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AHR-dependent misregulation of Wnt signaling disrupts tissue regeneration

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Abstract

The origins of molecular toxicology can be traced to understanding the interactions between halogenated aromatic hydrocarbons and the aryl hydrocarbon receptor (AHR). The physiological consequences of activation of the aryl hydrocarbon receptor are diverse, and we are just beginning to understand the importance of the AHR signal transduction pathway in homeostasis and disease. The many downstream targets that mediate these biological responses remain undefined. Studies have exploited the power of the zebrafish model to elucidate the mechanisms by which AHR activation disrupts biological signaling. Recent genomic analysis performed in a zebrafish tissue regeneration model revealed functional cross talk between AHR and the well established Wnt/ β -catenin signal transduction pathway. This review focuses on the development of the zebrafish model of AHR biology and the application of *in vivo* toxicogenomics to unravel molecular mechanisms.

1. Introduction

Polychlorinated dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs), and biphenyls (PCBs) belong to a family of lipophilic halogenated aromatic hydrocarbons that persist in the environment, posing a potential risk to humans. Numerous studies over the past two decades have concentrated on evaluating the toxicity of PAHs and related chemicals, and determining the molecular mechanisms by which these chemicals produce toxicity. One of the most studied aromatic hydrocarbons, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), elicits a wide array of toxicities in mammals such as epithelial hyperplasia and metaplasia, lymphoid involution, porphyria, wasting syndrome, tumor promotion, death, reproductive and developmental toxicities (reviewed in [1]). Despite the diversity of TCDD toxicities, much of it requires the aryl hydrocarbon receptor (AHR), a basic-helix-loop-helix (bHLH) transcription factor [2, 3].

The widely accepted mechanism of action of TCDD is that the ligand bound AHR translocates to the nucleus where it binds with its dimerization partner, Ah receptor nuclear translocator (ARNT). The AHR-ARNT complex binds to xenobiotic response elements (XREs) resulting in the transactivation of dioxin dependent genes and ensuing toxicity [reviewed in [4]. Near ubiquitous evolutionary conservation of the AHR across unrelated vertebrate species likely indicates an essential function other than responding to environmental chemicals [5]. Yet,

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despite considerable effort, activation of the AHR by endogenous ligands has not been definitively shown to contribute to normal physiology. However, evidence that AHR has normal physiologic functions is growing.

For instance, AHR null mice exhibit hepatic bile duct fibrosis and impaired spleen and lymph node development [6,7]. They also display small livers due to aberrant liver vascularization manifested as permanent portocaval vascular shunting [4,8]. The shunting may be a consequence of hypertension or the failure of normal vasoconstriction in the developing liver [9]. Cardiac hypertrophy and hypertension are also observed in early development of AHR null mice [7,10]. AHR knockout mice also display aberrant prostate and seminal vesicle development [11]

The most studied AHR responsive genes include CYP1A and CYP1B1 [12–14]. Considerable effort has defined other interacting/modulating factors including cytoplasmic AIP (XAP2, ARA9), P23, HSP90 (reviewed in [15]), nuclear factors including coactivators [15–18] and the estrogen receptor alpha [19]. The majority of research efforts have been aimed at understanding the first steps in toxic and adaptive responses to AHR ligands, namely AHR activation, nuclear translocation, dimerization, factor recruitment and direct transcriptional responses. Relatively few *in vivo* studies have been aimed at identifying the role of downstream regulated genes in toxicity.

2. AHR Biology in Zebrafish

2.1. Advantages of zebrafish as a research model

Zebrafish (*Danio rerio*) is an exceptional vertebrate model to unravel the mechanisms by which AHR activation produces diverse biological responses. First, molecular responses in zebrafish are relevant to human biology because the fundamental processes and signal transduction mechanisms are remarkably conserved across species. This, coupled with a nearly complete genome sequence provides the potential to integrate new discoveries at the biochemical, cellular and molecular levels with observations at the structural and functional level. Furthermore, much of the anatomy and physiology of fish is highly homologous to humans [20,21]. Another major advantage is that, unlike many other systems, zebrafish embryos develop externally and are optically transparent. This allows for simple microscopic observation of individual cells *in vivo* across a broad range of developmental stages. Dynamic changes in gene expression and detailed morphogenetic movements can be validated *in vivo* during development with the aid of transgenic zebrafish that express fluorescent reporter genes [22,23]. Several additional features of zebrafish biology including small size, rapid embryonic development and short life cycle (reviewed in [21,24,25]), make this model system logistically attractive for developmental studies. Zebrafish reach sexual maturity within 3–4 months, reproduce year round, and produce clutch sizes that range between 100 and 200 embryos per mating pair. Genetic screens have also been successfully used to identify genes essential for biological processes, and to date, large-scale zebrafish mutant searches have led to the identification of thousands of mutants with unique embryonic and larval development defects [26,27]. A recent study using mutant zebrafish has offered an explanation for pigment variation in human populations [28]. This demonstrates the rapidity by which results from zebrafish studies can be applied to understanding the role of human genes. Finally, this model system is amenable to rapid throughput assays [29–36].

2.2. AHR pathway members in zebrafish

The AHR signal transduction pathway in zebrafish is very similar to that of mammals, with the notable exception that zebrafish possess 3 *AHR* genes, *AHR1a* and *AHR1b* and 2 [37,38], while mammals have only one. Phylogenetic comparisons suggest that an early gene

duplication event with subsequent divergence during vertebrate evolution resulted in the two forms of the *AHR* [38]. Sequence analysis reveals that AHR1 in fish shares the greatest sequence similarity with the mammalian AHR. AHR1 and an AHR2 have been cloned in fathead minnow (FhAHR1 and FhAHR2) [39]. Full-length AHR2s have been described in zebrafish (zfAHR2) [40], the Atlantic tomcod [41], and rainbow trout (rtAHR α and rtAHR β) [42]. Full-length cDNAs for zebrafish AHR2 (zfAHR2) and ARNT2 (zfARNT2) have been cloned, and their translation products have been characterized [40,43]. zfAHR2 and zfARNT2b form a functional heterodimer *in vitro* that specifically recognizes dioxin response elements (DREs) in gel-shift experiments and induces DRE-driven transcription in response to TCDD exposure in COS-7 cells [40,43].

2.3. TCDD causes developmental toxicity in zebrafish larvae

Zebrafish larvae exposed to waterborne TCDD as early as 6 hours post fertilization (hpf) display a variety of deformities including pericardial edema, yolk sac edema, craniofacial malformations and mortality [44–47]. The earliest signs of TCDD toxicity observed in the cardiovascular system of zebrafish larvae were pericardial edema and reduced blood flow to the trunk at 72–77hpf. Yolk sac edema and reduced blood flow to gills and head were more apparent by 96hpf. It is noteworthy to mention that TCDD does not impact the initial development of the vasculature, but affects the maintenance of peripheral vascular beds after they form [45]. TCDD causes cardiac malformation and heart failure in zebrafish embryos through dramatic down-regulation of cell cycle progression genes, effectively stopping heart growth [48]. Cardiac valve development is also affected by TCDD. Zebrafish embryos exposed to TCDD fail to form functional heart valves in the developing heart, though TCDD does not prevent the initial specification of the presumptive valve locations [49].

Potent activation of AHR signaling by the TCDD ligand is thus seen to perturb a myriad of developmental processes. Defining the role of specific AHR-responsive genes in mediating these embryonic developmental responses is challenging because of the integrated complexity of developmental processes. To overcome this limitation, a post-embryonic development model of tissue regeneration was developed.

2.4. Tissue regeneration is inhibited by TCDD in zebrafish

Injury, disease and aging all result in a loss of tissue and reduced quality of life. Most adult tissues and organs, especially in mammals, have lost their potential for further growth and differentiation. Some organisms, however, have retained the ability to regenerate their tissues, organs and appendages (reviewed in [50]). Zebrafish caudal fin regeneration is a well recognized research model that has been utilized to understand the fundamental principles of tissue regeneration [51,52]. Zebrafish regenerate their caudal fins by a process referred to as epimorphic regeneration (Figure 1). Immediately after surgical amputation, epithelial cells begin to migrate over the injured site forming a wound cap, which is followed by the de-differentiation of cells proximal to the amputation plane into a cluster of pluripotent cells referred to as the blastema [51–53]. The blastema cells further proliferate and differentiate into the cell types required to complete the regenerative outgrowth. This complex process is tightly regulated by multiple signaling pathways, and intervention by external stimuli could alter the well orchestrated events during regeneration. Our laboratory has previously demonstrated that TCDD inhibits zebrafish fin regeneration at both adult and larval stages [54,55]. Morphological analysis demonstrated that TCDD affects several components involved in cellular differentiation and extracellular matrix composition in adult tissue regenerates [56]. The largest category of transcripts misregulated by TCDD were those related to ECM composition and metabolism. TCDD exposure resulted in repression of 34 of the 41 transcripts in this category suggesting that TCDD impairs the maturation of the ECM. This could occur by directly altering the expression of ECM genes or by impacting the genes responsible for controlling cell

differentiation and matrix maturation[56]. In mice TCDD exposure inhibited liver regeneration after partial hepatectomy [57]. The conserved inhibition of tissue regeneration by TCDD across species suggests that the AHR signaling pathway interacts with critical regenerative pathways.

3. Role of AHR-ARNT Factors in TCDD Mediated Toxicity in Zebrafish

Based on the *in vitro* studies using zfAHR-ARNT proteins with TCDD, it was presumed that TCDD toxicity is mediated through the activation of the AHR pathway. Antisense morpholino (MO) repression has been effective in characterizing the molecular players that mediate TCDD toxicity in zebrafish. With the availability of the nearly complete zebrafish genomic sequence, the functional role of any protein *in vivo* can be rapidly assessed *via* morpholino knockdown. [58]. Knockdown of zfAHR2 protein by MO revealed that TCDD-elicited developmental toxicity in zebrafish was completely zfAHR2 dependent [59]. The zfAHR2 MO microinjected animals (morphants) showed reduced expression of CYP1A confirming the inability of TCDD to activate the AHR pathway in the absence of zfAHR2 protein [59,60]. Previous *in vitro* studies suggested that zfARNT2 protein is the dimerization partner of zfAHR2, but TCDD elicited hallmark toxicity in both *zfarn2* morphants as well as *zfarn2* insertional mutant embryos indicating that zfARNT2 is not the dimerization partner of zfAHR2 [61]. Subsequent MO repression studies revealed that zfARNT1 and zfAHR2 are essential for TCDD to produce early life stage toxicity [62]. More recently, our laboratory used the caudal fin regenerative response as an endpoint of TCDD toxicity. In that study (Figure 2) zfAHR2 and zfARNT1 morphants completely regenerated their amputated caudal fin tissue in the presence of TCDD, and neither morphant had detectable vascular or epithelial CYP1A expression [56]. Collectively, the results in Figure 2 indicate that dimerization of zfAHR2-ARNT1 proteins is essential for TCDD to activate AHR signaling and block tissue regeneration [54]. We note the superficial similarity between inhibition of fin regeneration by TCDD and the induction of cleft palate in mice by TCDD. Both processes are affected by TCDD at a critical cell transformation stage (blastema to fin ray-forming cells in zebrafish [54], and epithelial to mesenchyme in the mouse palatal shelves (reviewed in [63])). However, the epithelial to mesenchyme transformation in the mouse palatal shelves is a single, discreet, differentiation inhibited by TCDD. This is in stark contrast to the complex regenerative differentiation of blastema mesenchyme to fin ray-forming cells, innervation and vascularization affected by TCDD [54,56]. Moreover, little is known about the molecular mechanism of TCDD induction of mammalian cleft palate, whereas in the fin, TCDD acts through the AHR and Wnt signaling pathways resulting in the misexpression of genes involved in extracellular matrix formation and remodeling [56,64].

4. Molecular Mechanisms of the Adverse Effects of AHR Activation in Zebrafish

Even though the transcriptional regulation of AHR has been widely studied, identification of the downstream genes ultimately responsible for AHR-dependent toxicities is still needed. The prototypical downstream AHR target genes are CYP1A1, CYP1A2, CYP1B1 and NQO1 [14,65,66] (reviewed in [12,13]). Recently, the CYP1C family, newly discovered in fish, was also shown to be an important AHR target in the eye and heart [67]. The significance of the CYP targets is still unclear. Increased expression of CYP1A by TCDD has been proposed to mediate toxic responses in zebrafish. However, antisense repression of CYP1A failed to prevent TCDD toxicity in developing zebrafish [60]. AHR2-dependent transcription of CYP1B is also induced by TCDD in developing embryos, but like CYP1A, antisense repression of CYP1B also failed to prevent TCDD developmental toxicity [68]. Collectively, at least for TCDD, the induction of CYP1A and 1B is a parallel transcriptional response, and the downstream AHR-dependent genes that modulate tissue specific toxicity remain largely unknown.

4.1 Genomic analysis to unravel signaling pathways impacted by AHR Activation

Toxicogenomic approaches have significantly advanced our understanding of the transcriptional changes following AHR activation in zebrafish. An expression-based toxicogenomic study performed on adult zebrafish with various AHR and estrogen receptor (ER) agonists demonstrated the potential for large-scale predictive and discovery chemical biology with the finding of biomarkers for potent AHR and ER agonists in multiple targeted tissues [69]. A study of heart specific transcriptional responses to TCDD aimed at understanding the molecular mechanisms leading to cardiovascular dysfunction identified genes important in xenobiotic metabolism, cell proliferation, heart contractility and heart development [48]. Gene expression studies performed in adult, regenerating fin tissue after exposure to TCDD revealed a cluster of genes important for xenobiotic metabolism, cellular differentiation and extracellular matrix composition [56]. Genomic analysis conducted in larval regenerating fin tissue after AHR activation led to the discovery of genes important for xenobiotic metabolism, cellular differentiation, signal transduction and extracellular matrix composition, similar to the results of the adult zebrafish study [56,70]. TCDD disrupts the proper synthesis of proteoglycans and collagens and the microarray analysis in both the adult and larval fin regeneration systems confirmed the same [56,64]. Genes including *sox9b*, different isoforms of collagens, cartilage link protein, and matrix metalloproteinases (MMPs) were differentially expressed in the regenerating fin tissue after exposure to TCDD [56,70]. Comparative genomic analysis performed in both the adult and larval fin regeneration systems revealed a concordance in the pattern of gene expression between these two models in response to TCDD, suggesting a common molecular mechanism of action. R-Spondin1, a secreted protein capable of promoting Wnt/ β -catenin signaling [71], was the most highly induced transcript while *Sox9b*, a transcription factor with a major role in chondrogenesis, was the gene most repressed by TCDD. A thorough analysis of the transcriptional responses to TCDD in both regeneration models identified numerous Wnt signaling pathway and target genes. Our current hypothesis is that TCDD inhibits epimorphic tissue regeneration by inappropriately activating Wnt signaling [70].

4.2. AHR activation and Wnt Signaling

Wnts are composed of a large family of highly conserved secreted factors with roles in development and normal homeostasis across taxa [72]. Their importance in development and human diseases has generated intense study, mostly focused on β -catenin-dependent (canonical) Wnt signaling (A number of Wnts appear to function independently of β -catenin, reviewed in [73]). In the nonstimulated condition, β -catenin accumulation is severely limited by a complex of proteins that include adenomatous polyposis coli (APC), Axin, and glycogen synthase kinase 3 β (GSK-3 β). Rapid phosphorylation by GSK-3 β , the signal for ubiquitination and targeting to the proteasome, normally prevents β -catenin accumulation. [74]. In canonical Wnt signaling, β -catenin accumulates cytoplasmically after Wnt binds to membrane associated Fzd/LRP receptor complexes, destabilizing the Axin-APC-GSK-3 β complex. Hypophosphorylated β -catenin accumulates and is translocated to the nucleus where it regulates target gene expression through interaction with the T cell-specific transcription factor/lymphoid enhancer-binding factor 1 (TCF/LEF) family of transcription factors [75]. There are a number of TCF/LEF target genes [76–79].

It is noteworthy that in addition to R-Spondin-1, a cluster of Wnt target genes were also altered by AHR activation in the regenerates of TCDD exposed zebrafish [56,70]. A mechanistic possibility is that R-Spondin1, as a Wnt ligand, is a TCDD-dependent upstream modulator of AHR-dependent signaling, thereby inhibiting tissue regeneration. If TCDD-elevated expression of R-Spondin1 is sufficient to inhibit regeneration, antisense repression of R-Spondin1 should restore it. This is exactly what was observed (Figure 3). R-Spondin1 morphants, in the presence of TCDD, regenerated their fin tissue indicating that induction of

the Wnt ligand during epimorphic regeneration inhibits normal regenerative signaling [70]. In support of this proposed mechanism, antisense repression of LRP6, the receptor for R-Spondin1, also blocked TCDD-dependent inhibition of tissue regeneration [70]. Collectively, we now know that knockdown of AHR signaling *via* zfAHR2 or zfARNT1, or of Wnt signaling *via* R-Spondin1 restores regeneration competency, otherwise lost upon TCDD exposure. This establishes a model of cross-talk between these two pathways. Implicit in the conservation of these pathways across species is that TCDD and related ligands may compromise wound healing in higher vertebrates, including humans.

4.2.1. Role of R-Spondins and Sox9—The R-Spondins are a new family of secreted proteins identified as ligands for the Fzd/LRP receptor complex and induce β -catenin/TCF dependent gene activation [71]. The family includes four members, each with a N-terminal signal peptide, two furin-type cysteine rich domains, a thrombospondin-type domain and a C-terminal basic amino acid rich domain [80,81]. Multiple studies in different species suggest that R-Spondin activity may function both within and outside of canonical Wnt signaling pathways [81,82]. Mutations in two R-Spondin genes have been associated with human disease. A rare autosomal recessive condition known as anonychia is linked to the mutation of R-Spondin4. A recessive syndrome characterized by XX sex reversal, palmoplantar hyperkeratosis and predisposition to squamous cell carcinoma, is due to the mutation of R-Spondin1 [83,84]. Mutation of R-Spondin1 causes XX sex reversal to male, perhaps by Sox9 promotion of testis development. Indeed, in the gonads of XX mice wildtype R-Spondin1 appears to normally down regulate the expression of Sox9 [83]. These examples are consistent with our microarray results from adult and larval zebrafish where TCDD-elicited AHR signaling resulted in dramatic R-Spondin1 induction, but strong repression of Sox9b transcription [56,70].

Sox9 is considered essential for commitment of mesenchymal cells to chondrocyte differentiation and cartilage formation [85,86]. A functional role of Sox9 was first discovered in humans with heterozygous mutations in and around the Sox9 gene resulting in skeletal malformations known as campomelic dysplasia (CD), often associated with XY sex reversal and other malformations of internal organs (reviewed in [87]). The two Sox9 paralogs in zebrafish, Sox9a and Sox9b, have partially overlapping developmental expression patterns and are thought to additively perform the conserved functions of Sox9 [88,89]. Sox9b homozygous mutant zebrafish can still regenerate their fin tissue, but do so with defective cartilaginous-like support structures and actinotrichia [70]. Studies using Sox9 mutant zebrafish and antisense translational repression determined that both Sox9 proteins are required for chondrogenic cell fate of cranial neural crest cells [86,88]. Thus, it is not surprising that TCDD exposure impairs chondrogenesis in an AHR2-dependent manner in the developing zebrafish head [46,59] to the extent that the TCDD-elicited head morphology is nearly identical to that of Sox9b morphants [88]. TCDD may also impair Sox9 function in mammals as evidenced by impaired bone growth and osteoblast differentiation in the rat [90,91].

4.2.2. Cross talk between SOX9 and Wnt/ β -Catenin signaling—Numerous studies have indicated that Wnt/ β -catenin signaling modulates Sox9 directly [92–95]. *In vivo* studies have revealed that over expression of Sox or inactivation of β -catenin led to a number of chondrocyte and bone defects in mice [96]. Inactivation of Sox9 in chondrocytes produces severe chondrodysplasia. Sox9 also inhibits the activation of β -catenin responsive genes and appears to increase the ubiquitination/proteasome dependent degradation of β -catenin. It has been proposed that Sox9 competes with Tcf/Lef for binding to β -catenin, leading to increased β -catenin degradation [96]. If this were a likely model, one might expect TCDD-elicited AHR signaling, with its subsequent downregulation of Sox9b transcription, to promote accumulation of β -catenin, stimulating the expression of β -catenin responsive genes. This is precisely what we have observed. A large number of β -catenin responsive genes were upregulated in the

regenerating tissues of TCDD-exposed adult and larval zebrafish [56,70]. Still elusive is the mechanism of regulation. Does AHR signaled induction of R-Spondin1 simply repress Sox9b transcription, or is an additional post-transcriptional or protein level regulation operative (Figure 4)? Further studies will help us to understand the complex regulation between AHR and Wnt signaling pathways.

4.2.3 The take-home message—TCDD-elicited AHR signaling results in the strong induction of R-Spondin1 and the reciprocally strong repression of Sox9 in larval and adult zebrafish. R-spondin is a Wnt ligand, and Sox9 is a negative regulator of β -catenin accumulation. Knockdown of AHR signaling *via* zfAHR2 or zfARNT1, or of Wnt signaling *via* R-Spondin1 restores the ability of TCDD-exposed zebrafish to regenerate their amputated caudal fin. Repression of Sox9 is accompanied by upregulation of numerous β -catenin-responsive genes. We propose that TCDD inhibits fin regeneration by AHR signaled misregulation of Wnt/ β -catenin signaling.

Conclusions

The field of toxicology is rapidly evolving from a descriptive to a mechanistic discipline. As toxicology is approached from a mechanistic perspective, we will be in a position to begin to unravel the complex environmental, genetic and biological interactions that define toxicity. There are inherent distinct advantages and disadvantages for all research models; however, with the advances in comparative genomics we now understand that remarkable similarities exist at the molecular level across taxa. Therefore, integrative approaches where the advantages of individual research models are exploited will speed the rate at which current information gaps are filled. Zebrafish share a number of similarities with well established rodent models at the genomics, cellular, physiologic and behavioral levels, but they also possess unique characteristics such as, external development, embryonic transparency, and amenability to efficient forward and reverse genetic manipulations. With the current tools in hand, there is a tremendous opportunity to exploit the unique advantages of zebrafish to improve human health.

The identification of the functional interaction between AHR and R-Spondin1 is relevant as proper regulation of Wnt/ β -catenin signaling is critical to maintain proper communication between epithelial and mesenchymal cells during embryonic development and during the early stages of tumor malignancies. R-Spondin1 functions proximally to Wnt/ β -catenin signaling at epithelial-mesenchymal transitions. Many tissues with the most sensitive developmental responses to TCDD require epithelial and mesenchymal interactions with the prostate and palate being particularly well-studied targets [63,97–100]. We propose that the AHR-dependent induction of R-spondin1 and the resulting downstream misregulation of Wnt/ β -catenin signal may alter individual susceptibility to other diseases. Finally, continued exploitation of the zebrafish model can help to define the genes downstream of AHR activation that are responsible for tissue specific responses.

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Figure 1. Schematic diagram depicting the events of epimorphic regeneration

Zebrafish regenerate the caudal fin after amputation through a distinctive three step process, a) the wound healing phase, b) the blastema phase and c) the regenerative outgrowth phase. Immediately after the amputation, the cells migrate over the wound to form the wound epithelium. This is followed by the de-differentiation of the cells beneath the wound epithelium to pluripotent cells to form the blastema. The blastemal cells proliferate and re-differentiate to complete the regenerative outgrowth phase.

Figure 2. TCDD mediated inhibition of regeneration of completely AHR2-ARNT1 dependent
Control morphants exposed to TCDD have impaired fin regeneration, whereas, zfAHR2 or zfARNT1 morphants regenerated the lost tissue after amputation. The expression of CYP1A is abundant in the trunk vasculature and epithelium of TCDD exposed control morphants, but not detectable in zfAHR2 or zfARNT1 morphants.

Figure 3. Misexpression of R-Spondin1 signaling through LRP6 is responsible for impairment of regeneration by TCDD
Antisense repression of R-Spondin1 or LRP6 blocks the inhibitory effect of fin regeneration by TCDD.

Figure 4. Mechanistic model

Activation of the AHR pathway results in the improper expression of R-Spondin1, which acts through LRP6 to activate Wnt/ β -catenin signaling. **A)** Over-activation of Wnt/ β -catenin signaling causes the stabilization of β -catenin, resulting in the misexpression of various Wnt target genes. **B)** β -catenin could also be regulated at the post-transcriptional level by direct interactions with Sox9 controlling the expression of these proteins. The inhibitory effect on regeneration after AHR activation could be due to an interplay between these events.