/kg bw)
Figure 6. AHR-dependent effects of TCDD on the development of the epidermal barrier. Time-mated mice were treated by gavage with either corn-oil or TCDD (10 μg/ kg bw) at E12 or E13 and sacrificed at E15 and P1 or E16, respectively. (A) Representative photographs of fetuses following the X-gal skin permeability assay. (B) Quantitation of photographs from X-gal staining by densitometry (n = 5). All values are means ± SDs. *p < 0.05 compared to vehicle control; significance was determined with two-way ANOVA, followed by the Tukey test. (C) Representative images of formalin-fixed, paraffin-embedded tissue sections at P1 stained with H&E. Scale bar = 50 µm. Double-headed arrows indicate the epidermal thickness. (D) Quantitation of epidermal thickness at P1 by microscopy (n = 5-6). All values are means ± SDs. *p < 0.05 compared to vehicle control; significance was determined with two-way ANOVA, followed by the Tukey test. Note: bw, body weight; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; E, embryonic day; P, postnatal day; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; SD, standard deviation.

Cutaneous effects of in utero and lactational exposure of mice to TCDD

AHR-CA mice exhibit AD-like phenotypes with frequent scratching, dysfunctional barrier and increased skin inflammation (Tauchi et al., 2005). To investigate if ligand-mediated activation of AHR produces similar effects, mice exposed to TCDD in utero and through lactation were assessed for development of AD-like pathologies. Dams were treated with 5 µg/ kg bw dose of TCDD because the dose of 10 µg/ kg bw impairs mammary development and lactation in the dams, ultimately affecting the survival of the pups (Vorderstrasse et al., 2004). Pups exposed to TCDD developed epidermal acanthosis at birth that was AHR dependent (Figure 6D) and dose-dependent (Figure 7A). Interestingly, the epidermal thickening observed in the skin of treated mice at P1 did not persist at any other time (Figure 7B). TCDD-exposed mice were followed from birth to P135, but they did not display any visible signs of skin lesions at any time (Figure 7B). The treated mice were similar in size and weighed about the same as the controls (data not shown). The TEWL values in treated mice were similar to controls, indicative of a normal undisrupted barrier (Figure 7D). As compared to control mice, treated mice showed increased tendency to scratch at P35-49, though at low frequency
Next, the mice were evaluated for the presence of skin inflammation, particularly focusing on the Th2 immune response, which is induced in cases of AD. None of the Th2 cytokines (IL-4, 5, 13) were detected in skin of the treated mice (data not shown). Further, serum IgE levels were lower in treated mice at P35 as compared to controls (Figure 7F). In summary, TCDD-exposed mice did not show any signs of atopy.
Figure 7. Cutaneous effects of *in utero* and lactational exposure of mice to TCDD. Timemated mice were treated by gavage with either corn-oil or TCDD [5 μg/ kg (A-F) or 10 μg/ kg (A) bw] at E12 and pups were examined for cutaneous effects at the indicated postnatal days. (A) Histological analyses of murine pup skin at P1 following the indicated treatments. Representative images of formalin-fixed, paraffin-embedded tissue sections stained with H&E, followed by quantitation of epidermal thickness by microscopy (n = 5-7). Scale bar = 50 μm. Double-headed arrows indicate the epidermal thickness. All values are means ± SD. *p < 0.05 compared to vehicle control, #p < 0.05 compared to 5 μg/ kg bw TCDD group; significance was determined with one-way ANOVA, followed by the Tukey test. (B) Quantitation of epidermal thickness by microscopy at the indicated times (n = 4-6). All values are means ± SDs. *p < 0.05 compared to vehicle control, significance was determined with a two-tailed Student’s *t* test. (C) Representative image of the appearances of the corn-oil- and TCDD-treated mice at P35 (n > 20). (D) Barrier function measured by TEWL (n = 6 mice). (E) Frequency of scratching measured following the indicated treatments and time of exposure (n = 8-15). All values are means ± SDs. *p < 0.05 compared to vehicle control, significance was determined with a two-tailed Student’s *t* test. Note: TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; bw, body weight; E12, embryonic day 12; P, postnatal day; H&E, hematoxylin and eosin; SD, standard deviation; ANOVA, analysis of variance; TEWL, transepidermal water loss.

**Effects of TCDD on Topical MC903-induced AD-like dermatitis**

TCDD-exposed animals in this study did not develop any phenotype related to AD. Additionally, TCDD-exposed animals had lower IgE levels at P35 as compared to the control animals (Figure 7F). To determine if the absence of atopy in TCDD-exposed mice is due to an immunosuppressive effect of TCDD (Quintana et al., 2008), an established model of MC903-induced AD-like dermatitis was used (Li et al., 2006).

MC903 was topically painted on the ears of corn-oil- and TCDD-exposed mice for 14 consecutive days and resulted in visible scaling and reddening of ears (Figure 8A). MC903-applied ears were significantly thicker and showed inflamed histology with epidermal hyperplasia in both the corn-oil- and TCDD-treated mice (Figure 8B and 8C). MC903 treatment also induced increased serum IgE levels and IL-4 transcript levels in
both the corn-oil- and TCDD-treated animals, indicative of AD-like inflammatory pathology (Figure 8D, E). Overall, there was no significant difference in the response to MC903-induced dermatitis between corn-oil- and TCDD-exposed animals.

Figure 8. Effects of TCDD on topical MC903-induced AD-like dermatitis. MC903 (2 nmol in 25 µL EtOH) or EtOH (vehicle control) was applied once daily for 14 days to ears of mice exposed to either corn-oil or TCDD (5µg/ kg bw) in utero. (A) Representative images of the gross appearance of MC903-treated ears at the end of the treatment. (B) Thickness of the ears measured with a micrometer on indicated days of MC903 treatment (n = 5). All values are means ± SDs. *p < 0.05 compared to vehicle control; significance was determined with two-way ANOVA, followed by the Tukey test. (C) Representative images of H&E staining of ear sections at day 14. Scale bar = 50 µm, * identifies epidermal hyperplasia. (D) Total serum IgE (n = 5) at the end of MC903 treatment. All values are means ± SDs. *p < 0.05 compared to vehicle control; significance was determined with a two-tailed Student’s t test. (E) IL-4 mRNA levels measured by qPCR in skin tissue (n = 5) at the end of MC903 treatment. All values are means ± SDs. *p < 0.05 compared to vehicle control; significance was determined with two-way ANOVA, followed by the Tukey test. (D) Note: TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; EtOH, ethanol; bw, body weight; H&E, hematoxylin and eosin; IL-4, interleukin-4; SD, standard deviation; ANOVA, analysis of variance.
**Effects of TCDD on sebaceous gland morphogenesis**

Chloracne is the hallmark of TCDD toxicity in humans. Cutaneous lesions of chloracne include epidermal thickening and metaplastic changes of sebocytes, resulting in atrophy or loss of sebaceous glands. The effects of TCDD on the sebaceous units in exposed murine skin was studied. As observed in the H&E-stained samples, TCDD-treated skin had visibly smaller sebaceous glands compared to control skin indicating sebaceous hypoplasia at P13 and P21 (Figure 9A). The sebaceous gland hypoplasia returned to control levels by P35. Additionally, four out of the eleven TCDD-exposed animals developed keratinous cyst-like structures in the epidermis at P21 (Figure 9B). None of the nine control mice skin analyzed had these structures. In addition to the H&E analysis of the sebaceous glands, Oil Red O staining of the sebum in the sebaceous glands confirmed that TCDD-exposed samples at P13 had less prominent sebaceous glands compared to the control mice (Figure 10A). The difference was even greater at P21 (Figure 10A). At P21, the sebaceous glands in TCDD-treated skin were about 30% of the size of the sebaceous glands in control skin but returned to control levels by P35 (Figure 10B).
Figure 9. Sebaceous hypoplasia in TCDD-exposed mice. Time-mated mice were treated by gavage with either corn-oil or TCDD (5 μg/ kg bw) and pups were analyzed for effects on sebaceous glands. (A) Representative images of H&E staining of corn-oil- and TCDD-treated skin at P13, P21 and P35. Scalebar = 50 μm. Arrowheads point towards sebaceous glands. (B) Serial sections of TCDD-treated mice skin stained with H&E at P21 showing development of keratinous cyst. Scalebar = 50 μm. Magnified images in boxes, scalebar = 20 μm. Note: TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; bw, body weight; H&E, hematoxylin and eosin; P, postnatal day.
Figure 10. Effects of TCDD on sebaceous gland morphogenesis. Time-mated mice were treated by gavage with either corn-oil or TCDD (5 μg/ kg bw) and pups were analyzed for effects on sebaceous glands. (A) Representative images of Oil Red O staining of corn-oil- and TCDD-treated frozen skin sections at P13, P21 and P35. Scalebar = 50 μm. Arrowheads point towards sebaceous glands stained with Oil Red O. (B) Quantitation of the area of Oil Red O-stained sebaceous gland expressed as percentage of control. Each symbol represents individual animal (n = 4-8). Longer horizontal lines indicate the mean. *p < 0.05 compared to vehicle control; significance was determined with a two-tailed Student’s t test. Note: TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; bw, body weight; P, postnatal day.
Clearance of TCDD in animals

The effects on the sebaceous glands observed in TCDD-exposed skin at P13-21 was short-lived which returned to control levels by P35. Since the half-life of TCDD in mice is relatively short (11 days) (Gasiewicz et al., 1983), we wanted to determine if the reversible effects on sebaceous glands and the lack of atopy is due to rapid clearance of TCDD from these mice. We therefore examined the expression of cytochromes CYP1A1 and CYP1B1, established biomarkers of AHR activation. Both CYP1A1 and CYP1B1 mRNA levels were increased in the skin of TCDD-treated animals until P13 and decreased to control levels by P35 (Figure 11A). Recently it was reported that the AHR-CA mice that develop AD phenotype, express increased levels of ARTN gene, encoding artemin, a neurotrophic factor that leads to hypersensitivity to pruritus (Edamitsu et al., 2019; Hidaka et al., 2017). Further, it was shown that ARTN gene is regulated by the AHR (Edamitsu et al., 2019). We measured transcript levels of ARTN in control and TCDD-treated skin. ARTN mRNA levels were increased in TCDD-exposed skin at P13 and P21 that returned to normal levels by P35 similar to CYP1A1 and CYP1B1 levels (Figure 11A). Immunohistochemical analyses showed that increases in CYP1A1 and CYP1B1 protein levels were highest at P13 and P21 (Figure 11B, C). Further, cell-specific localization of these proteins was also analyzed. Staining intensity for each sample was manually scored on a scale of 0 to 3 for CYP1A1 and 0 to 4 for CYP1B1. A score of 0 was given when no signal was observed, and a score of 3 (in case of CYP1A1) and a 4 (in case of CYP1B1) when the highest signal was observed. CYP1A1 and CYP1B1 were expressed maximally at the infundibulum, junctional zone and sebaceous glands. CYP1B1 was also maximally expressed throughout the epidermis and isthmus.
(Figure 11C). The TCDD-mediated increases in expression of both proteins decreased to control levels by P35.
Figure 11. Biomarkers in response to TCDD-mediated AHR activation. Time-mated mice were treated by gavage with either corn-oil or TCDD (5 μg/ kg bw). Expression of CYP1A1 and CYP1B1 were analyzed as biomarkers of AHR activation in the pups. Expression of ARTN, a neurotropic factor implicated in AD, was also measured. (A) Murine skin CYP1A1, CYP1B1 and ARTN mRNA expression measured by qPCR at the indicated time of exposure and treatment (n = 4-6). All values are means ± SDs. *p < 0.05 compared to vehicle control; significance was determined with a two-tailed Student’s t test. (B) Representative images of immunohistochemical staining of CYP1A1 and CYP1B1 at P21 following the indicated treatment. Scale bar = 20 μm. Arrows point to E, epidermis, IF, infundibulum, IS, isthmus and SG, sebaceous gland. (C) CYP1A1 and CYP1B1 immunostaining intensity scores for corn-oil- and TCDD-treated samples. Staining intensity at different areas of localization was scored manually on a scale of 0 to 3 (CYP1A1) or 4 (CYP1B1), 0 being absence of staining and 3 or 4 being highest intensity of staining for CYP1A1 or CYP1B1 respectively. Each symbol represents an individual skin sample (n = 4-7). Images in boxes are representative immunostaining of TCDD-treated sample at P6 (E, SG) and P21 (IF, IS) as per localization of maximum intensity. Scale bar = 20 μm. Red and blue horizontal bars indicate the mean. *p < 0.05 compared to vehicle control; significance was determined with the Mann–Whitney U test. Note: TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; AHR, aryl hydrocarbon receptor; bw, body weight; CYP, cytochrome P450; ARTN, artemin; AD, atopic dermatitis; qPCR, quantitative PCR; P, postnatal day.

Effects of TCDD on microbiome assembly

Skin is a habitat to diverse microorganisms that contribute to the establishment of a protective barrier to prevent invasion by other pathogens (Grice & Segre, 2011). To determine the effects of AHR activation on the assembly and shifts of the skin microbiome during development, 16s rRNA gene sequencing was performed on the microbiome isolated from skin swabs. Shannon diversity index was determined to evaluate overall microbial community across treatments and time-points. Compared to corn-oil-treated skin, TCDD-exposed skin exhibited significantly increased bacterial diversity at P21 (Figure 12A). No significant shifts in bacterial community were observed between the treatment groups at any other time point (Figure 12A). Comparing the relative abundance of the most prominent taxa in each treatment group, *Staphylococcus* represented the most abundant genera across all time points in both the
control and treatment groups (Figure 12B). Further, at P21, relative abundance of
*Staphylococcus* was reduced and that of *Allobaculum* was increased in TCDD-treated
samples as compared to corn-oil-treated samples (Figure 12B). Finally, the differential
abundance analysis identified 358 OTUs as significantly abundant in TCDD-treated
samples and 76 OTUs that were significantly abundant in corn-oil-treated samples
(Figure 12C).
A

B

C

Log2 fold change (TCDD/Corn-oil) vs Log2 mean abundance

- TCDD: 358
- Corn-oil: 76
- NS

Genus:
- Ruminococcus
- Allobaculum
- Bacillus
- Corynebacterium
- Defini
- Lactobacillus
- Methylobacterium
- Oscillospira
- Propionibacterium
- Ruminococcus
- Staphylococcus
- Turicibacter
**Figure 12.** Effects of TCDD on murine skin microbiota revealed by 16S rRNA gene sequencing. Time-mated mice were treated by gavage with either corn-oil or TCDD (5 μg/ kg bw) and pups were analyzed for effects on the skin microbiome. (A) Alpha diversity of skin microbiota as measured by Shannon index (y-axis) following the indicated treatments and time. *p < 0.05, significance was analyzed with Wilcoxon rank sum test. (B) Stacked bar plot of mean relative abundance (y-axis) of the top 12 genera identified in the skin microbiota following the indicated treatments and time. (C) MA-plot comparing differentially enriched OTUs between corn-oil- or TCDD-exposed skin microbiota at P21. Shown is log2 mean abundance (x-axis) and log2 fold-change of normalized OTU counts (y-axis). Color of points indicates whether OTUs were significantly increased in TCDD (red), corn-oil (blue), or not significant (NS; grey). Top 30 most significant OTUs that have a genus-level taxonomy assignment are highlighted. Note: TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; rRNA, ribosomal RNA; bw, body weight; OUT, operational taxonomic plot; P, postnatal day.

**TCDD-responsive cells in skin**

Sebaceous glands are maintained by a population of stem and progenitor cells that reside in specific niches of the hair follicle. To determine if these cells are possible targets of TCDD, skin samples at P21 were immunostained with CYP1A1 and CYP1B1, as well as markers of skin progenitor cells. Since CYP1A1 is specifically induced at the infundibulum along with CYP1B1 (Figure 11B-C, 13A, g-h), triple immunofluorescence staining was performed to study co-localization of CYP1A1 and CYP1B1 with leucine-rich repeats and immunoglobulin-like domains protein 1 (LRIG1), a marker of sebocyte progenitor cells located at the infundibulum and junctional zone. As shown in Figure 13A (i-k), LRIG1 co-localized with both CYP1A1 and CYP1B1. In TCDD-exposed skin, CYP1B1 also co-localized with leucine rich repeat containing G protein-coupled receptor 6 (LGR6), at the infundibulum and bulge region (Figure 13B, e-h). LGR6 is a well-established marker of sebaceous stem cells that reside specifically in the isthmus (Jaks et al., 2010; Page et al., 2013; Snippert et al., 2010) but is also expressed in the progenitor cells in the bulge, sebaceous glands and epidermis (Fullgrabe et al., 2015). To verify the specificity of LGR6 staining in our study, LGR6 antibodies from two different sources
(detailed in Figure legend) were used to co-stain skin samples. Co-localization of LGR6 signals with the two LGR6 antibodies confirmed the specificity of LGR6 antibody (Figure 13C).
Figure 13. Expression of skin stem cell progenitor markers and AHR biomarkers in skin. Time-mated mice were treated by gavage with either corn-oil or TCDD (5 μg/ kg bw) and pups were analyzed for specific localization of response to TCDD. Corn-oil- or TCDD-exposed mice skin at P21 were labelled with antibodies for markers as indicated. DAPI (blue) stains nuclei. (A) CYP1A1, CYP1B1 and LRIG1 expression in corn-oil- and TCDD-treated skin samples. Images show control skin (a-d) and TCDD-exposed skin (e-k). (B) CYP1B1 and LGR6 expression in corn-oil- and TCDD-treated skin. Images show control skin (a-d) and TCDD-exposed skin (e-h). Boxed areas in (a) and (e) are shown at higher magnification in (d) and (h) respectively. (C) LGR6 expression (a-d) as detected with co-staining with two anti-LGR6 antibodies. LGR6 (SC) indicates staining with anti-LGR6 antibody (sc-393010, Santa Cruz Biotechnology) and LGR6 (Pt) indicates staining with anti-LGR6 antibody (17658-1-AP, Proteintech). Anti-LGR6 antibody from SantaCruz was used for co-localization studies in panel B. Scalebar = 25 μm. Note: AHR, aryl hydrocarbon receptor; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; bw, body weight; P, postnatal day; CYP, cytochrome P450; LRIG1, leucine rich repeats and immunoglobulin-like domains protein 1; LGR6, leucine rich repeat containing G protein-coupled receptor 6.

Summary

In this study, we investigated the effects of perinatal exposure to TCDD on the development of epidermal barrier and susceptibility to skin conditions like chloracne and AD in C57BL/6J mice. TCDD exposure resulted in AHR dependent acceleration of epidermal barrier formation in utero and diffuse epidermal hyperplasia at birth. However, this effect did not persist to other times. The animals grew with no visible signs of eczema and did not develop any AD-related pathology such as skin lesions, increased serum IgE and Th2 immune response. Because TCDD is immunosuppressive, we used a vitamin D analogue (MC903) to induce AD-like inflammation in skin. No significant differences in response to MC903 were observed between TCDD-exposed and the control animals. Animals in both groups exhibited visible skin inflammation with epidermal hyperplasia and elevated Th2 immune response in response to MC903. TCDD-exposed animals exhibited sebaceous gland hypoplasia, reminiscent of chloracne, at P13-21. Additionally, four of the treated animals developed keratinous cysts indicating
chloracne-like lesions. These effects were reversed by P35. Similarly, analysis of the skin microbiome showed increased bacterial diversity in TCDD-exposed skin at P21 that was no longer evident by P35. To determine if TCDD is cleared rapidly in these animals causing transient effects, we examined levels of CYP1A1 and CYP1B1, biomarkers of AHR activation. Both RNA and protein expression of CYP1A1 and CYP1B1 increased in the TCDD-exposed skin at P13-21 that returned to control levels by P35. To identify specific cell-types targeted by TCDD, we studied localization of CYP1A1 and CYP1B1 expression. Specifically, both CYP1A1 and CYP1B1 protein expression co-localized with LRIG1+ progenitor cells at the infundibulum. CYP1A1 was detected only there, while CYP1B1 was also expressed throughout the epidermis, sebaceous glands, isthmus, and bulge indicating multiple cell-type targets of TCDD. CYP1B1 protein also colocalized with LGR6+ progenitor cells at the infundibulum and bulge.
Chapter 4: Knockdown of the Aryl Hydrocarbon Receptor increases CDKN1B
(p27KIP1) and promotes differentiation in keratinocytes.

Introduction

The epidermis is a stratified squamous epithelium consisting of a basal layer, a suprabasal layer, a granular layer, and an uppermost stratum corneum layer. Keratinocytes in the basal layer are highly proliferative and are undifferentiated. As the process of differentiation is initiated, the basal keratinocytes exit from the cell cycle and subsequently move into the suprabasal layers. On terminal differentiation, these cells are anucleated and filled with keratins to form the corneocytes that are surrounded by a proteinaceous structure called the cornified envelope (CE). This is a highly insoluble structure formed by crosslinking of precursor proteins like involucrin (IVL), loricrin (LOR), small proline-rich region proteins (SPRRs) and others by calcium-dependent transglutaminases (TGM) (Nemes & Steinert, 1999). Stratum corneum is composed of 10–15 layers of corneocytes surrounded in a lipid matrix that collectively provide the barrier function of the skin (Candi et al., 2005).

This entire process of cell exiting the cell cycle and terminally differentiating is critical for continuous renewal of epidermis and so the balance between proliferation and differentiation of the keratinocytes must be tightly regulated to maintain epidermal homeostasis (Fuchs & Raghavan, 2002). The role of the AHR in regulating this balance is not well understood. In several studies, we and others have shown a physiological role of the AHR in keratinocyte differentiation and development of the epidermal barrier. Ligand activation of the AHR upregulates several genes involved in the epidermal differentiation process and ceramide synthesis as well as accelerates barrier formation in
vivo (Loertscher et al., 2002; Loertscher et al., 2001; Muenyi et al., 2014; Sutter et al., 2011; Sutter et al., 2009). To understand how the AHR affects human keratinocyte differentiation, we used short hairpin RNAs to decrease AHR protein levels.

Results

AHR knockdown using short hairpin RNA (shRNA)

The immortalized keratinocyte cell line, N/TERT-1 cells, were transduced with lentiviral shRNA particles resulting in approximately 93% reduction in AHR mRNA and 80% reduction in AHR protein levels (Figure 14A, B). Reducing AHR levels blocked TCDD-mediated induction of CYP1A1 mRNA (Figure 14C) further validating AHR knockdown in these cell line.
**Figure 14.** AHR knockdown (KD) in N/TERT-1 cells. N/TERT-1 cells were stably transduced with empty vector or a shRNA to knockdown AHR expression. (A) AHR mRNA expression as measured by qPCR in AHR KD as compared to empty vector N/TERT-1 cells (n = 3). All values are means ± SDs. *p < 0.05 compared to vector control; significance was determined with a two-tailed Student’s t test. (B) AHR protein levels in AHR KD as compared to empty vector N/TERT-1 cells (n = 3). All values are means ± SDs. *p < 0.05 compared to vector control; significance was determined with a two-tailed Student’s t test. (C) CYP1A1 mRNA induction in AHR KD as compared to empty vector N/TERT-1 cells. Cells were treated with either 0.1% DMSO or 10 nM TCDD for 24 hours (n = 3). All values are means ± SDs. *p < 0.05 compared to vehicle control and †p < 0.05 compared to vector control; significance was determined with two-way ANOVA, followed by the Tukey test. Note: KD, knockdown; shRNA, short hairpin RNA; AHR, aryl hydrocarbon receptor; SD, standard deviation; CYP, cytochrome 450; DMSO, dimethyl sulfoxide; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; ANOVA, analysis of variance.

**AHR knockdown increases keratinocyte differentiation in N/TERT-1 cells**

Expression levels of different markers of keratinocyte differentiation were determined in the AHR knockdown in N/TERT-1 cells. Knocking down of the AHR resulted in upregulation of FLG, LOR, and hornerin (HRNR) mRNA (Figure 15A-C). Similarly, protein levels of FLG, IVL and TGM1 also were significantly higher in the AHR knockdown cells as compared to control vectors (Figure 15 D-F). Further, knocking down the AHR in these cells also significantly increased cornified envelope (CE) formation, an indicator of terminal differentiation (Figure 15G).
Figure 15. AHR knockdown increases keratinocyte differentiation in N/TERT-1 cells. Control vectors and AHR knockdown cells were grown to 48 hours post confluence before treating them with or without basal media for an additional 24 hours or 48 hours. (A-C) FLG, LOR, and HRNR mRNA expression as measured by qPCR 24 hours post indicated treatments (n = 4). All values are means ± SDs. *p < 0.05 compared to vector control and †p < 0.05 compared to same treatment in vector control; significance was determined with two-way ANOVA, followed by the Tukey test. (D-F) Relative protein levels of FLG, IVL, and TGM1 in AHR KD and control vectors 48 hours post treatment. For all immunoblots, representative images are shown. Quantitation of protein levels was completed by densitometry, normalized with levels of ACTB (n = 4). All values are means ± SDs. *p < 0.05 compared to vector control and †p < 0.05 compared to same treatment in vector control; significance was determined with two-way ANOVA, followed by the Tukey test. (G) Total percentage of CEs as measured in cells treated with either 0.1% DMSO or 10 nM TCDD for 5 days. All values are means ± SDs. *p < 0.05 compared to vehicle control and †p < 0.05 compared to vector control; significance was determined with two-way ANOVA, followed by the Tukey test. Note: AHR, aryl hydrocarbon receptor; FLG, filaggrin; LOR, loricrin; HRNR, hornerin; qPCR, quantitative PCR; SD, standard deviation; ANOVA, analysis of variance; TGM1, transglutaminase1; KD, knockdown; ACTB, actin beta; CE, cornified envelope; DMSO, dimethyl sulfoxide; TCDD, 2,3,7,8- tetrachlorodibenzo-p-dioxin.

CDKN1B is upregulated in AHR knockdown N/TERT-1 cells

As these cells grow slowly and reports by others (Frauenstein et al., 2013; Pollet et al., 2018) link AHR knockdown to elevation of cyclin-dependent kinase inhibitors, we measured this family of proteins. Levels of CDKN1B (p27 KIP1) were increased in AHR knockdown cells (Figure 16C). Taken together, these findings indicate that knocking down AHR upregulates p27 in N/TERT-1 cells to promote proliferation to differentiation switch.
Figure 16. CDKN1B is upregulated in AHR knockdown N/TERT-1 cells. Control vectors and AHR knockdown cells were grown to 50% confluence before treating them with basal media for 24 hours. Relative CDKN1B protein levels in control vector or AHR KD. Representative image of immunoblot shown. Quantitation of protein levels was completed by densitometry, normalized with levels of ACTB (n = 3). All values are means ± SDs. *p < 0.05 compared to vector control; significance was determined with a two-tailed Student’s t test. Note: CDKN1B, cyclin dependent kinase inhibitor 1B; AHR, aryl hydrocarbon receptor; ACTB, actin beta; SD, standard deviation.

Summary

A genetic knockdown of the AHR was generated to evaluate the influence of loss of AHR on differentiation of keratinocytes. Overall, knockdown of the AHR in keratinocytes resulted in increased CDKN1B levels and increased keratinocyte terminal differentiation. Ongoing studies explore the relationship between AHR knockdown and elevated CDKN1B in relation to increased keratinocyte differentiation.
Chapter 5: Discussion

Decades of AHR research have identified a spectrum of TCDD-induced health hazards. Studies show activation of the AHR during critical periods of development can have long lasting effects. In mice, in utero exposure to TCDD adversely affects the development of multiple organs and tissues, including cardiac, neuronal, prostrate, and kidney development (Aragon et al., 2008; Carreira et al., 2015; Kimura et al., 2015; Vezina et al., 2009). These findings are in line with the DOHaD hypothesis that postulates that exposure to certain environmental influences during critical periods of development determines long-term health (Barker, 2007).

Skin is a continuously renewing organ that protects the inside of the body from all external insults while keeping water loss at a minimum through the epidermal permeability barrier. In mice, the development of the epidermis begins at E9 with a single layer of epidermal cells. This single layer begins stratification at E13, and a complete barrier is formed around E16-17 (Hardman et al., 1998; O'Shaughnessy & Cristiano, 2004). In utero, human fetuses are exposed to maternal dioxins through the placenta (Schecter et al., 1998). Additionally, since dioxins accumulate in fat tissue due to their lipophilicity, breast milk is an additional source of exposure for breastfed newborns (Nau et al., 1986). Breast feeding for 6 months contributes to about 12% and 14% of total cumulative dioxin content until 25 years in boys and girls, respectively (Patandin et al., 1999). Thus, for a 70-year lifetime, an individual who has been breast-fed accumulates 3-18% higher dioxin content than an individual who has not been breast-fed (Lorber & Phillips, 2002). Previous studies demonstrated epidermal barrier acceleration and defects in the skin of fetuses exposed to TCDD in utero, but mice past the age of P1 were not
investigated (Muenyi et al., 2014; Sutter et al., 2011). To investigate the effect of TCDD on neonatal epidermal barrier health and increased susceptibility of AD in adulthood we treated pregnant dams with TCDD on gestational day 12, exposing the pups in utero and via lactation to TCDD. TCDD-mediated AHR activation resulted in epidermal acanthosis at birth, however this effect did not persist into any other stages of development and the pups did not show signs of atopy. These results are contrary to the transgenic mice that express constitutively active AHR (AHR-CA) in the epidermis and develop an AD-like phenotype in adulthood (Tauchi et al., 2005). Two major differences between these two models may explain the discrepancy in the outcomes. The first is a difference between systemic versus localized activation of the AHR. In utero TCDD exposure activates the AHR systematically in multiple tissues, while the AHR-CA model specifically activates the AHR in keratinocytes. Further, it is known that AHR activation induces various dose- and cell type-dependent responses (Jackson et al., 2015). The second difference between these two models is the duration of AHR activation. The AHR-CA mice develop eczematous changes of the skin at 5 weeks of birth (Tauchi et al., 2005). Here, using the very sensitive biomarkers, CYP1A1 and CYP1B1, TCDD-mediated activation of the AHR was observed to be highest at P13 and P21. By P35, both CYP1A1 and CYP1B1 levels in TCDD-exposed skin returned to control levels indicating that the AHR was no longer activated, likely due to the relatively shorter half-life of TCDD in mice (11 days) (Gasiewicz et al., 1983). Prolonged AHR signaling such as in the AHR-CA mice appears necessary for mice to develop AD. Notably, such differences in outcomes of transient and sustained AHR activation have been reported in processes such as cell cycle transit where transient activation allows progression whereas sustained activation results in cell cycle
arrest (Mitchell & Elferink, 2009). It should be noted, that although prolonged activation of the AHR appears to be necessary for the skin effects studied here, other effects of in utero and lactational exposures, such as T-cell impairment, are maintained long after TCDD is cleared and have been attributed to persistent changes in DNA methylation (Burke et al., 2021; Winans et al., 2015).

Skin conditions like AD contain dysbiotic microbiome that contribute to disease pathogenesis (Kong et al., 2012). Hypothesizing an AD-like effect in TCDD-treated mice, the microbial community was analyzed to determine if the skin microbiome is altered as reported in cases of AD. TCDD-mediated AHR activation during development did not affect initial microbiome assembly as compared to controls. However, by P21, TCDD-exposed mice skin exhibited increased microbial diversity. The influence of TCDD on the skin microbiome is understudied but reports on gut microbiome in mice have shown TCDD exposure results in dysbiotic gut microbiota and alterations in microbiota-host metabolic homeostasis (Lefever et al., 2016; Stedtfeld et al., 2017; Tian et al., 2020). Here, TCDD-exposed mice showed sebaceous hypoplasia with altered sebaceous gland morphology and function at P21. Sebaceous glands are attached to the hair follicle and release lipids-rich sebum through the follicular duct to the skin surface. Sebum not only provides a hydrophobic coating protecting and lubricating the skin, but also affects the bacterial composition of skin (Skowron et al., 2021). Levels of long-chain unsaturated free fatty acids are correlated with bacterial composition, in particular with *Propionibacteria* and *Corynebacteria* abundance (Baurecht et al., 2018). Similarly, the anoxic environment in the sebaceous glands support the growth *Propionibacterium acnes*, a facultative anaerobic bacteria found commonly in the skin (Grice & Segre, 2011;
Further, the breakdown of sebum and release of fatty acids along with antimicrobial components by the skin microbial community controls microbial colonization (Sanford & Gallo, 2013). As the microbial community is greatly dependent on the maintenance of the skin topography and immune responses (Prescott et al., 2017), the resulting TCDD-mediated epidermal changes at P21 might contribute to the altered skin microbiome. The converse may also be true, whereby TCDD-mediated alterations of the microbiome might contribute to epidermal changes.

Sebaceous hypoplasia is one of the key features of chloracne, the most consistent indicator of TCDD toxicity in humans (Suskind, 1985). In mouse skin, the first sebocytes become evident shortly after birth (Paus et al., 1999). Soon, a cluster of sebocytes is formed that generates prominent sebaceous glands at P6-9 (Niemann & Horsley, 2012; Paus et al., 1999). Specific stem and progenitor cells present at the basal layer and periphery of sebaceous glands drive the continuous regeneration of sebocytes and sebum production (Jensen et al., 2009; Niemann & Horsley, 2012). Involution of sebaceous glands in TCDD-treated mice started at P13, with the greatest reduction in gland size observed at P21. These changes coincided with the levels of CYP1A1 and CYP1B1 mRNA and protein levels, which were also maximum at P21. Moreover, as CYP1A1 and CYP1B1 levels in mice skin returned to control levels by P35, the sebaceous glands were also restored by this time point, showing the effect on sebaceous glands was reversible. This indicates that the effects were not long-lasting, but rather dependent on the extent of AHR activation. Similar involution of sebaceous glands have been reported, when adult (4-8 weeks) haired mice were topically exposed to TCDD for 2 weeks (Puhvel & Sakamoto, 1988) and 5 weeks (Fontao et al., 2018). Additionally, we observed that out of
eleven total TCDD-exposed mice studied, four developed keratinous cyst-like structures, indicative of chloracne-like lesions. Since this effect was not commonly observed, the differences could be due to individual differences in susceptibility to TCDD. Such chloracne-like lesions have only been observed after a 2-year gavage with 3,3',4,4'-tetrachlorazobenzene (Ramot et al., 2009).

The observed involution of sebaceous glands in each of these studies, irrespective of mode of exposure, could indicate a particular group of skin cells with enhanced sensitivity to TCDD. During homeostasis, sebaceous glands are maintained by progenitor cells harbored in specific niches of hair follicle, particularly LRIG1+ cells in the junctional zone and LGR6+ cells in the isthmus (Jensen et al., 2009; Snippert et al., 2010). To determine if any of these cell populations were targeted by TCDD, we co-stained skin samples with these markers of progenitor cells together with CYP1A1 and CYP1B1. As reported previously, CYP1A1 colocalized with LRIG1+ cells at the infundibulum/junctional zone (Fontao et al., 2018). Further, CYP1B1 also colocalized with LRIG1+ cells at the junctional zone and isthmus. Since CYP1A1 specifically localized at the infundibulum/junctional zone, we only studied the co-localization of CYP1B1 with LGR6. CYP1B1 expression colocalized with LGR6 at the infundibulum and bulge. This indicates that not only the LRIG1+, but also LGR6+ progenitor cells are targets of TCDD. Further, immunohistochemical staining shows elevated expression of CYP1B1 in epidermis, infundibulum, isthmus, sebaceous glands and bulge, indicating more than one cellular target of TCDD.

The AHR is involved in pathways critical to cell cycle, differentiation and apoptosis (Puga et al., 2009). Thus, stem cells that require a balance between self-renewal
and differentiation are sensitive targets of AHR. Accordingly, B lymphocyte-induced maturation protein1 (Blimp1), a c-Myc repressor and marker of sebocyte precursor cells, is reduced in the human sebocyte cell line SZ95 by AHR knockdown (Ikuta et al., 2010). Blimp1 regulates expression of c-Myc to maintain sebaceous homeostasis and either Blimp1 deletion or c-Myc overexpression, results in enlarged sebaceous glands (Cottle et al., 2013; Horsley et al., 2006; Lo Celso et al., 2008). Additionally, TCDD treatment induces atrophy of SZ95 sebocytes (Ju et al., 2011). Lineage tracing studies in mice have shown that Blimp1+ sebocytes are progeny of the LRIG1+ and the LGR6+ progenitor cells (Horsley, 2011; Kretzschmar et al., 2014). Thus, alteration of Blimp1 and c-Myc signaling by AHR activation in LRIG1+ and LGR6+ sebaceous progenitor cells may be causing the observed sebaceous atrophy. Clarification of the role of AHR activation in the commitment to differentiation of these progenitor cells would improve the understanding of the molecular mechanisms underlying chloracne.

The AHR is the mediator of TCDD toxicity in the skin and other tissues. Particular to the keratinocytes, TCDD upregulates keratinocyte terminal differentiation in vitro (Kennedy et al., 2013; Sutter et al., 2011; Sutter et al., 2009) however the role of the AHR in normal physiology of the skin is not well understood. We generated a genetic knockdown of the AHR in the keratinocytes. Surprisingly, AHR knockdown in immortalized human keratinocytes resulted in increased keratinocyte differentiation. Moreover, these AHR knockdown cells grew slower as compared to controls. Studies with other cell lines have reported that knocking down AHR resulted in increased expression of CDKN1B (p27Ki67), a cyclin-dependent kinase inhibitor and a cell cycle regulator that controls cell cycle progression (Frauenstein et al., 2013; Pollet et al., 2018).
Similar to these studies, levels of p27$^{\text{Kip1}}$ was found to be elevated in the AHR knockdown cells in our study. Upregulation of cyclin dependent kinase inhibitors like p21$^{\text{Cip1/WAF1}}$ and p27$^{\text{Kip1}}$ has been associated with growth arrest and onset of terminal differentiation in keratinocytes (Hara et al., 2011; Hauser et al., 1997; Kolly et al., 2005). The increased expression of p27$^{\text{Kip1}}$ mRNA in keratinocytes was due to suppression of JNK/c-Jun signaling (Hara et al., 2011). Further studies are necessary to explore the relationship between AHR knockdown, elevated p27$^{\text{Kip1}}$, cell cycle, and keratinocyte differentiation. Overall, our findings show that either activating AHR with TCDD or compromising AHR in keratinocytes, both results in increased keratinocyte differentiation. This indicates an endogenous role of AHR in keratinocyte differentiation and that modulating AHR signaling disturbs this function.
Chapter 6: Conclusions and outlook

For a long time, AHR has been studied as a mediator of dioxin toxicity. More recently, AHR is being investigated for its involvement in physiological functions, development, and homeostasis. However, the outcomes of AHR activation are often dependent on the cell type and context and so they can result in dissenting effects. Moreover, the discovery of non-canonical AHR signaling pathways adds to the complexity of AHR target genes and their involvement in toxic and normal physiological processes. Similarly, the role of AHR in driving inflammatory skin diseases like AD has been inconsistent. AD is a chronic pruritic inflammatory skin disease that affects up to 25% of children and 3% of adults (Carroll et al., 2005). Although the pathogenesis of AD is not completely understood, it is often associated with defective barrier and immune dysregulation (Boguniewicz & Leung, 2011). Increasing evidence suggests a correlation between chronic exposure to air pollution and inflammatory skin diseases (Ahn, 2014; Brauer et al., 2007; Mancebo & Wang, 2015). However, little is known about how environmental pollutants cause in these diseases. Coal tar, one of the oldest therapies for AD, is rich in polycyclic aromatic hydrocarbons (PAH) and activates the AHR (van den Bogaard et al., 2013). More importantly, topical application of the bacteria derived AHR agonist, tapinarof, alleviates the clinical symptoms of AD and is currently in clinical trials of being developed as a therapeutic agent for psoriasis and AD (Bissonnette et al., 2018). In contrast, POPs and PAHs can exacerbate inflammatory skin conditions in an AHR-dependent manner (Hidaka et al., 2017). Activation of the AHR by either diesel exhaust particles or DMBA induces expression of artemin, resulting in epidermal hyper-innervation and increased inflammation in mice (Hidaka et al., 2017). Moreover,
keratinocyte-specific activation of the AHR in transgenic mice causes these mice to develop AD-related pathologies postnatally (Hidaka et al., 2017; Tauchi et al., 2005). On the contrary, our study shows that activation of the AHR with TCDD, the most toxic agonist, during development resulted in chloracne-like phenotype and did not increase susceptibility of the animals to develop AD. Clearly, AHR serves an interesting candidate to study as it is both a toxic and therapeutic target.

Our findings show that perinatal exposure to TCDD resulted in sebaceous hypoplasia in mice at postnatal day 21 indicating sebocytes in skin are sensitive targets of AHR. Sebaceous hypoplasia is a prominent feature of chloracne, a dioxin-induced skin toxicity in humans. Molecular mechanisms of chloracne are still to be elucidated, but AHR activation in the sebaceous progenitor cells as observed in our study indicate that these progenitor cells could be susceptible targets. The AHR may be involved in regulating the balance between proliferation and differentiation of the sebocytes. Further studies are required to illuminate the underlying mechanisms.
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