INAPPROPRIATE AHR ACTIVATION BLOCKS TISSUE REGENERATION BY MODULATING WNT SIGNALING

Mathew, L.K, Sengupta, S.S., Andreasen, E.A., and Tanguay R.L.[1, 2]

[1]Department of Environmental and Molecular Toxicology and [2]Environmental Health Sciences Center, Oregon State University, 1007 ALS, Corvallis, OR, 97331

Abstract
We have exploited the ability of zebrafish to regenerate lost or damaged tissues, a process that is dependent on cellular differentiation, proliferation and migration to identify interactions between AHR and other signaling pathways. We discovered that AHR activation inhibits regeneration in adult and larval zebrafish, demonstrated that the block in regeneration is AHR dependent. Comparative gene expression analysis between adult and larval regenerates revealed a number of conserved gene expression changes occur in response to AHR activation. Specifically, SRY-box 9 (Sox9) transcription factor is the earliest and most down-regulated transcripts in regenerating tissues. Sox9 is a master regulatory factor with important developmental functions. We also identified that one of the most highly induced genes in both regeneration models is R-sponidn1 (R-Spo1), which is a ligand for the Fdz/LRP receptors that increases β-catenin/TCF dependent gene activation. Using *in vivo* antisense morpholinos, we report that of R-Spo1 induction is necessary for TCDD to block tissue regeneration. We also demonstrate that R-Spo1 is significantly induced following TCDD exposure in human prostate cancer epithelial cells. We propose that the AHR-dependent induction of R-Spo1 and the resulting downstream misregulation of Wnt/ β -catenin signaling play a key role in responses to TCDD in vertebrate tissues.

Introduction
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. As a result, injury to a tissue or organ usually results in permanent damage (from scarring to disability). Some organisms; however, have retained the ability to regenerate their tissues, organs and appendages. By understanding the molecular and genetic pathways that coordinately function to accomplish regeneration in these "lower" animals, we will be in a stronger position to understand why mammals fail to regenerate tissue in response to injury. Probing the regenerative process by chemical genetic screening has the potential to reveal the signaling pathways required for vertebrate tissue regeneration. Thus, as proof of concept, TCDD was used as a chemical probe to evaluate interactions between the AHR and other signal transduction pathways. To that end, we previously reported that TCDD inhibits epimorphic fin regeneration in early life stage and adult zebrafish in an AHR dependent manner (1,2).

Epimorphic regeneration involves the reprogramming and migration of cells that differentiate and restore a tissue to its original form. In recent years the emergence of the zebrafish model has reinvigorated the field of adult fin regeneration (3). The zebrafish adult caudal fin (tail fin) consists of boney rays attached to the skeleton by ligaments. Each ray, referred to as a lepidotrichia, is comprised of two hemirays. The area between the hemirays of a lepidotrichia contains blood vessels, nerves and mesenchymal cells. After partial amputation of the caudal fin, an apical epithelial wound cap (AEC) forms over the clot at the amputation site. The AEC cells are derived from non-proliferating local epithelial cells that migrate laterally over the wound. Once the epithelial cap is formed, there is communication between the mesenchymal cells and the AEC leading to mesenchymal cellular proliferation and migration from sites distal to the wound plane and accumulation under the epithelial cap forming the blastema within 48 hpa. Outgrowth begins at 2 days post amputation (dpa) until the regenerative event is complete at about 14 dpa.

Early life stage regeneration: Many of the experimental advantages of zebrafish reside at the earliest life stages. Recently it was reported that the embryonic fin is capable of complete regeneration (2-5 days post fertilization) (4). There is mounting evidence that there are significant similarities at the cellular and molecular level between adult and early life stage regeneration, despite the fact that the tissues are structurally different. Morphologically, the

larval fin regenerates by a process that resembles that of the adult. A wound epithelium covers a pool of highly proliferating blastema-like cells as in adult regeneration (4). Also not surprisingly, as in the adult, chemical inhibition of FGFR1 abrogates fin regeneration (1,4). There is growing evidence to suggest that heart regeneration in newts and zebrafish also share a high degree of commonality with fin regeneration (5). Both tissues regenerate through a wound formation, cellular proliferation and wound healing to complete outgrowth. Gene expression of *msxB* and *msxC* encoding homeo-domain containing transcription factors are reinduced in regenerating zebrafish hearts as early as 3 days post amputation (dpa) and also in regenerating fin blastema (6).

Wnt/β-catenin signal transduction pathway. There is a significant body of evidence indicating the importance of Wnt/β-catenin signaling in development, normal cellular function, and in diseases. Wnts are composed of a large family of highly conserved secreted factors that play roles in development and in normal homeostasis across taxa (7). Wnts have been associated with a number of different biological responses, and the majority of work has focused on β-catenin-dependent (canonical) Wnt signaling. For canonical Wnt signaling, there is cytoplasmic accumulation of β-catenin and its subsequent nuclear translocation and transcriptional activity. The levels of β-catenin protein are under tight control by a regulated degradation pathway. Recent studies illustrate that a proper balance of Wnt/β-catenin signaling is also found to be critical for the formation and proliferation of blastema cells that is required for regeneration (8,9). Interestingly, overexpression of wnt5b inhibits regeneration, whereas wnt5b mutants elicit an accelerated regenerative growth implicating the significance for a tight regulation of these signaling molecules (9).

Materials and Methods

TCDD dosing and fin amputation. Adult male zebrafish (*Danio rerio)* (AB strain, Eugene, OR) were anesthetized with tricaine methanesulfonate (MS-222) and their caudal fins were amputated at the bifurcation of the fin rays. Fish were then injected with phosphatidylcholine liposomes (vehicle control) alone or liposomes incorporated with TCDD (>99% pure, Chemsyn, Lenexa, KS) prepared as previously described (10). Three groups of tissue were obtained from vehicle control or TCDD exposed fish at 1, 3, and 5 days post amputation (dpa). For the larval studies, 48 hpf larvae were anesthetized with MS-222, followed by amputated with a diamond blade. Fish were waterborne exposed to TCDD (0.5 ng/ml) for 1 hour and allowed to recover in chemical free water for up to three days. Two groups of larvae were obtained from vehicle or TCDD exposed fish at 2 and 3 dpa. Due to tissue limitation, each group consisted of 150 individual regenerates. Total RNA was purified with TRI reagent (Molecular Research Laboratories, Cincinnati, OH) according to the manufacturer's instructions. Total RNA was DNase treated with RQ1 DNase (Promega, Madison, WI) according to the manufacture's protocol and RNA quantity and quality was determined by UV absorbance. Ribosomal RNA abundance and degree of degradation was determined in electropherogram patterns using the 2100 Bioanalyzer and RNA 6000 Nano chips (Agilent Technologies, Palo Alto, CA).

Affymetrix microarray processing. Probe synthesis, hybridization and scanning were conducted by the Center for Gene Research and Biocomputing at Oregon State University, Corvallis OR. Biotinylated cRNA was synthesized from the double stranded cDNA using T7 RNA polymerase and a biotin-conjugated pseudouridine containing nucleotide mixture provided in the IVT Labeling Kit (Affymetrix, Santa Clara, CA). Prior to hybridization, the cRNA was purified, fragmented and 10 µg from each experimental sample was hybridized to zebrafish genome arrays (Zebrafish430_2) according to the Affymetrix GeneChip Expression Analysis Technical Manual (701021 Rev. 5). Each sample was conducted in independent biological triplicate.

Morpholino microinjection. Zebrafish R-Spo1 morpholino (Gene Tools, Corvallis, OR) was designed to target the Intron 1-Exon2 junction (5'GTGCTTACTGATGGAGAAAAGACAG3') and the 3' end was fluorescein tagged to assess microinjection success. A standard morpholino (Gene Tools, Corvallis, OR) (5'CTCTTACCTCAGTTACAATTTATA 3') was used as a control (Control-MO). Morpholinos were diluted to 1.5 mM in 1X Danieau's solution (58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO4, 0.6 mM Ca(NO3)2, 5 mM HEPES, pH 7.6) and 1-3 nl was injected at the 1–2 cell stage. Embryos were screened for fluorescence at 24 hpf to assess for successful injection. Regeneration progression was assessed daily until 3 dpa.

Human prostate cell culture studies. Non-tumorigenic human prostate epithelial cells (BPH-1), androgen-dependent tumorigenic prostate epithelial cells (LNCaP) and androgen-independent tumorigenic prostate epithelial cells (PC-3) were grown to 50% confluency, and then were exposed to DMSO, 1 or 10 nM TCDD for 24 hours prior to RNA isolation. To determine if transcriptional activation was required to lead to R-Spo1 mRNA elevation, LNCaP cells were exposed to ActinomycinD (1 μg/ml) one hour prior to TCDD exposure. For each sample, quantitative RT-PCR was performed using human R-Spo1 and CY1B1 primers. The gene expression experiments were conducted in triplicate.

Results and Discussion

Comparative Toxicogenomic Approach Reveals Misregulation of Wnt Signaling by AHR Activation Gene. To reveal the gene expression changes that result from TCDD exposure, Affymetrix microarray analysis was conducted using RNA isolated from larval and adult regenerates exposed to vehicle or TCDD. Since AHR activation inhibits regeneration at both life stages, we speculated that there would be common molecular responses to TCDD exposure. The transcriptional response to TCDD in larval fin regeneration system was compared with our previously published adult fin gene expression data (10). The genes that were differentially expressed by TCDD exposures in both adult and larval regenerates were therefore identified. Approximately, 50 common transcripts were enhanced and 150 transcripts were repressed due to AHR activation in the two regenerative models. Transcripts related to extracellular matrix metabolism, cell adhesion and migration were the most differentially expressed genes after TCDD exposure in both the models.

Misregulation of Wnt Signaling by AHR activation is mediated through R-Spondin1. R-Spo1, a novel ligand for the Wnt receptor was one of the most highly TCDD-induced genes at all the time points analyzed in both regeneration models. Since R-Spo1 is an upstream modulator of Wnt signaling, we hypothesized that AHR-dependent misexpression of R-Spo1 may dictate the downstream gene expression changes that collectively result in impaired extracellular matrix remodeling and cellular differentiation, adhesion and migration. If the induction of R-Spo1 is absolutely required for TCDD to block the regenerative growth, antisense repression of R-Spo1 should permit regeneration even in the presence of TCDD. A splice junction morpholino was designed against the R-Spo1 gene in the I1E2 boundary. The control and R-Spo1 microinjected embryos (morphants) at 48hpf were amputated and exposed to vehicle or TCDD and raised for 3 days. The control and R-Spo1 morphants exposed to vehicle regenerated whereas, TCDD blocked the regeneration of control morphants. On the other hand, partial suppression of R-Spo1 using splice blocking morpholinos abrogated the TCDD-dependent block of regeneration, suggesting that inappropriate induction of R-Spo1 is required for TCDD to impair regeneration.

We recently reported that SOX9b message is induced during normal regeneration suggesting that it plays a role in normal fin regeneration. Importantly, in the presence of TCDD, SOX9b was the earliest and most strongly reduced transcript in the adult and larval regenerates (10,11). SOX9 is considered essential for commitment of mesenchymal cells to chondrocyte differentiation and cartilage formation (12,13). Recent human genetic studies revealed that mutations in two R-Spo genes have been associated with human disease. A rare autosomal recessive condition known as anonychia is linked to the mutation of R-Spo4 and another recessive syndrome characterized by XX sex reversal, palmoplantar hyperkeratosis and predisposition to squamous cell carcinoma is due to the mutation of R-Spo1 (14,15). Mutation of R-Spo1 causes the XX sex reversal to male and this impact is speculated to be due to the inappropriate induction of SOX9 promoting the testis development. The expression pattern of R-Spo1 and SOX9 is also inversely expressed in the gonads of mice with two X chromosomes suggesting that R-Spo1 down regulates the expression of SOX9 in the gonads (14). Using Sox9b knockout fish, we have similarly concluded the TCDD-dependent repression of Sox9b is downstream of R-Spo1 as Sox9b$^{-/-}$ animals regenerate their amputated caudal fins. It is noteworthy that the regenerates of Sox9b $^{-/-}$ animals display defects in actinotrachial fibril formation, which later give rise to the cartilaginous fin rays.

TCDD induces R-Spo1 transcription in human prostate cancer cells. The mesenchymal-epithelial signaling in the mouse UGS is essential for prostatic bud formation to occur, but the various signaling pathways involved are now just beginning to be discovered. Since AHR signal transduction has been evaluated in human prostate cancer cells (16,17), and that the prostate development is particularly responsive to TCDD we have evaluated the expression of R-Spo1 in non-tumorigenic human prostate epithelial cells (BPH-1), androgen-dependent tumorigenic prostate epithelial cells (LNCaP) and androgen-independent tumorigenic prostate epithelial cells (PC-3). The three cell lines were exposed to DMSO, 1 or 10 nM TCDD for 24 hours prior to RNA isolation and qRT-PCR analysis using human R-Spo1 and CY1B1 specific primers. TCDD exposure leads to significant induction of CYP1B1 in all three cell lines. Most importantly, R-Spo1 was significantly induced by 1 nM TCDD in LnCaP cells. To determine if the LNCaP response was at the level of transcription, ActinomycinD (1 μg/ml) was added one hour prior to exposure to inhibit transcription in the presence and absence of TCDD. TCDD significantly increased R-Spo1 expression, and the inhibition of transcription completely blocked the elevation of the R-Spo1 mRNA. These data suggest that the AHR dependent transcriptional induction of R-Spo1 is conserved between zebrafish and humans and the tissue regeneration models offer unique opportunities to dissect the mechanism of the crosstalk between Wnt and AHR signaling.

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