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Ah receptor signals cross-talk with multiple developmental pathways

Alvaro Puga*, Craig R. Tomlinson, Ying Xia

Center for Environmental Genetics and Department of Environmental Health, University of Cincinnati Medical Center, P.O. Box 670056, Cincinnati, OH 45267-0056, USA

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Dedicated to the memory of Prof. Jaques Gielen, devoted editor, eminent scientist, gentleman and good friend.

Abstract

For many years, the Ah receptor (AHR) has been a favorite of toxicologists and molecular biologists studying the connections between genes and the changes in the control of gene expression resulting from environmental exposures. Much of the attention given to the Ah receptor has focused on the nature of its ligands, many of which are known or suspected carcinogens, and on the role that its best studied regulatory product, the CYP1A1 enzyme, plays in toxic responses and carcinogen activation. This understandable bias has resulted in a disproportionate amount of Ah receptor research being directed at toxicological or adaptive end points. In recent times, it has become evident that Ah receptor functions are also involved in molecular cascades that lead to inhibition of proliferation, promotion of differentiation, or apoptosis, with an important bearing in development. Developmental and toxicological AHR functions may not always be related. The ancestral AHR protein in invertebrates directs the developmental fate of a few specific neurons and does not bind xenobiotic ligands. The mammalian AHR maintains normal liver function in the absence of exogenous ligands and, when activated by dioxin, cross-talks with morphogenetic and developmental signals. Toxic end points, such as the induction of cleft palate by dioxin in mice embryos, might be at the crossroads of these signals and provide important clues as to the developmental role of the AHR.

Keywords: Ah receptor; TGF-β; Retinoic acid; GABA; MAP kinases

1. Introduction

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor that directs the expression of many phase I and phase II detoxification genes. Because several AHR ligands are known or suspected carcinogens, much of the attention given to the Ah receptor has focused on pro-proliferative responses resulting from its activation. In an extension of this bias, a disproportionate amount of AHR research has been directed at the analysis of toxicological or adaptive end points. It has become evident that just as often the stimuli that make the AHR function are resolved into molecular cascades leading to inhibition of proliferation, promotion of differentiation or apoptosis. Over the years, evidence has accumulated to indicate that many AHR ligands also activate signal transduction pathways that run hand in hand with the detoxification pathways and probably in combinatorial interaction with them. Many AHR ligands stimulate signaling cascades similar to those initiated at the cell surface by growth factors, hormones, and neurotransmitters.

Rarely does the Ah receptor act alone. Many factors modulate AHR activity, even in the induction of *CYP1A1,* its best-understood target. In most cases, Ah receptor ligands elicit responses by mechanisms that combine signals triggered by the Ah receptor itself with signals from other pathways [1]. In recent years, it has also become evident that in many instances the AHR does not need a recognizable ligand to activate its signaling pathway. We have focused this Commentary on the cross-talk between the AHR and signaling pathways that have substantial developmental consequences. Connections with some of these pathways, like the TGF-β pathway, were recognized many years ago [2], whereas other connections, like those established with GABAergic neuronal pathways, have just been found [3]. In all cases, recent data from knockout and other genetically modified

* Corresponding author. Tel.: +1 513 558 0916; fax: +1 513 558 0925.
E-mail address: alvaro.puga@uc.edu (A. Puga).

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mice provide new insights on the role of the AHR in development.

2. The Ah receptor signaling pathway

The AHR is a member of the PER-ARNT-SIM (PAS) family of basic-region-helix-loop-helix (bHLH) transcription factors [4]. In vertebrates, the AHR is found in the cytosol in association with HSP90 chaperones and several HSP90 accessory proteins and immunophilin-like proteins (XAP2/ARA9/AIP and p23) [1]. Upon ligand binding, the AHR translocates to the nucleus where it complexes with ARNT/HIF-1β [5]. AHR/ARNT heterodimers stimulate the transcription of genes in the cytochrome P450 *CYP1* family, as well as of several Phase II detoxification genes [6,7], via transactivation through enhancer domains known as AHR-, dioxin-, or xenobiotic-response elements (AhREs, DREs or XREs) [8]. Gene transactivation mediated by the AHR represents an adaptive response required for the detoxification of foreign compounds. However, this effect does not adequately explain the range of outcomes resulting from exposure to AHR ligands in different systems, particularly regarding gene repression effects seen for TGF-β2 [2,9], fibrinogen γ chain and plastin mRNAs [10], cyclin A [11], and others [12]. In mammalian cells, activation of the AHR by ligand results also in the rapid transcriptional activation of a large number of genes whose products control a broad spectrum of cellular functions [13–15]. This is likely due to interactions between the AHR and transcription factors other than ARNT, some of which are involved in the control of complex cellular programs, like cell division and cell fate [14–18]. To be functional, the Ah receptor has an absolute requirement for nuclear translocation [19] but its inhibitory interactions with cell cycle regulatory proteins, although taking place in the nucleus, do not require interaction with ARNT or DNA binding at the cognate AhRE/DRE/XRE motifs [20].

AHR ligands include planar polycyclic and halogenated aromatic hydrocarbons and diverse classes of plant-derived chemicals. It has been hypothesized that the AHR/ARNT transcriptional complex and the genes that it regulates evolved for defense against an increasingly diverse array of plant toxins and that as a result it is unlikely that it serves endogenous physiological functions [21]. Often enough, however, the mammalian AHR needs no exogenous ligand-dependent activation to be functional ([22] and references therein). Undoubtedly, activity in those cases might result from the presence of unknown endogenous ligands. In fact, over the past few years there has been a considerable volume of literature describing candidate endogenous ligands, including an unknown CYP1Al substrate [22], various tryptophane photooxidation products [23], lypoxin A4 [24], indirubin [25], bilirubin and biliverdin [26], and an indole derivative isolated from porcine lung [27]. It is possible that the biological functions of the AHR depend on the presence of these or other endogenous ligands, which might differ from tissue to tissue and from one developmental stage to another. If that were the case, the toxic effects of the xenobiotic ligands might simply be due to derailment or intensification of normal AHR functions. Alternatively, xenobiotic ligands could have effects of their own, unrelated to endogenous AHR functions, as might be the case of the metabolic activation of promutagenic ligands that are also substrates for the CYP1A1 and CYP1B1 cytochrome P450s. Most likely, a combination of endogenous and exogenous functions is closer to reality. To complicate matters, recent studies in *Caenorhabditis elegans* have shown that the worm AHR protein does not respond to activation by the classical xenobiotic ligands of the vertebrate AHR [28]. These studies have shown that the ancestral function of the AHR protein is to regulate neuronal differentiation during worm development [29] and to direct the fate and gene expression phenotype of two of the worm's 26 GABAergic neurons [3]. As its mouse counterpart [30], the *C. elegans* AHR requires interaction with ARNT for proper function.

3. The transforming growth factor-beta (TGF-β) signaling pathway

The TGF-β cytokines are members of a family of signaling proteins that include the activins, the nodal subfamily, the bone morphogenetic proteins, growth and differentiation factor, and the Mullerian inhibiting substance subfamily. TGF-β signaling plays a critical role in cellular physiology and development by inhibiting cell proliferation, promoting apoptosis, inducing differentiation, and specifying developmental fates in vertebrates and invertebrates [31]. In mice and humans, TGF-βl, −2 and −3 have diverse functions in embryonic and adult tissues for immune cell function, epithelial cell growth, and extracellular matrix deposition. TGF-β signaling is initiated by the binding of processed TGF-β dimers to at least three separate types of dimeric TGF-β receptors. Formation of liganded TGF-β receptor complexes induces the phosphorylation of the Smads, the intracellular TGF-β signal transducers. Following phosphorylation, Smads translocate to the nucleus and bind as heterodimers to Smad-binding elements on gene promoters to regulate transcription.

TGF-β is secreted as a dimer from most cell types as a complex in a latent form due to its non-covalent association with two latency-associated proteins (LAP). LAP, in turn, is bound to latent TGF-β binding protein (LTGBP) by a disulfide bond. The amino terminus of LTBP anchors the complex to the extracellular matrix, and TGF-β activation is accomplished when TGF-β is released from the LAP–TGBP complex. Extracellular TGF-β activators include proteases such as plasmin, cathepsin, and calpain [32,33]; thrombospondin 1, a glycoprotein that inhibits angiogenesis [34], and matrix metalloproteinase 2 (MMP-2) [35].

The extracellular matrix itself appears to play a major regulatory role in TGF-β signaling. Decorin, a small extracellular matrix proteoglycan, binds TGF-β and modulates its activity [36–40]. TGF-β can also stimulate other signaling pathways, such as phosphoinositol-3-kinase (PI3K), mitogen-activated protein kinases (MAPKs), and PP2A/p70s6K. There is much overlap in TGF-β expression but little redundancy in activity, probably due in part to the differential distribution in the extracellular matrix of the proteins involved in processing theTGF-βs [41].

When deleted in mice, the three *Tgfb* genes produce quite different phenotypes. *Tgfb1* null mice die of an autoimmune disease with an average survival of only 20 days. *Tgfb2* knockout mice have severe heart, skeletal, ear and eye defects and die at birth; and *Tgfb3* knockout strain die within the first 18 h after birth due to a severe cleft palate that affects breathing. Interestingly, conditional inactivation in cranial neural crest of the *Tgfbr2* gene, encoding the TGF-β IIR receptor, also causes cleft palate [42]. The abnormal expression of TGF-β has been implicated in fibrosis, cancer, and cardiomyopathy [41].

4. Ligand-independent AHR activity and TGF-β signaling

The mammalian AHR needs no exogenous ligand-dependent activation to be functional [22], and this appears to be the case for its role in the regulation of the expression of some TGF-β isoforms. This is not to say that ligand-dependent AHR activation is not needed for the AHR to function in those cases, but that, if a ligand is needed, it is provided endogenously by the cells or tissues in question and its identity is unknown. In studies comparing $Ahr^{-/-}$ mice to wild type, levels of TGF-βl and -β3 but not of -β2 were higher in the liver and increased in conjunction with increased hepatocyte apoptosis and portal fibrosis [43]. In $Ahr^{-/-}$ mouse embryonic fibroblasts (MEF), decreased cell proliferation rate, increased 4N DNA content, and greater rates of apoptosis occurred along with a striking increase in TGF-β3 [44]. In addition, two cell cycle proteins important in the G2/M transition, Cdc2 and Plk, were down regulated in the $Ahr^{-/-}$ cells. Ectopic expression of TGF-β3 in wild type MEFs also caused reduced levels of these two proteins, suggesting that the AHR controlled the levels of crucial cell cycle proteins through regulation of TGF-β3 [44]. Independent data indicates that at least Cdc2 is a known regulatory target of TGF-β in myeloid cells [45]. Thus, the above studies lead to the conclusion that in the absence of the AHR the increase in TGF-β3 signaling activity inhibits cell proliferation and induces apoptosis.

Comparison of global gene expression profiles of $Ahr^{+/+}$ and $Ahr^{-/-}$ vascular smooth muscle cells (vSMC) from mouse aorta [9] has shown that, as in MEFs, TGF-β3 mRNA levels are elevated in $Ahr^{-/-}$ vSMCs, and that approximately a dozen known TGF-β-regulated and accessory genes were coordinately deregulated. For example, decorin mRNA levels increased more than 30-fold while thrombospondin-1 mRNA levels were significantly lowered. Cell-specific responses have also been detected in cells from *Ahr* knockout mice. In $Ahr^{-/-}$ MEFs, the mRNA and protein levels of LTBP-1, the extracellular TGF-β anchor protein, were found to be elevated while the levels of the extracellular protease MMP2 were decreased relative to wild type cells [46]; however, in $Ahr^{-/-}$ vSMCs, the results were precisely the opposite, with LTBP-1 mRNA levels being decreased and MMP2 increased [9], suggesting that for some genes, a cell-type specific response may occur when the *Ahr* gene is lacking.

5. Ligand-dependent AHR activity and TGF-β signaling

In human SCC-12F keratinocytes, a cell line derived from a non-turnorigenic squamous cell carcinoma, exposure to TCDD caused a decrease in transcription of TGF-β2 mRNA [2] by a mechanism dependent on a region near the TATA box of the TGF-β2 promoter, and possibly mediated by a tyrosine kinase pathway [47]. TGF-β2 protein levels in the same cell line were also lower relative to untreated cells, while the levels of plasminogen activator inhibitor-2 (PAI-2) increased [48]. PAI-1 and -2 inhibit the activation of plasmin, one of the extracellular enzymes that activate TGF-β. In agreement with this work [49], TCDD was shown to cause PAI-1 and PAI-2 mRNA levels to increase in several other cell types [50].

Although the regulation of genes by the TGF-β signaling pathway has been well studied, relatively less is known about the regulation of the *Tgfb* genes themselves. Human and mouse *Tgfb* promoters are very similar, but do not have canonical AHR response motifs. The mouse *Tgfb1* promoter has several Sp1 and AP-2 binding sites [51–53]; the mouse *Tgfb2* promoter contains a TATA box, a CRE/ATF site, and an E-box [54–56] and the mouse *Tgfb3* promoter possesses a TATA box, CRE site, and two AP-2 elements [57]. To this day, there has not been a molecular explanation for the repression of TGF-β2 expression by TCDD; however, the involvement of AHR in this regulation is suggested by the finding that the mRNA levels of TGF-β2 and of several known TGF-β-regulated genes are higher in $Ahr^{-/-}$ than in wild type vSMCs exposed to TCDD or B[*a*]P [9,58,59]. Thus, ligand-induced AHR activation represses TGF-β2 transcription and induces PAI-1 and PAI-2 expression, which might further suppress TGF-β signaling.

The primary conclusion from all the studies above is that signaling through the AHR regulates the expression of all three *TGFB* genes; however, this conclusion needs to be considered in light of several caveats. The first of these is that signaling through the AHR is not synonymous with TCDD-dependent activation of the AHR. The data shows that as far as the role of the AHR is concerned, TGF-β

expression depends primarily on the presence or absence of the Ah receptor, which might indicate dependence on endogenous ligand(s) or total ligand independence. Thus, $Ahr^{+/+}$ cells express normal levels of all three TGF-β's whereas $Ahr^{-/-}$ cells have elevated levels of β1 and β3 but retain normal levels of β2. Quite to the contrary, TCDD treatment of $Ahr^{+/+}$ cells with TCDD represses TGF-β2 expression without affecting the others, while, lacking such suppression, TCDD treatment of $Ahr^{-/-}$ cells results in the up-regulation of TGF-β2 but has no effect on the already high levels of expression of β1 and β3. What molecular mechanisms account for this complicated regulatory matrix remains a mystery.

6. Retinoic acid: a connection between AHR and TGF-β?

Retinoic acid (RA), derived from Vitamin A_1 or retinol, is a morphogen involved in vertebrate limb and organ development [60] and gene regulation in the skin [61,62]. All-*trans-RA* initiates signaling by binding the retinoic acid receptor (RAR) in association with the auxiliary 9-*cis*-retinoic acid receptor (RXR). The liganded RAR/RXR complex recognizes specific promoter response elements (RARE) to regulate transcription of target genes. The transcriptional regulatory role of the transcription factor AP-2 is intimately linked to retinoic acid-induced differentiation since AP-2 is induced by retinoids [63,64]. The retinoids can trigger cell differentiation and block malignant progression, perhaps because they also negatively regulate POU-domain factors such as Oct-3 [65] and AP-1-responsive genes [66].

Retinoids are three-fold higher in the livers of $Ahr^{-/-}$ mice than in wild type [67], suggesting that the AHR regulates RA levels. Accumulation of retinoids was due to a decreased rate of catabolism, as shown by a significant reduction of oxidized RA end products in the $Ahr^{-/-}$ mice. Decreased RA catabolism was due to a drop in the level of cytochrome P450 CYP2C39, a newly identified retinoic acid 4-hydroxylase, in $Ahr^{-/-}$ mice relative to wild type [68]. Feeding the $Ahr^{-/-}$ mice a diet deficient in Vitamin A reduced liver fibrosis and brought down the elevated levels of TGF-β1, -β2 and -β3 found in $Ahr^{-/-}$ mice fed a control diet [69], suggesting that increased RA levels in $Ahr^{-/-}$ mice might be a major inducer of the fibrosis and developmental abnormalities observed in the livers of these mice. All-*trans-RA* signals affect the expression of TGF-β in many systems. For example, they increase TGF-βl, -β2, and -β3 mRNA and protein levels in the epithelium of the rat [62,70], and increase TGF-β2 mRNA levels and expression in cultured mouse and human keratinocytes [61]. By enhancing plasminogen activator levels, retinoids can also induce the proteolytic activation of TGF-β [71] and be the primary cause of the fibrosis and abnormal liver development seen in $Ahr^{-/-}$ mice.

In rats, TCDD has also been shown to alter the endogenous metabolism of retinoids and to induce a dose-dependent increase of all-*trans*-retinoic acid that is accompanied by a decrease in *cis-RA* isomers in all tissues tested [72]. TCDD treatment also increased significantly the enzymatic activity of CYP1A1, although no experimental evidence connected the elevated RA levels with CYP1A1 activity. CYP2S1 is yet another AHR-dependent cytochrome P450 induced by TCDD in cultured cells [73] and responsible for metabolism of all-*trans-RA* in the skin [74]. CYP2S1 levels were not measured in the experiments with $Ahr^{-/-}$ mice, but it stands to reason to suspect that they would be critical in the reduction of RA catabolism in these mice. TCDD exposure of $Ahr^{+/+}$ mice induces plasminogen activator inhibitors-1 and -2 and might also be responsible for reducing the circulating levels of activated TGF-β. It appears that the homeostatic levels of all-*trans*-retinoic acid are regulated by both TCDD as well as by *Ahr* ablation. When the *Ahr* gene is present, TCDD treatment elevates the homeostatic levels of all-*trans-RA,* by increasing TCDD-inducible retinaldehyde dehydrogenases and perhaps CYP1A1/1A2 activities; on the other hand, when the *Ahr* gene is absent, RA levels accumulate due to a drop in CYP2C39 activity and a reduction of catabolism. In either case, all-*trans-RA* levels increase and cause a concomitant increase in RAR/RXR signaling.

7. Do TCDD, AHR, TGF-β and RA converge on the regulation of mouse palate fusion?

The developmental toxicity of TCDD has been known for quite some time. Induction of cleft palate in mice is one of the best characterized in vivo effects of TCDD. Cleft palate occurs with peak incidence when TCDD is administered between gestational days GD11 and 12 (reviewed in [75,76]). In mammals, the medial edge epithelium of the opposing palatal shelves adhere to each other to form an epithelial seam which then disrupts by apoptosis and cell migration accompanied by epithelium-to-mesenchyme transition (EMT) [77]. TCDD treatment blocks cell death and causes the medial epithelium to continue proliferating and not to transform into mesenchyme. The opposing palatal shelves come in contact but fail to fuse [76].

Interestingly, TGF-β3 knockout mice also have cleft palate. In these mice, the opposing palatal shelves continue to proliferate and fail to fuse due to impaired adhesion of the medial epithelium and lack of filopodia such that eventually one shelf overlaps the other [78,79]. The effects of gestational TCDD exposure on TGF-β-regulated genes have not been measured, but given the similarity of the developmental outcome, it seems reasonable to propose that TCDD-AHR and TGF-β3 signals come together in the palatal shelves and that activation of AHR signals might down-regulate TGF-β3 signals and be responsible for the failure of the palatal shelves to fuse. AHR protein expres-

sion is significantly higher in the epithelial as compared to the mesenchymal cells and is down-regulated by TCDD exposure [80]. TGF-β3 signaling through the Smads activates transcription of genes involved in EMT during palate development [81] and induces chondroitin sulphate proteoglycans that mediate palatal shelf adhesion [82,83]. EMT signals from TGF-β2 [84], which is down-regulated by TCDD in cultured cells, are also likely to contribute to the induction of cleft palate through this signal convergence.

Retinoic acid administration also causes cleft palate formation and coadministration of RA and TCDD is more effective than either compound singly [75,85,86]. As discussed, RA induces AP-2 activity, normally not in adult tissues [63,64], but in facial mesenchyme during development and in extra-embryonic lineages [87,88]. AP-2 is a critical factor in morphogenesis, essential for cranial closure and craniofacial development [89–91]. RA and AP-2 regulate TGF-βl and -β3 at the level of transcription [61,92,93]. Interestingly, RA-induction of cleft palate in mice is accompanied by differential regulation of TGF-β isoforms. Steady state mRNA levels of all three isoforms increased rapidly and transiently after RA exposure, and these increases are followed by decreases in intracellular and extracellular forms of TGF-β protein [94]. These changes in TGF-β isoforms preceded the changes in mesenchyme morphology, suggesting that they may be mediators of the RA effects on mesenchyme development.

Regulation of ETM transformation, extracellular matrix deposition, cell movement, and differentiation by TGF-β isoforms seems to be the most likely pathway activated by TCDD and RA signals. Both these agents cause a defect in palate fusion and they might do so by similar mechanisms. Paradoxically, intracellular and extracellular levels of TGF-β isoforms follows the transient increase of TGF-β mRNA levels in palatal shelves of RA-treated mice. Similarly, AHR activation by TCDD may cause a decrease in circulating levels of TGF-β protein by repression of TGF-β mRNA synthesis, induction of PAI-1 and -2, and activation of the ERK family of MAP kinases [95], since ERK blocks TGF-β signaling by phosphorylation of serines in the Smad1 inhibitory domains [96]. Overall, not enough TGF-β might perturb normal EMT progression required for epithelial fusion during palate development.

8. What role for GABA?

The gene coding for AHR has been cloned in many vertebrates and invertebrates (reviewed by [97]). The *C. elegans* AHR, like all invertebrate AHR tested to date, does not respond to xenobiotic activation nor does it bind TCDD or any of the other classical AHR ligands [28]. This observation lent impetus to the idea that perhaps the ancestral function of the AHR was unrelated to xenobiotic toxicity and that it would be worthwhile to find out what the function of the AHR was in invertebrates. In recent papers, the orthologous *C. elegans* Ah receptor protein was shown to direct the fate and gene expression phenotype of two of the worm's 26 GABAergic neurons [3] and to regulate neuronal development [29]. GABA (λ-aminobutyric acid) is the principal inhibitory neurotransmitter, synthesized from glutamic acid by glutamate decarboxylase (GAD) in GABAergic neurons. GAD and GABA are also expressed in non-GABAergic neurons, particularly during embryonic and fetal development [98,99].

Both genetic and teratological studies have shown that GABA signaling is involved in normal craniofacial development [100,101]. Retinoids induce the differentiation of embryonal carcinoma cells into neurons [102] and regulate the expression of GABA receptors and glutamate decarboxylase in these cells [103,104]. What brings GABA and the Ah receptor together in mammals is the interesting fact that mice with a homozygous deletion of the GABA-A receptor β3 subunit also have a cleft palate [105] and so do mice with a homozygous deletion of the glutamate decarboxylase GAD67 gene [106,107]. The palatal cleft in these mice is wider than the cleft of TGF-β3 knock-out mice and resembles more the cleft induced by TCDD exposure, suggesting that the absence of GABA leads to premature arrest of cell proliferation. Inhibition of cell proliferation is one of the functions of the activated AHR that has been recently uncovered [14,15], suggesting that the relationship between AHR and GABA in mice and worms is much too intimate to be coincidental. Little more evidence connecting the two has accumulated throughout the years, although, however little there is, it is tantalizing enough. In the preoptic area of the rat brain, AHR expression colocalizes with expression of GAD67 [108]. GABA receptor ligands have been shown to induce CYP1A1 mRNA [109] and the promoters of the GAD65 and GAD67 glutamate decarboxylase genes contain several AHR response elements [110], although none of these have been tested for their ability to be regulated by the AHR.

9. Conclusions

The salient features of the ideas developed in this Commentary are graphically summarized in Fig. 1. Palate development is an extremely dynamic process that involves neural crest cell migration, epithelial-to-mesenchyme transition, cell adhesion and reorganization, differentiation, proliferation and apoptosis. It is highly unlikely that one single signaling pathway will be responsible for all these biological processes. In fact, expression of many genes with important developmental roles—loricrin, GAD, GABA-A receptor β3, *frizzled*, *wnt-5a* and others—has recently been found to be temporally regulated during normal palate development [111]. Given the limited amount of information available, it might seem premature

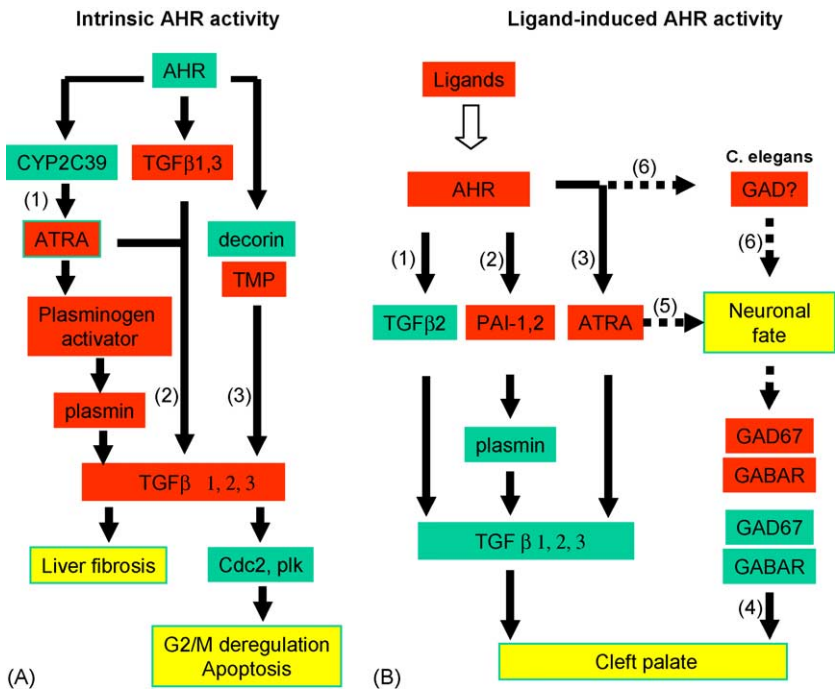

Fig. 1. Molecular pathways converging on AHR in the control of cell and tissue functions. (A) Intrinsic AHR activity refers to its activity in the absence of an exogenous ligand. This is not meant to exclude the likelihood that this activity depends on the presence of an endogenous ligand. AHR ablation alters the expression or activity of TGF-β through at least three mechanisms: (1) AHR ablation causes transcriptional down-regulation of CYP2C39, which leads to increased all-*trans*-retinoic acid (ATRA) and the subsequent induction of TGF-β expression and activity; (2) AHR ablation itself results in elevated levels of TGF-β1 and -β3, and (3) Lack of AHR alters the expression of TGF-β regulatory proteins and therefore the enhancement of TGF-β activity. Deregulated TGF-β signaling is likely to be responsible at least in part for cell cycle abnormalities, apoptosis in MEFs and fibrosis in liver resulting from AHR ablation. (B) Ligand-induced AHR activity refers to effects resulting from the presence of a known exogenous ligand. We have made the gratuitous assumption that the role of AHR in GABA signaling might depend-hence the dotted lines-on the presence of an endogenous ligand. Ligand-induced AHR activity leads to cleft palate during mouse development, possibly through decrease of TGF-β signaling by the following mechanisms: (1) transcriptionally down-regulation of TGF-β2; (2) up-regulation of PAI, the plasminogen activator inhibitors; and (3) decrease of TGF-β protein resulting from AHR-induced ATRA accumulation. Cleft palate also results from not enough GABA, as in (4) GAD67 or GABA-A β3 receptor knockout mice. ATRA promotes neuronal differentiation (5) and stimulation of GAD67 and GABA receptor expression. The *C. elegans* AHR is also involved in the control of neuronal cell differentiation (6) perhaps through regulation of GABA signaling. *Red and green boxes* denote up- and down-regulation, respectively. *Yellow boxes* denote biological or physiological end points.

to speculate on the connections between different signal transduction pathways and their possible role in the developmental toxicity of Ah receptor ligands. As far as GABA signaling is concerned, there is good evidence that not only loss-of-function of GABA signaling can induce cleft palate, but that gain of GABA function, through administration of diazepam to pregnant mice, does the same thing [112], suggesting that there is a process during palatogenesis that requires a specific range of GABA signaling for normal development. This might be a good example of the identity of effects resulting from either too much or too little of a good thing.

In the field of developmental toxicology, we are most ignorant of the consequences that exposure to environmental agents during embryonic life bring about in the adult. When thinking about dioxins in this light, we often overlook the fact that they are stored in humans in fat tissues and that, due to its long biological half-life, TCDD accumulates in the body throughout the lifetime of the individual. In pregnant and lactating women, however, increased metabolism and mobilization of accumulated dioxins and related organochlorinated compounds stored in fat tissues exposes the fetus and the newborn to such high levels of these compounds that they are readily detectable much later in adulthood [113]. Science is beginning to unravel the developmental effects that these exposure levels create for the exposed infants [114], but we are largely ignorant of the effects to the adult cardiovascular, nervous, reproductive or immune systems, to name a few, that this early exposure to dioxins may cause. By now, it should be self-evident that we had better find out.

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