Genetic and Pharmacological Analysis Identifies a Physiological Role for the AHR in Epidermal Differentiation

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Stimulation of the aryl hydrocarbon receptor (AHR) by xenobiotics is known to affect epidermal differentiation and skin barrier formation. The physiological role of endogenous AHR signaling in keratinocyte differentiation is not known. We used murine and human skin models to address the hypothesis that AHR activation is required for normal keratinocyte differentiation. Using transcriptome analysis of $Ahr^{-/-}$ and $Ahr^{+/+}$ murine keratinocytes, we found significant enrichment of differentially expressed genes linked to epidermal differentiation. Primary $Ahr^{-/-}$ keratinocytes showed a significant reduction in terminal differentiation gene and protein expression, similar to $Ahr^{+/+}$ keratinocytes treated with AHR antagonists GNF351 and CH223191, or the selective AHR modulator (SAhRM) SGA360. *In vitro* keratinocyte differentiation led to increased AHR levels and subsequent nuclear translocation, followed by induced *CYP1A1* gene expression. Monolayer cultured primary human keratinocytes treated with AHR antagonists also showed an impaired terminal differentiation program. Inactivation of AHR activity during human skin equivalent development severely impaired epidermal stratification, terminal differentiation protein expression, and stratum corneum formation. As disturbed epidermal differentiation is a main feature of many skin diseases, pharmacological agents targeting AHR signaling or future identification of endogenous keratinocyte-derived AHR ligands should be considered as potential new drugs in dermatology.

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INTRODUCTION

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor and member of the bHLH/PAS (basic Helix-Loop-Helix/Per-Arnt-Sim) family (Shimizu *et al.*, 2000; Omiecinski *et al.*, 2010). The AHR regulates metabolism of drugs and environmental toxicants. Compounds such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and polycyclic aromatic hydrocarbons, as well as plant polyphenols and tryptophan photoproducts, are primarily agonists, although some flavonoid antagonists have been described (Murray *et al.*, 2010a). The AHR resides in a cytoplasmic multiprotein complex, which translocates to the nucleus upon agonist binding. There the receptor dissociates from HSP90 and dimerizes with the aryl hydrocarbon nuclear translocator (ARNT) to transactivate target genes primarily through dioxin response elements (DRE) in promoters of responsive genes (Omiecinski *et al.*, 2010). In humans, TCDD toxicity causes chloracne, which is associated with epidermal hyperproliferation and hyperkeratinization (Poland and Knutson, 1982), and increased expression of genes critical for the formation of the cornified envelope (Greenlee *et al.*, 1985; Loertscher *et al.*, 2001; Sutter *et al.*, 2009; Sutter *et al.*, 2011). In cell culture, TCDD induces the expression of genes in the epidermal differentiation complex, causing aberrant differentiation of keratinocytes (Geusau *et al.*, 2005; Sutter *et al.*, 2009; Sutter *et al.*, 2011). Six- to eight-month-old $Ahr^{-/-}$ mice exhibit alopecia associated with dystrophic and degenerating hair follicles, ulceration, and regenerative hyperplasia (Fernandez-Salguero *et al.*, 1997). We recently demonstrated in atopic dermatitis that coal tar therapy activates the AHR through an unknown agonist(s) and restores defective differentiation and barrier function (Van den Bogaard *et al.*, 2013). Together, these data suggest that dysregulation of normal AHR function could be important in the pathogenesis of chronic skin diseases with aberrant epidermal differentiation.

In addition to pathological AHR agonists such as TCDD, other AHR ligands can act either as full antagonists or selective modulators of AHR function with overlapping but

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Abbreviations: AHR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; DRE, dioxin response element; SAhRM, selective aryl hydrocarbon receptor modulator; TCDD, 2,3,7,8-Tetrachloro-p-dibenzodioxin

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nonidentical effects on AHR cellular activities in the cell (Boitano *et al.*, 2010; Murray *et al.*, 2010b; Smith *et al.*, 2011; Choi *et al.*, 2012; Lahoti *et al.*, 2013). These too may have significant therapeutic potential in suppressing some or all of the AHR-driven pathways in human disease. However, despite this extensive characterization of the pathogenesis and molecular biology of cutaneous responses to TCDD (Chiaro *et al.*, 2008a; Veldhoen *et al.*, 2009; Schroeder *et al.*, 2010), the role of the AHR in normal epidermal differentiation and homeostasis and the potential role of AHR ligands in therapy for cutaneous disease is poorly understood. Here we show that *in vitro* expression of differentiation genes and proteins is suppressed in $Ahr^{-/-}$ keratinocytes, that the AHR undergoes nuclear translocation during *in vitro* differentiation, and that AHR antagonists and selective modulators can block differentiation of human and mouse keratinocytes in monolayer culture and in human skin equivalents. These data underscore a significant physiological role of the AHR in normal epidermal differentiation.

RESULTS

The AHR regulates epidermal differentiation, attachment, and inflammatory cytokine gene expression

To identify AHR-dependent genes, we compared gene expression between $Ahr^{+/+}$ and $Ahr^{-/-}$ keratinocytes cultured in 0.05 mM Ca^{2+} growth medium using Affymetrix ST arrays and ArrayStar 11 Software (DNASTAR, Madison, WI). We identified 391 genes whose expression was altered by at least 1.5-fold ($P<0.05$) in $Ahr^{-/-}$ keratinocytes relative to $Ahr^{+/+}$. We used the DAVID Bioinformatics Software (Huang *et al.*, 2009b) to identify functional annotation clusters within the group of differentially regulated genes, and consistent with initial analysis the top functional annotation clusters were extracellular matrix and adhesion (Enrichment score 7.2, 5.4; $P=7.3\times10^{-7}$ and 9.7×10^{-8}, respectively) and keratinocyte, epidermal cell differentiation (Enrichment score 3.3; $P=2.1\times10^{-7}$). Of the top downregulated transcripts in $Ahr^{-/-}$ keratinocytes, 22 were linked to epidermal differentiation, including those for structural proteins, proteins involved in the formation of the cornified envelope, proteases and protease inhibitors, and the transcription factor *Pou2f3* (Skin1; Supplementary Table S1 online). Thirteen of the top upregulated transcripts in $Ahr^{-/-}$ keratinocytes encoded proteins associated with extracellular matrix and adhesion (Supplementary Table S2 online). Expression of genes linked to skin inflammatory diseases, *Il33*, *Il36g*, and thymic stromal lymphopoietin (*Tslp*), was downregulated in $Ahr^{-/-}$ keratinocytes, whereas the expression of *Il24* and *Il18r* was induced (Supplementary Table S1 and S2 online).

We compared the expression of representative epidermal differentiation genes in $Ahr^{+/+}$ and $Ahr^{-/-}$ keratinocytes cultured in proliferation medium or 24 hours after switching to differentiation medium (0.12 mM Ca^{2+}). In the absence of the AHR, both basal and induced expression levels of *Krt1, Lor, Ivl, Dsc1*, and the transcription factor *Pou2f3* were significantly reduced (Figure 1). Induction of differentiation with elevated calcium also increased the expression of the well-characterized AHR target gene *Cyp1a1* in $Ahr^{+/+}$ keratinocytes, but it was blocked in $Ahr^{-/-}$ keratinocytes (Figure 1). As expected, TCDD caused a significant induction of *Cyp1a1* in $Ahr^{+/+}$, but not in $Ahr^{-/-}$, keratinocytes (data not shown). Similarly, both *Il33* and *Il36γ* were significantly repressed in $Ahr^{-/-}$ keratinocytes relative to $Ahr^{+/+}$ in cells cultured in proliferation medium (Supplementary Figure S1 online).

AHR antagonists and selective modulators block epidermal differentiation in monolayer culture

AHR ligands that act as either full antagonists or selective modulators have been identified. GNF351 is a full antagonist of DRE and non-DRE AHR function, it interacts directly with the AHR ligand binding pocket, and it competes with a well-characterized photoaffinity AHR ligand for binding to the AHR, with an IC_{50} of 62 nM (Smith *et al.*, 2011). GNF351 blocks AHR target gene induction by TCDD, but it has no agonist activity for either DRE-dependent or -independent functions of the AHR. SGA360 is a selective AHR modulator (SAhRM), as it blocks TCDD-induced DRE-mediated AHR activity, but it has agonist-like activity for non-DRE mediated AHR functions (Patel *et al.*, 2009b; Tanos *et al.*, 2012). We treated $Ahr^{+/+}$ and $Ahr^{-/-}$ keratinocytes with TCDD or $Ahr^{+/+}$ differentiating keratinocytes with GNF351 or SGA360, and we measured changes in gene expression by

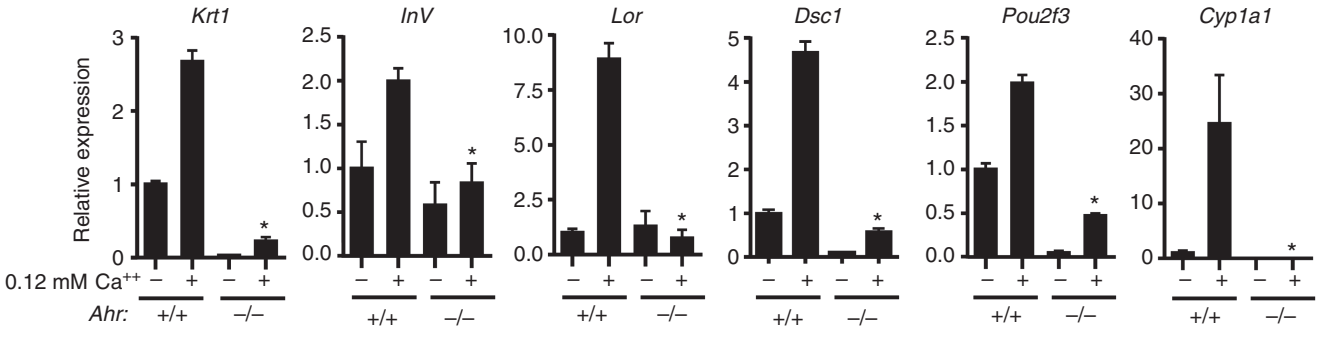

Figure 1. Downregulation of differentiation gene expression in $Ahr^{-/-}$ mouse keratinocytes. Expression of indicated genes during calcium-induced differentiation of primary $Ahr^{+/+}$ and $Ahr^{-/-}$ keratinocytes was determined by quantitative PCR from triplicate cultures, and it was repeated twice. Expression was normalized to *Gapdh*. *Krt1,* Keratin 1; *Lor*, Loricrin; *Ivl*, Involucrin; *Dsc1*, Desmocollin 1; *Pou2f3,* POU Class 2 Homeobox 3, *Cyp1a1*; Cytochrome P450 1A1. *significantly different from $Ahr^{+/+}$ $P<0.05$.

quantitative real-time reverse-transcriptase–PCR. Consistent with its known effects, TCDD induced the expression of genes involved in epidermal differentiation under proliferation conditions (Sutter *et al.*, 2011), which was blocked in $Ahr^{-/-}$ keratinocytes (Figure 2a). Both GNF351 and SGA360 suppressed the expression of early (*Krt1*, *Pou2f3*) and late (*Ivl*, *Lor*, *Dsc1*) differentiation genes in both basal and differentiation culture conditions. Similarly, GNF351 completely blocked the induction of *Cyp1a1* in differentiating keratinocytes, supporting the concept that the AHR becomes activated during normal epidermal differentiation (Figure 2a). In addition, both *Il33* and *Il36g* were significantly downregulated in $Ahr^{+/+}$ keratinocytes treated with either GNF351 or SGA360 (Supplementary Figure S1 online). To test whether similar effects occurred in human keratinocytes, we treated differentiating human primary keratinocytes with the AHR antagonists GNF351 and CH223191 and observed a comparable reduction in expression of *CYP1A1*, *FLG*, hornerin (*HRNR*), and *LOR* relative to the untreated control differentiating keratinocyte cultures. There was a trend toward induced epidermal differentiation with the AHR agonist indirubin, but this was not statistically significant (Figure 2b). FICZ (6-Formylindolo(3,2-b)carbazole), an AHR agonist generated in the skin from tryptophan by UV light (Fritsche *et al.*, 2007), also induced the expression of some, but not all, differentiation genes in human keratinocytes (Supplementary Figure S2 online). There was minimal toxicity of these AHR ligands in mouse or human keratinocytes (Supplementary Figure S3 online). Immunoblot analysis confirmed that genetic or pharmacological inactivation of AHR blocked the induction of keratin 10 and loricrin protein expression in mouse (Figure 2c) and of involucrin, loricrin, and pro-filaggrin in human keratinocytes (Figure 2d).

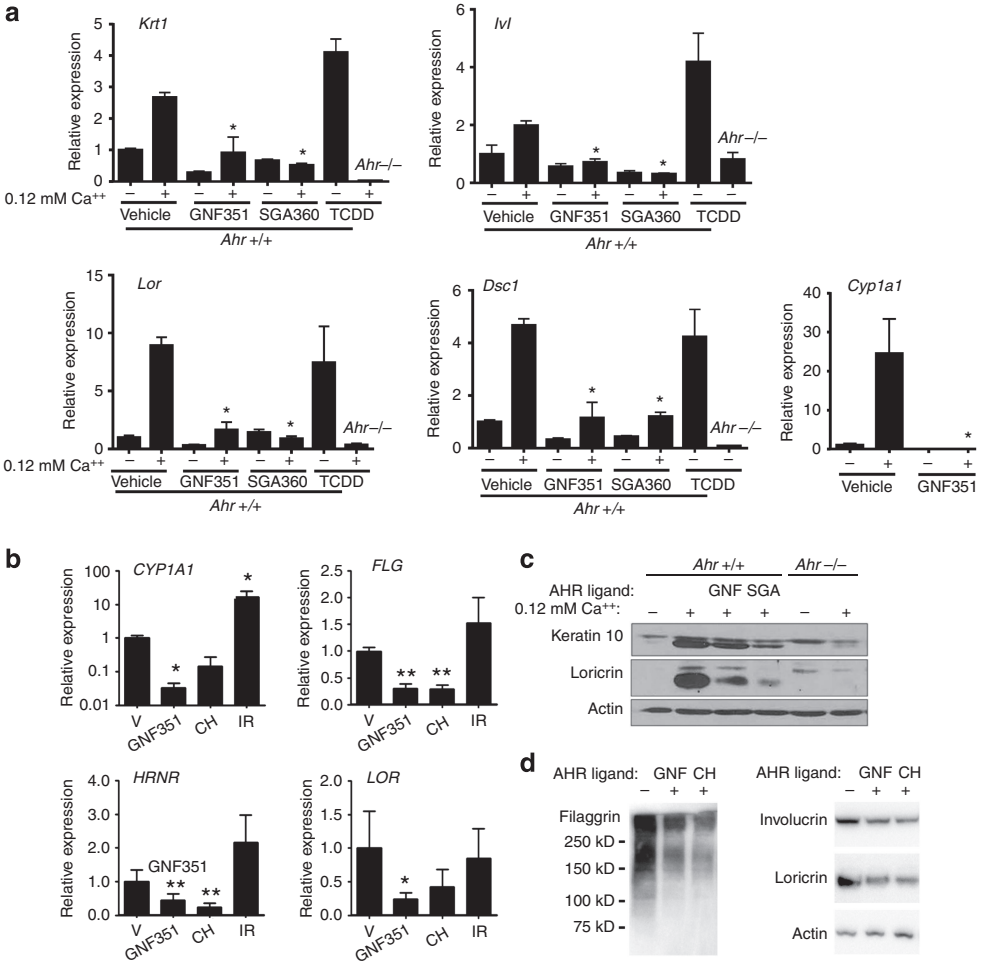

Figure 2. Aryl hydrocarbon receptor (AHR) antagonists and selective modulators suppress epidermal differentiation in monolayer culture. (**a**) Effect of 2,3,7,8-Tetrachloro-*p*-dibenzodioxin (TCDD; 10 nM), GNF351 (500 nM), and SGA360 (10 μM) on gene expression in proliferating (0.05 mM $CaCl_2$) or differentiating (0.12 mM $CaCl_2$) primary mouse keratinocytes (triplicate, repeated twice). (**b**) Effect of GNF351 (500 nM), CH223191 (CH, 5 μM), and indirubin (IR, 50 nM) on differentiation gene expression in primary human keratinocytes (two separate experiments, total of $n = 5$ donors). (**c**) Immunoblot analysis showing the effect of *Ahr* ablation, GNF351, or SGA360 on differentiation-induced expression of keratin 10 and loricrin in primary mouse keratinocytes. (**d**) Immunoblot analysis showing the effect of GNF351 and CH223191 (CH) on pro-filaggrin (FLG), involucrin (IVL), and loricrin (LOR) in monolayer cultured primary human keratinocytes. *$P < 0.05$ compared with vehicle control.

AHR antagonists suppress epidermal differentiation and stratum corneum thickness in human skin equivalents

To further examine the effect of AHR antagonists on epidermal differentiation, we generated epidermal skin equivalents using human primary keratinocytes cultured on plastic inert filters. We tested the effect of antagonists added at different time points during generation of the human skin equivalents. When the keratinocytes were in submerged culture (proliferation/attachment phase) or when monolayers were initially brought to the air–liquid interface, the addition of GNF351 or CH223191 substantially suppressed the stratification process and the formation of the stratum corneum (Figure 3a). Expression of late differentiation markers involucrin and filaggrin was strongly reduced, but the early differentiation marker keratin 10 was less affected (Figure 3b). Addition of antagonists during the last phase of air–liquid interface culture (from day 4 or 7 onward) resulted in thinning of the stratum corneum, but it did not affect involucrin or filaggrin expression (Figure 3b). However, when skin equivalents were generated using de-epidermized dermis, treatment with GNF351 4 days after transfer to the air–liquid interface reduced the expression and number of cell layers expressing loricrin and filaggrin, whereas the expression of keratin 10 was delayed (Figure 4). As AHR antagonists were added during the proliferation phase of the skin equivalent development (submerged culture), we tested whether they affected keratinocyte proliferation. There was a significant reduction in the percentage of Ki67-positive cells and cell number after treating proliferating monolayer cultures of human keratinocytes with AHR antagonists for 48 hours (Figure 5, Supplementary Figure S4 online). In contrast, skin equivalents generated on inert filters and treated with GNF351 during the submerged phase or at day one of transfer to the air–liquid interphase had more Ki67-positive basal cells at the end of the skin equivalent development compared with untreated cultures (Figure 3b).

Increased AHR nuclear localization during epidermal differentiation *in vitro*

To confirm whether the observed AHR dependence of differentiation gene expression was associated with AHR nuclear translocation, we isolated nuclear and cytoplasmic protein from primary mouse keratinocytes at specific time

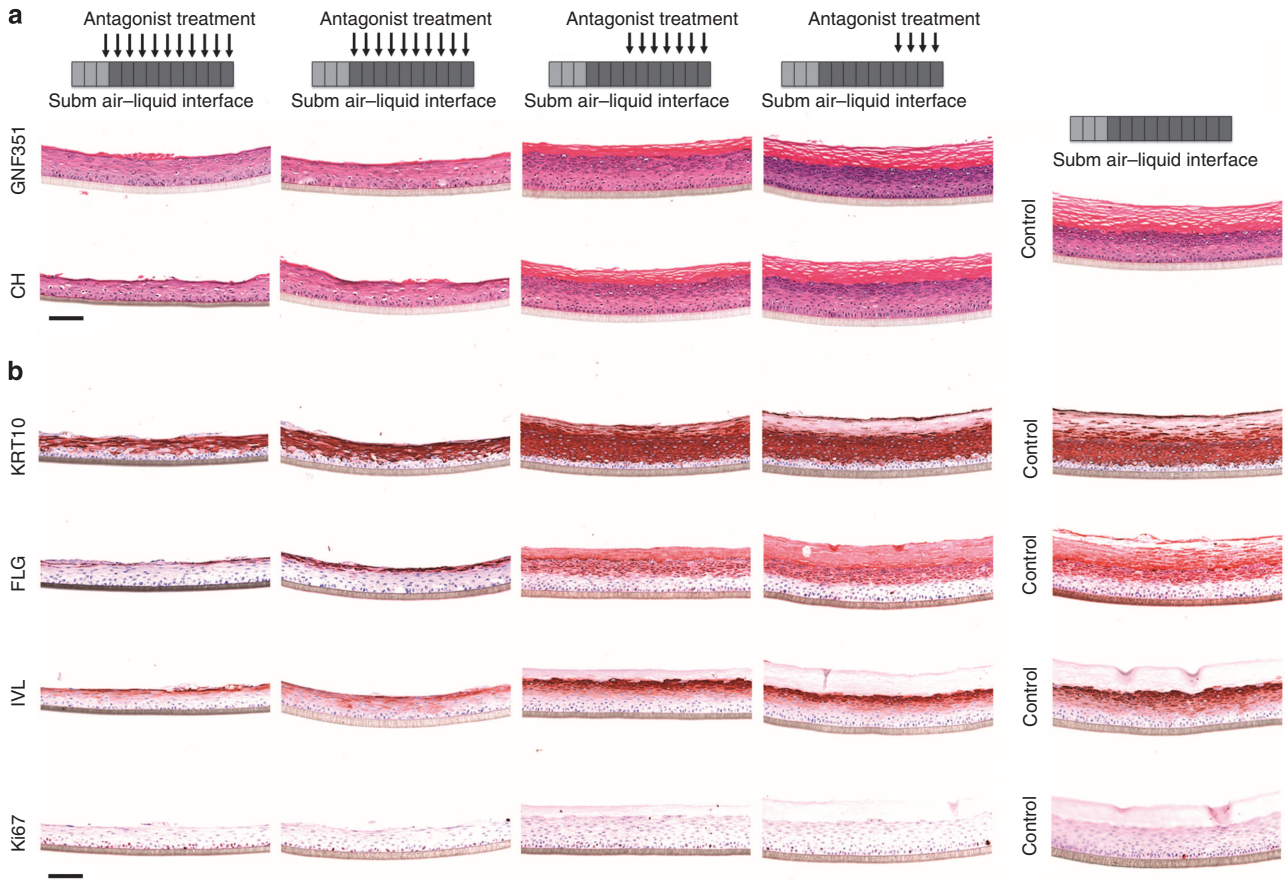

Figure 3. Epidermal stratification defects and reduced stratum corneum thickness caused by aryl hydrocarbon receptor (AHR) inactivation. Human skin equivalents (epidermis only) were generated on plastic inert filters. At indicated time points (arrows) during skin equivalent development (each block represents one day of culture), AHR antagonists were added to the culture medium. All skin equivalents were harvested at day 10 of air–liquid interface culture. (**a**) Hematoxylin and Eosin staining of skin equivalents treated with GNF351 (500 nM) or CH223191 (CH) (5 μM). (**b**) Immunohistochemical staining of Keratin 10 (KRT10, early differentiation), filaggrin (FLG, terminal differentiation), involucrin (IVL, terminal differentiation), and Ki67 (proliferation) of skin equivalents treated with GNF351, as depicted in Figure 2a ($n = 2$ keratinocyte donors). Scale bar = 100 μm.

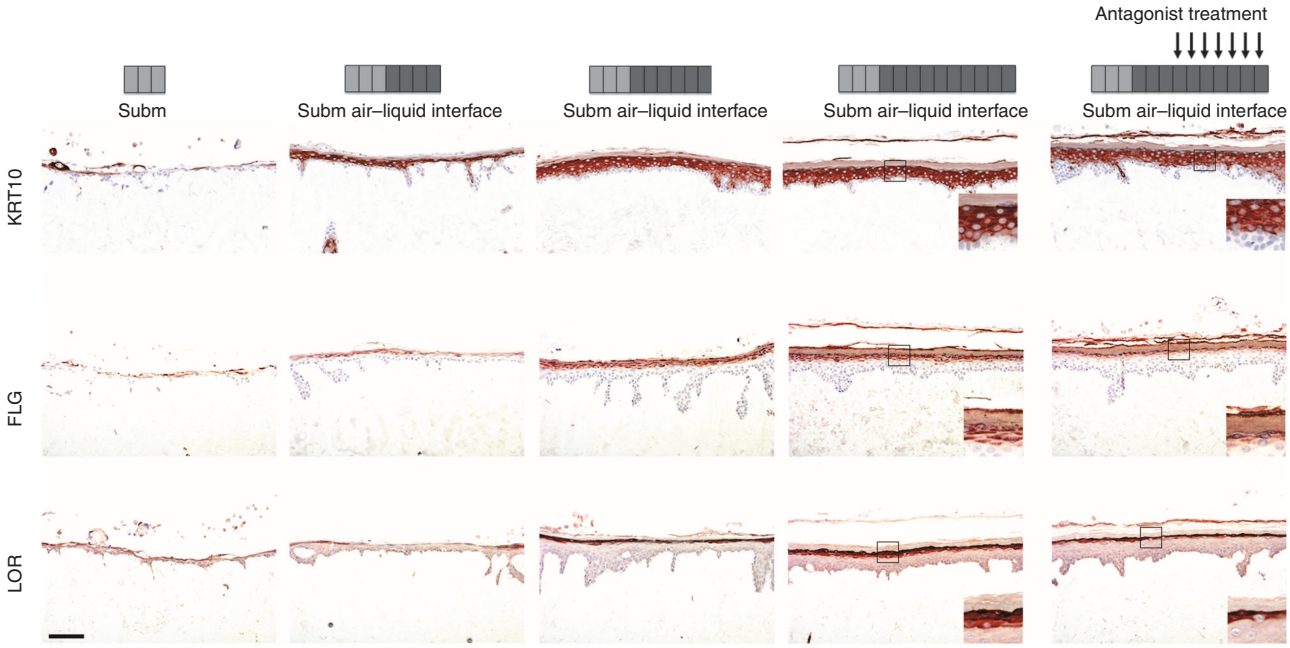

Figure 4. Reduced terminal differentiation protein expression caused by aryl hydrocarbon receptor (AHR) inactivation. Human skin equivalents were generated using de-epidermized dermis, and expression of keratin 10 (KRT10), filaggrin (FLG), and loricrin (LOR) was followed in time by harvesting the skin equivalents directly after submerged culture, and after 4, 6, and 10 days of air–liquid interface culture (each block represents one day of culture). Treatment with GNF351 (500 nM, arrows) was initiated at day 6 and sustained until day 10 of air–liquid interface culture. Magnification inlays show epidermal differentiation protein expression affected by AHR inactivation ($n = 2$ keratinocytes donors). Scale bar = 100 μm.

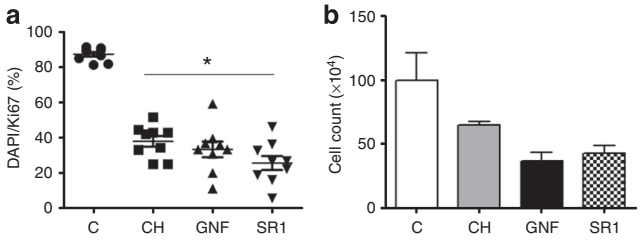

Figure 5. Aryl hydrocarbon receptor (AHR) antagonists suppress human keratinocyte proliferation. Monolayer cultures of human primary keratinocytes ($n = 3$ keratinocyte donors, * $P < 0.05$) were treated with AHR antagonists (GNF: GNF351, 500 nM; CH: CH223191, 5 μM; SR1, 500 nM) for 48 h during the proliferation stage of the culture. (**a**) Quantification of Ki67-positive cells and (**b**) total cell count of antagonist-treated keratinocytes as compared with untreated cells.

points after induction of differentiation with elevated medium calcium. Under basal proliferating conditions, AHR protein expression was detected in the cytosolic extracts, but not in nuclear extracts (Figure 6a). Six hours after induction of differentiation, there was an increase in nuclear AHR reaching a maximum at 12 hours that was sustained through 48 hours. AHR levels in cytosolic extracts also increased 12 hours after induction of differentiation, and this was sustained through 48 hours (Figure 6a). ARNT levels retained in the nucleus also increased during differentiation, whereas slightly reduced levels were detected in *Ahr*$^{-/-}$ keratinocytes (Figure 6a). Treatment with GNF351 for 24 hours enhanced nuclear AHR levels under differentiation conditions, whereas SGA360 increased cytosolic AHR levels and prevented nuclear retention. In proliferating keratinocytes, AHR agonists such as TCDD and ICZ (indolo[3,2-b]carbazole) caused rapid nuclear localization followed by significant loss of the protein from both the nuclear and cytoplasmic compartments, as expected, most likely owing to proteosomal degradation (Ikuta *et al.*, 1998; Davarinos and Pollenz, 1999; Ikuta *et al.*, 2000). GNF351 caused a slower and sustained increase in nuclear AHR and cytoplasmic AHR, whereas SGA360 completely blocked the presence of AHR retained in nuclear extracts and increased cytoplasmic AHR levels.

DISCUSSION

The AHR is the mediator of TCDD toxicity in the skin and other tissues. TCDD causes induction of keratinocyte terminal differentiation *in vitro*, and it accelerates skin barrier function *in utero* (Loertscher *et al.*, 2001; Sutter *et al.*, 2011; Muenyi *et al.*, 2014), suggesting that AHR activation by exogenous ligands can cause pathological dysregulation of keratinocyte differentiation. In contrast, recent studies point to the beneficial effects of AHR activation in skin and other tissues and its potential as a therapeutic target (Qiu *et al.*, 2012; DiMeglio *et al.*, 2014). However, the role of the AHR in normal skin physiology is poorly understood. Here we show that normal epidermal differentiation is regulated by AHR signaling in both murine and human keratinocytes: *Ahr*$^{-/-}$ mouse keratinocytes have defects in differentiation gene expression; AHR antagonists and SAhRMs suppress differentiation gene

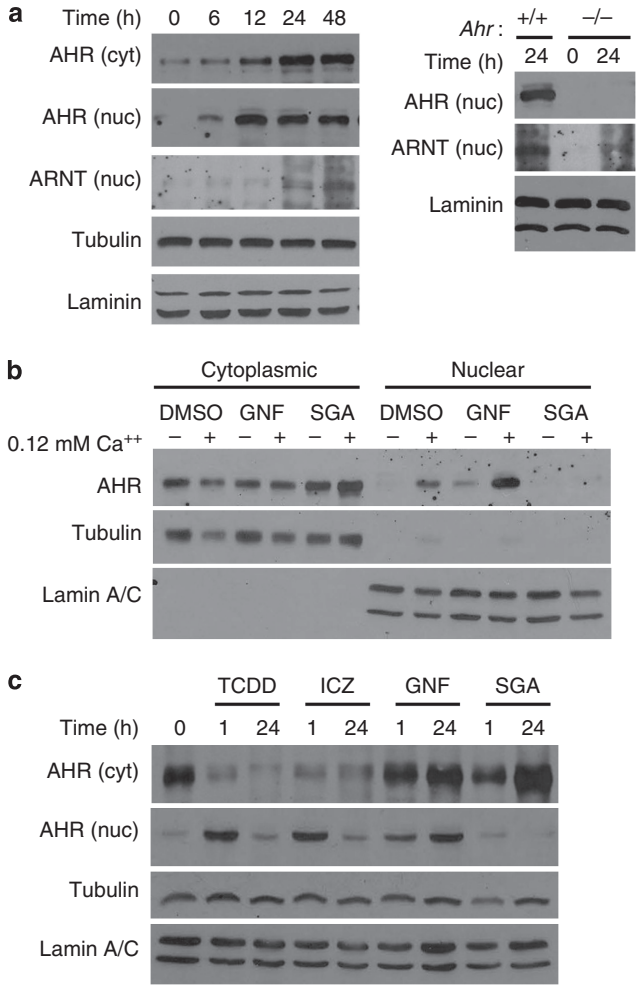

Figure 6. Nuclear localization of aryl hydrocarbon receptor (AHR) during epidermal differentiation. (**a**) Immunoblot of AHR and aryl hydrocarbon receptor nuclear translocator (ARNT) after induction of differentiation with elevated calcium medium. (**b**) Immunoblot of cytoplasmic and nuclear AHR in primary mouse keratinocytes induced to differentiate in the presence of GNF351 or SGA360. (**c**) Immunoblot showing time course of nuclear and cytoplasmic AHR levels in response to AHR ligands in primary mouse keratinocytes cultured in proliferation medium.

expression in monolayer culture of human and mouse keratinocytes and epidermal differentiation and stratification in human skin equivalent models. These data suggest a physiological role for the AHR during epidermal differentiation and stratification amenable to manipulation by pharmacological AHR antagonists or SAhRMs.

Our results are in concordance with earlier reports of elevated CYP1A1 enzyme levels in differentiated keratinocytes in the absence (Sadek and len-Hoffmann, 1994) or presence of xenobiotics (Reiners *et al.*, 1992), and the response to TCDD was higher in differentiated keratinocytes (Wanner *et al.*, 1995; Swanson 2004). In addition, retinoic acid, which interferes with epidermal differentiation, suppresses AHR induction during keratinocyte differentiation (Wanner *et al.*, 1995). In contrast, lentiviral knockdown of the AHR in 3D skin equivalents had no effect on epidermal morphology (Forrester *et al.*, 2014), but this may be owing to insufficient AHR inactivation (40% knockdown) compared with the AHR antagonists used here. The ability of AHR antagonists to suppress proliferation in human keratinocyte monolayers is consistent with cell cycle arrest and reduced proliferation caused by AHR knockdown in HaCaT cells (Kalmes *et al.*, 2011). As the AHR can activate the EGFR/extracellular signal–regulated kinase pathway through c-Src (Fritsche *et al.*, 2007; Sutter *et al.*, 2009), it is possible that AHR antagonist– and SAhRM-induced growth inhibition is indirect. However, this is unlikely, as keratinocyte cultures were supplemented with EGF. The increased numbers of Ki67-positive cells in skin equivalents treated with AHR antagonist at early stages of skin equivalent development is most likely owing to some type of feedback response to the disturbed differentiation or retention of cells with a basal cell phenotype owing to suppressed differentiation.

We observed increased levels of nuclear AHR in mouse keratinocytes after induction of differentiation. Translocation and activation of AHR is unlikely to be due to the indirect mechanism reported by Wincent *et al.* (2012), whereby inhibition of CYP1A1 increases culture media levels of the agonist FICZ, as differentiation induces CYP1A1 enzyme activity (Reiners *et al.*, 1990; Jones and Reiners, 1997). We hypothesize rather that keratinocyte differentiation generates endogenous AHR ligands, which drive translocation and activation. It is possible that differentiation-induced prostaglandin synthesis could mediate the observed nuclear translocation of the AHR, as prostaglandins and other arachidonic acid metabolites can act as AHR agonists (Seidel *et al.*, 2001; Chiaro *et al.*, 2008b), and increased Cox-2 expression is associated with epidermal differentiation *in vitro* and *in vivo* (Cameron *et al.*, 1990; Evans *et al.*, 1993; Leong *et al.*, 1996; Xu *et al.*, 2008), although this remains to be determined directly. Surprisingly, GNF351 treatment in differentiation and basal culture conditions also increased the nuclear AHR levels after 24 hours, whereas SGA360 blocked AHR retention in the nucleus. This is in direct contrast to AHR agonists TCDD and ICZ, which cause rapid nuclear translocation, followed by loss of receptor, as previously reported, suggesting that AHR nucleocytoplasmic shuttling and degradation induced by differentiation and AHR antagonists are distinct from that driven by exogenous agonists (Ikuta *et al.*, 1998; Davarinos and Pollenz, 1999; Ikuta *et al.*, 2000). Our results also show distinct mechanisms of action of the pure antagonist GNF351 and the SAhRM SGA360, as GNF351 appears to allow AHR nuclear translocation but to prevent AHR-mediated gene expression, whereas SGA360 blocks AHR nuclear translocation. Similar distinctions have been made for the regulation of inflammatory gene expression by SAhRMs, which is DRE-independent and involves the inhibition of AHR interaction with proinflammatory pathways, although pure antagonists also block nuclear retention of the AHR in other cell types (Patel *et al.*, 2009a; DiNatale *et al.*, 2010; Smith *et al.*, 2011; Tanos *et al.*, 2012). Computational analysis has indicated the presence of DRE in promoters for many terminal differentiation genes, and functional DREs have been identified in the human FLG gene (Sutter *et al.*, 2011). Our expression results reveal that keratinocyte differentiation gene expression

is suppressed upon ablation or pharmacological inhibition of AHR activity. In addition, nuclear localization of the AHR during differentiation suggests that endogenous AHR ligands drive translocation and binding of AHR/ARNT complexes to DREs present in the promoter region of differentiation genes, although this remains to be directly determined through chromatin immunoprecipitation and chromatin-immunoprecipitation sequencing. Interestingly, ablation of ARNT in mouse skin or knockdown in human keratinocytes induces the expression of a number of epidermal differentiation genes (Geng *et al.*, 2006; Robertson *et al.*, 2012), although *Flg* and *Lor* are reduced in $Arnt^{-/-}$ skin (Geng *et al.*, 2006), similar to our findings with $Ahr^{-/-}$ keratinocytes. However, this aberrant induction of differentiation gene expression may be indirect through downregulation of the EGFR ligand amphiregulin in the absence of ARNT (Robertson *et al.*, 2012). It is possible that some effects of AHR ablation or antagonism on differentiation could be indirect, through downregulation of the transcription factor Pou2f3 (Skn-1a) critical for epidermal proliferation and differentiation (Andersen *et al.*, 1997; Takemoto *et al.*, 2010).

We also observed upregulation of a significant number of genes encoding extracellular matrix and cell attachment proteins in $Ahr^{-/-}$ keratinocytes relative to $Ahr^{+/+}$, but it is not known whether this represents secondary changes owing to long-term ablation of AHR function, possible function of AHR as a repressor of gene expression, or reprogramming of primary keratinocyte epithelial phenotype. In $Ahr^{-/-}$ keratinocytes, we found a significant downregulation of three cytokine genes that are important in different inflammatory skin diseases *Il33*, *Il36g*, and *Tslp* (Shigeno *et al.*, 2009; Larson *et al.*, 2010; Carrier *et al.*, 2011; Hueber *et al.*, 2011; Balato *et al.*, 2012; Tortola *et al.*, 2012). Interestingly, suppression of *Il33* and *Il36g* expression by GNF351 and SGA360 suggests that AHR antagonists and selective modulators may be useful therapeutics for regulating skin inflammation. The observed upregulation of the proinflammatory cytokine IL24 (Kunz *et al.*, 2006; Kumari *et al.*, 2013) in $Ahr^{-/-}$ keratinocytes indicates that this may be more complex within a treatment setting.

Similar to our recent finding that AHR activation mediated by coal tar restores disturbed epidermal differentiation and improved skin barrier function in atopic dermatitis (Van den Bogaard *et al.*, 2013), this study opens an additional avenue for the development of AHR antagonists or selective modulators that can regulate AHR signaling in keratinocytes to modulate epidermal differentiation and inflammation.

MATERIALS AND METHODS

Isolation and culture of primary keratinocytes

Primary keratinocytes were isolated from newborn $Ahr^{+/+}$ and $Ahr^{-/-}$ mice, PCR-genotyped (Schmidt *et al.*, 1996), and cultured as described in 0.05 mM $CaCl_2$ medium (Markell *et al.*, 2011). Cells were treated with 10 nM TCDD, 500 nM ICZ, 200 nM GNF351, and 10 μM SGA360 or DMSO for the indicated times in either 0.05 or 0.12 mM $CaCl_2$-containing medium. All animal studies were conducted using protocols approved by the Penn State University IACUC. Human keratinocytes were isolated from abdominal skin (Rheinwald and Green, 1975) in accordance with the Declaration of Helsinki Principles, approval by Radboud University Medical Center, and written informed patient consent. For submerged culture, keratinocytes were cultured in KGM (Lonza, Slough, UK) and differentiated by growth factor depletion (Van Ruissen *et al.*, 1996). Cells were treated with 50 nM indirubin (IR), 500 nM GNF351, 5 μM CH223191, 500 nM SR1, and 0.1 and 1 μM FICZ at indicated time points or DMSO as vehicle control.

Microarray analysis

RNA isolated from four independent pooled $Ahr^{+/+}$ primary keratinocyte cultures and three $Ahr^{-/-}$ cultures were reverse transcribed and hybridized to Affymetrix Mouse Gene ST 2.0 arrays in the Penn State Genomics Core Facility according to the manufacturers' protocol. Arrays were scanned using a GeneChip Scanner 3000 7G (Affymetrix, Santa Clara, CA) and analyzed using the ArrayStar 11 Software (DNASTAR, Madison, WI) with RMA background correction and quantile normalization. Mean $\log_2$ signal was used to compare gene expression between groups and significantly different genes identified using a 1.5-fold cutoff and *P*-value <0.05 using a one-sided equal variance Student's *t*-test. Functional annotation clustering with genes identified as differentially expressed using ArrayStar 11 was conducted using the DAVID Bioinformatics Software (Huang *et al.*, 2009a; Huang *et al.*, 2009b). GEO accession number is GSE62490.

Human skin equivalent development

Human primary keratinocytes were seeded onto plastic inert filters (ThinCerts, Greiner Bio-one, Breda, The Netherlands) in CnT-PR medium (CELLnTEC, Bern, Switzerland). After 48 hours, cells were switched to CnT-PR-3D barrier (CELLnTEC) for 24 hours, and then cultured at the air–liquid interface for 10 days. Human skin equivalents using de-epidermized dermis were generated as described previously (Van den Bogaard *et al.*, 2012). Skin equivalents were treated, as indicated, with 500 nM GNF351 or 5 μM CH223191.

Immunostaining

Formalin-fixed paraffin-embedded skin equivalents were stained with hematoxylin and eosin (H&E; Sigma, Sigma-Aldrich, Zwijndrecht, The Netherlands) or processed for immunohistochemistry. Human keratinocytes on glass coverslips were treated twice over 48 hours with AHR antagonists (GNF351, 500 nM; CH223191, 5 μM; SR1, 500 nM), fixed with 4% paraformaldehyde, permeabilized with 1% Triton-X/PBS, and stained for Ki-67. Nuclei were counterstained with DAPI.

RNA and quantitative real-time reverse-transcriptase–PCR

Total RNA was isolated from keratinocytes using Ribozol, and quantitative real-time reverse-transcriptase–PCR was performed, as described, in triplicate (Markell *et al.*, 2011) and normalized to Gapdh (mouse) or RPLP0 (human). Intron-spanning primer sequences for analyzed genes were obtained using Primer 3 software (Rozen and Skaletsky, 2000) with Genebank sequence information.

Western blot analysis

Proteins were isolated from mouse keratinocytes as described (Cheng *et al.*, 1990; Hogan *et al.*, 2013), and from human keratinocytes with radioimmunoprecipitation assay buffer. Separated proteins were detected by ECL (Markell *et al.*, 2011).

Statistical analysis

For gene expression, statistical significance was determined between genotypes or between treatment and control using Student's *t*-test and GraphPad Prism4 (GraphPad Software, La Jolla, CA) with significance determined as a *P*-value <0.05.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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