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IDENTIFICATION OF STAGE SPECIFIC GENE MODULATION DURING EARLY THYMOCYTE DEVELOPMENT BY WHOLE GENOME PROFILING ANALYSIS FOLLOWING ARYL HYDROCARBON RECEPTOR ACTIVATION

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Running title: Stage specific gene regulation by AHR activity in thymocytes

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The abbreviations used are: AHR, aryl hydrocarbon receptor; TCDD, tetrachlorodibenzo-p-dioxin; TN, triple negative; TCR, T cell antigen receptor; RAG, recombinase activating gene; GCOS, Genechip operating system; IPA, ingenuity pathway assist; MTAP, 5 –methythioadenosine phosphorylase.

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Abstract:

The Aryl Hydrocarbon Receptor (AHR) is a basic helix-loop-helix transcription factor, implicated as an important modulator of the immune system and early thymocyte development. Previously we have shown that AHR activation by the environmental contaminant and potent AHR agonist, 2,3,7,8 Tetrachlorodibenzo-p-dioxin (TCDD) leads to a significant decline in the percentage of S phase cells in the CD3-CD4-CD8- triple negative stage 3 (TN3) and TN4 T-cell committed thymocytes 9-12 hours after exposure. In the more immature TN1 or TN2 stage cells no effect on cell cycle was observed. In order to identify early molecular targets, which could provide insight into how the AHR acts as a modulator of thymocyte development and cell cycle regulation, we performed gene profiling experiments using RNA isolated from four intrathymic progenitor populations where the AHR was activated for 6 or 12 hours. This microarray analysis of AHR activation identified 108 distinct gene probes that were significantly modulated in the TN1-4 thymocyte progenitor stages. While most of the genes identified have specific AHR recognition sequences, only seven genes were altered exclusively in the two T-cell committed stages of early thymocyte development (TN3 and TN4) in which the decline of S phase cells is seen. Moreover, all seven of these genes were reduced in expression and five of these seven are associated with cell cycle regulatory processes. These 7 genes are novel targets for modulation by the TCDD activated AHR and may be involved in the observed cell cycle arrest and suppression of early thymocyte development

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The Aryl hydrocarbon receptor (AHR), a member of the basic helix loop helix–PAS family of transcription factors, has been implicated as an important modulator of immune system development and function (Stevens et al., 2009; Stockinger et al., 2009). Studies using AHR null mice suggest that AHR dependent gene transactivation is required for normal vascular development, circadian rhythms and hematopoietic stem cell regulation (Fernandez-Salguero et al., 1996; Garrett and Gasiewicz, 2006; Schmidt et al., 1996). AHR agonists regulate tolerogenic signaling in autoimmunity models, cause immune suppression in infectious disease models, and modulate cell cycle regulation during early thymocyte development (Funatake et al., 2005; Hogaboam et al., 2008; Laiosa et al., 2003; Quintana et al., 2008; Veldhoen et al., 2008).

Given the requirement for the thymus in establishing central tolerance, we have had an interest in identifying the molecular mechanisms by which AHR dependent gene modulation within thymocyte progenitors modulates cell cycle regulation in specific stages of thymocyte ontogeny. We previously, have shown that AHR activation by TCDD causes a dramatic reduction in cell cycle progression within 9-12 hours of *in vivo* exposure to TCDD within a subset of cells called TN3 and TN4, but not their immediate thymocyte precursors TN1 or TN2 (Laiosa et al., 2003). Identification of the TN3 and TN4 stage thymocytes as proximal targets of AHR activation is important as the TN3-TN4 developmental transition is a critical checkpoint during thymocyte development where the T-cell antigen receptor β (TCRβ) gene locus is rearranged and the resulting protein trafficked to the cell surface in a complex known as the pre-TCR. If the pre-TCR is functional, the cell becomes committed to the αβ-T-lineage, the TCRβ rearrangement machinery is turned off, and cell survival is driven by pre-TCR dependent signals. Pre-TCR dependent survival signals drive entry into the cell cycle, lead to differentiation into TN4 stage cells, and eventual up-regulation of the CD4 and CD8 TCR co-receptors. An important question that remains from this earlier work is how does AHR dependent signal transduction modulate cell cycle in the pre-TCR dependent stage of T cell development? Is it due to direct modulation of cell cycle regulating

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genes expressed in the TN3 and TN4 subsets; indirectly through modulation of other immune specific growth regulatory genes; or are there other unique gene regulatory pathways modulated by the AHR in these cells that affect cell cycle progression and thymocyte maturation?

In order to begin to address the mechanism by which AHR activation effects cell proliferation and thymocyte differentiation, we conducted gene profiling experiments to identify candidate genes and gene pathways modulated specifically in the TN1-TN4 stages of early thymocyte development. Our approach is distinct from previous gene profiling investigations, which focused on either whole thymus preparations, or on a single population of cells within the thymus (McMillan et al., 2007; Svensson and Lundberg, 2001). For example, the gelsolin like protein scinderin (adseverin 5) was identified as a thymus specific marker induced by TCDD activation of AHR in all thymocyte subsets, including the TN1-TN4 stages (Svensson et al., 2002). In another study genes associated with the KLF2 regulon were modulated by AHR activation by TCDD in a thymocyte population not seen in normal mice (McMillan et al., 2007). However, these investigators utilized a higher dose of TCDD than in most previous studies, and they also conducted their *in vivo* studies in mice deficient for the recombinase activating gene-2 (RAG2) (McMillan et al., 2007). RAG2-/- mice have a complete block in thymocyte development at the TN3 stage owing to their inability to rearrange the T-cell antigen receptor β locus and inability to express a functional pre-TCR (Levelt et al., 1993; Mombaerts et al., 1992). Consequently, it is possible that the gene expression changes observed in this study may be secondary to the aborted pre-TCR dependent survival signaling in these mice.

Our genetic profiling approach differed from these earlier studies because it did not bias the results using genetically modified mice, a pre-selected cell population, or extracts from the entire heterogeneous mixture of thymocytes. In order to determine the actual early gene regulation events modulated by an activated AHR that could explain the cell cycle effects in the pre-TCR stage of thymocyte development, we performed sixteen separate gene-profiling experiments using RNA obtained from sorted pooled TN1&TN2 cells; TN3; TN4A; and TN4B subsets at 6 and 12 hours after exposure (Figure 1). Gene expression changes between treatment, thymocyte population and time were compared

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yielding potential insights into how persistent AHR activation modulates cell cycle regulation during early thymocyte development.

Materials and Methods:

Mice and treatment protocol:

Four to five week old C57BL/6J ($AHR^{b/b}$) mice were purchased from the Jackson Laboratory and allowed to acclimate for one full week after arrival at the Upstate Medical University. Mice were maintained on a 12-h dark, 12-h light cycle and were provided food (5001 rodent diet, Purina Mills, St. Louis, MO) and water ad libitum. All mice were housed and cared for according to protocols approved by the animal welfare committees at both SUNY Upstate Medical University and University of Rochester.

TCDD was obtained from Cambridge Isotopes (Andover, MA). A stock solution in the solvent p-dioxane (Sigma-Aldrich, Milwaukee, WI) was diluted to an appropriate concentration in olive oil (F. Berio, Lucca, Italy) to yield a treatment solution containing 6 µg/ml TCDD. Mice were injected i.p. with either 30 µg/kg TCDD in olive oil per kilogram of body weight, or olive oil alone in a volume of 0.1 ml per 20 g of body weight six or twelve hours before euthanasia. Injections were scheduled such that euthanasia and tissue harvest could be completed for one group of five mice, before the second group was started (one hour apart). Thymocytes were pooled from five mice per group and each time point was conducted four times for TCDD and four times for control.

Flow cytometry staining and cell sorting:

Mice were euthanized by CO_2 asphyxiation, followed by cervical dislocation at the appropriate time point after TCDD exposure. For each experimental group, thymi from five mice were removed, pooled, and cell suspensions made in cold MEM containing 5% FBS plus 100 U/ml penicillin and 0.1 mg/ml streptomycin (P/S; Life Technologies, Rockville, MD). Debris was eliminated by passing the cell suspension through Pasteur pipettes containing 80-gauge nylon mesh (Tetko, Kansas City, MO). Cell

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yield and viability were enumerated with a Neubauer hemocytometer (Reichert, Buffalo, NY). Cell viability was determined to be >95% from all cell suspensions by trypan blue dye exclusion.

Pooled thymocytes were washed with PBS+2mM EDTA in preparation for enrichment of CD4 negative cells. Specifically, thymocytes were labeled with magnetic-microbead conjugated anti- CD4 (clone RM4-5, rat IgG2a) (BD Biosciences, San Jose, CA) according to the manufacturer's instructions. After labeling was complete, cells were washed, resuspended in running buffer (PBS+2mM EDTA+0.5% FBS), and separated using the BD Imag cell separation magnet. Enriched cells were counted and prepared for fluorochrome labeling and fluorescence activated cell sorting.

In preparation for cell sorting, CD4 negative thymocytes were Fc blocked (clone 2.4G2) and subsequently stained with a cocktail of PE-Texas Red conjugated CD3 (clone 145-2C11), CD4 (clone L3T4), CD8 (clone 53-6.7), αβ TCR (clone H57-597), γδ TCR (clone GL-3), CD11b (clone M1/70), CD11c (clone HL3), GR-1 (clone RB6-8C5), NK1.1 (clone PK136), B220 (clone RA3-6B2) and Ter119. Additionally, cells were stained with PE- conjugated CD24 (HSA; clone M1/69), FITC- conjugated CD25 (clone 7D4), and APC-conjugated CD44 (clone IM7). All antibodies were purchased from BD Biosciences, (San Jose, CA). After TN enriched thymocytes were labeled with fluorescence-conjugated antibodies directed at thymocyte surface antigens, cells were separated into four triple negative (TN) populations designated TN1+2, TN3, TN4A, and TN4B, utilizing a BD FACSVantage cell sorter and DiVa (5) software.

Microarray Studies

To evaluate the molecular mechanisms underlying effects of AHR activation following TCDD exposure on thymocyte differentiation, we performed comprehensive gene expression profiling with the mouse 430 2.0 GeneChip (Affymetrix). RNA for these analyses was obtained from three independent replicate preparations involving four FACS-sorted fractions (TN1+2, TN3, TN4A, TN4B) at two time points (6 hr and 12 hr) following exposure to either TCDD or vehicle. Thus, 48 RNA samples (3 replicates x 4 fractions x 2 time points x 2 treatments) were used for microarray analysis. The RNA from

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these preparations was purified using the RNeasy kit (Qiagen, Valencia, CA) and the quality and yield examined using the RNA NanoChip (Agilent Technologies, Santa Clara, CA). Amplification and labeling of the 48 RNA samples were performed using the WT-Ovation™ RNA Amplification System (NuGen, San Carlos, CA), and processing of the GeneChips performed according to standard protocol (GeneChip Expression Analysis Technical Manual 701021 rev 5, Affymetrix, Santa Clara, CA). After scanning, the microarray images were analyzed using GeneChip Operating System (GCOS) software to obtain performance metrics, and normalized using the RMA method during import into GeneSpring GX 9.0 (Agilent).

Significant differences in transcript expression were determined using a three-way analysis of variance (ANOVA) comparing treatment x fraction x time (2 x 4 x 2 ANOVA). The nominal significance for the main effect that was obtained was adjusted for multiple testing of the 45,101 probes on the 430 2.0 array using the Bonferroni-Holm method (with α=0.05). Because our primary aim in this report was to determine those genes with significant TCDD-induced expression changes, we focused our analysis on those probes with a significant main effect of treatment and/or a significant interaction of treatment and time after correction (n=108 total probes).

IPA Functional Analysis

To further understand the biological relevance of the statistically significantly changed probes, we used Ingenuity Pathway Assist (IPA) to explore their relationships and known annotations. Enrichment scores and/or P values were automatically generated by the software for the top implicated Functional Networks, as well as Bio Functions, and Canonical Pathways based on a comparison of the uploaded 108 probes compared with the annotated content of the 430 2.0 GeneChip.

Real Time Quantitative RT-PCR Validation

From the list of genes with significant changes in expression due to treatment, as well as genes known to be differentially expressed in specific fractions, we derived a set of eight genes to use for

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validation of the microarray findings (Acpp (acid phosphatase, prostate), CD44, CD8a, Cyp1b1, PtcrA (Pre-TCRa), Scin (Scinderin), Tparp (TCDD-inducible poly(ADP-ribose) polymerase), and Trpm1). Primers for each of these genes were obtained from previous reports or custom-designed using Primer3 software. Once the optimal conditions were obtained for each primer set, the quantification reactions were performed on the same RNA fractions prepared for the microarrays. Thermal cycling was performed on a BioRad iCycler iQ5 in triplicate reactions on 96 well plates. Ribosomal protein S6 (Rps6) was chosen as the internal reference gene and differences in expression were calculated using the delta Ct method. Statistical significance was determined using a repeated measures ANOVA on all 10 genes, with a Scheffe post-hoc test.

Results:

Thymocyte cell numbers are unchanged by TCDD exposure

In order to identify the AHR dependent modulation of gene expression which potentially mediates alterations in early thymocyte cell cycle regulation and T-cell development, we sorted early stage thymocytes from vehicle or TCDD exposed mice, extracted RNA from each pool of cells, and performed a gene profiling experiment on the RNA from each stage of early thymocyte development. As shown in Figure 1, the sorting gates chosen were designated TN1&2, TN3, TN4A, and TN4B. Importantly, there were no differences in frequency among the four populations between vehicle or TCDD-exposed groups at either time point (data not shown and reference (Laiosa et al., 2003)).

The sorting gates shown in Figure 1 separate the thymocyte progenitors four ways consisting of a pooled TN1&TN2 population, and the three T-committed populations: TN3, TN4A and TN4B to identify gene expression changes in each subset dependent on AHR activation. Cell sorting based on CD44, CD25 and HSA resulted in populations that were at least 98% pure.

As further confirmation of our purity, RT-PCR was performed on RNA isolated from each population using genes known to be differentially expressed during early thymocyte development. Specifically, the gene expression patterns of CD44, preTα (Ptcra), and CD8 were analyzed in each sorted

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population to confirm that CD44 expression is enriched in the TN1&TN2 subset, preTα is enriched in the TN3, with lower expression in TN4, and CD8 expression is relegated to TN4A and TN4B. Indeed, as shown in Table 1, expression levels of the developmentally regulated genes were considerably higher in the appropriate stages compared to the same message levels in developmental stages where expression is predicted to be extinguished. Notably, CD44 gene expression in the TN1&2 population was almost completely absent in the TN3, TN4A, and TN4B populations. Furthermore, the gene encoding the invariant preTα (Ptcra) was 31 fold higher in TN3 than in TN1&TN2. Finally, CD8 expression in TN4A was 15.9 fold enriched compared to its expression in TN1&TN2, and 12.6 fold enriched compared to CD8 expression in TN3. These data demonstrate the fidelity of RNA obtained from our four-way cell sorting procedure.

108 probes are modulated by AHR activation and are developmentally regulated in early thymocyte progenitors:

In order to identify candidate genes responsible for the profound block in DNA synthesis that occurs in the TN3 and TN4 stages of thymocyte development following exposure to TCDD, we performed gene expression profiling experiments using purified RNA from each thymocyte progenitor subset. Significant differences in transcript expression were determined using a three-way analysis of variance (ANOVA) comparing treatment x fraction x time (2 x 4 x 2 ANOVA). The nominal significance for the main effect that was obtained was adjusted for multiple testing of the 45,101 probes on the 430 2.0 array using the Bonferroni-Holm method (with α=0.05). In order to determine those genes with significant TCDD-induced expression changes, we focused our analysis on those probes with a significant main effect of treatment and/or a significant interaction of treatment and time after correction (n=108 total probes). This analysis identified 108 of 45,101 probes for which a significant interaction of treatment and time was observed. Of the 108 probes significantly modulated, there are 21 duplicate probes indicating that the gene expression of 87 unique genes and/or expressed sequence tags are modulated six to twelve

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hours after exposure to TCDD compared to vehicle exposed mice within intrathymic hematopoietic progenitor cells. The 87 genes, their symbol, and unigene identification are listed alphabetically in Table 2.

From the identification of the 108 gene probes demonstrating a significant interaction of treatment and time our analysis was further refined to identify potential changes in expression, which were dependent on the stage of thymocyte differentiation. As shown in Figure 2, a heat map was generated to display the relative expression of each of the 108 probes within each thymocyte progenitor population at six and twelve hours after exposure to either vehicle or TCDD. Display of the relative changes for the affected probes reveals a striking diversity in responses to TCDD that is both time and population dependent. For example, probes corresponding to Cyp1b1, Lgals3, Ahrr, and Scin all show a significant increase in gene expression across all four populations (Figure 2). Notably, Cyp1b1, Lgals3, Ahrr, and Scin were among the most highly TCDD-inducible probes on the array. In comparison to probes found to be induced in all four sorted populations, we identified seven probes on the array, that showed a significant interaction of treatment and fraction. Specifically, probes associated with Hspa4, Hspa9, Mtap, Nolc1, Pprc1, Tmem97, and Wdr12 revealed TCDD-modulated changes that occurred primarily in the TN3, TN4A, and/or TN4B populations with little to no modulation occurring in TN1&2 (Figure 3). Moreover, the expression levels of the seven probes where gene modulation was limited to the TN3, TN4A, and/or TN4B subsets is lower following TCDD exposure compared with vehicle treated controls. It should be noted that the identification of genes with an interaction of treatment and fraction were not selected for *a priori*.

Validation of the results from the gene profiling experiment was performed using real time reverse transcriptase PCR (RT^2-PCR) with primers for four genes modulated by TCDD. Specifically, Acpp, Cyp1b1, Scin, and Tparp were analyzed by RT^2-PCR for potential changes induced by TCDD in each sorted thymocyte sub-population six hours after exposure. As shown in Figure 4, in each population the fold changes induced by TCDD detected by microarray were similar to the fold changes detected by

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RT^2-PCR. It should be noted that only genes that reported a significant treatment effect of TCDD are plotted. For example, the Cyp1b1 comparison is not plotted in the TN1&2 or TN4A subsets because there was not a significant treatment effect difference for Cyp1b1 in these two subsets. Additionally, RMA normalization is known to underestimate true changes, particularly for low expressing genes. This underestimation may account for the discrepancies between RT^2-PCR and microarray expression data that are observed for scinderin in the TN4A and Cyp1b1 and Tparp in the TN4B population.

IPA Functional Analysis

We explored functional relevance of 87 significantly changed genes using IPA. Results for the top five implicated Functional Networks, Bio Functions (which includes three classes: Diseases and Disorders; Molecular and Cellular Functions; Physiological System Development and Function), and Canonical Pathways are all displayed in Table 3. Examination of these data showed that the top ranked Functional Network (Endocrine System Development and Function, Lipid Metabolism, Small Molecule Biochemistry) was highly similar to the second ranked Functional Network. In addition, the top Bio Functions in the Molecular and Cellular Function class and Physiological System Development and Function class were also highly similar and related to 5th ranked Functional Network (Cell Morphology, Cellular Development, Cellular Growth and Proliferation). Because of these redundancies, we elected to display biological interaction networks for the 1st and 5th ranked Functional Networks (Figure 5).

Discussion:

We conducted *in vivo* gene profiling analysis in defined stages of early thymocyte development to identify candidate genes that could potentially explain the alterations in the development of thymocyte progenitors following AHR activation by TCDD. In TCDD exposed mice, we identified 108 unique gene probes whose corresponding 87 gene products were modulated, relative to vehicle treated mice. Our rationale for analyzing gene expression within individual early thymocyte subsets was based on our earlier findings demonstrating stage specific alterations in DNA synthesis in the TN3 and TN4 stages of

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thymocyte development as measured by BrdU incorporation following TCDD exposure (Laiosa et al., 2003). Moreover, given the striking functional and developmental differences which exist within the defined stages of thymocyte development it was anticipated that our approach might reveal subtle yet critical differences in AHR dependent gene regulation in a stage specific manner. Specifically, we anticipated the possibility of detecting differences in AHR regulated gene expression between the T-cell committed TN3 and TN4 stages, compared with the non-T cell committed TN1&TN2 stages of thymocyte development.

In support of our approach, we identified a number of genes, which have been implicated as targets of AHR activation. Specifically, Cyp1a1, Cyp1b1, Lgals3, and the AHR repressor have all previously been identified in a number of cell types to be induced following exposure to AHR agonists (Mimura et al., 1999; Okey et al., 2005; Puga et al., 2005). In addition, we found TCDD exposure led to the induction of scinderin and KLF2, two genes previously identified as AHR dependent hematopoietic targets in the thymus (McMillan et al., 2007; Svensson and Lundberg, 2001; Svensson et al., 2002).. The elevated thymus expression of scinderin following AHR activation identifies this protein as an interesting candidate potentially involved in mediating TCDD-induced changes in T-cell development and cell cycle regulation. Indeed, loss of scinderin expression has been associated with megakaryoblastic leukemia cells, and the forced expression of scinderin within these oncogenic cells dramatically reduced cell proliferation (Zunino et al., 2001). The decrease in the megakaryocyte proliferation was attributed at least in part due to a loss of filimentous actin (F-actin) which is important for cell division (Zunino et al., 2001). Given this potential cell cycle regulatory role of scinderin, the earlier identification and our confirmation of scinderin expression in immature thymocytes following AHR activation raises the hypothesis that AHR activation in TN3 and TN4 stage thymocytes induces scinderin, leading to a decrease in F-actin and a rapid loss of cell proliferation.

While confirmation of gene products previously shown to be induced by TCDD in the thymus provided confidence that our approach and methodology was valid, new insights into potential mechanisms of AHR-dependent changes in T-cell development might be obtained from the identification

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of gene expression changes in a stage specific manner. Indeed, our analysis resulted in the identification of seven gene products whose expression varied only slightly in the TN1&2 subset, but, underwent significant modulation in the TN3, TN4A and/or TN4B subset. Moreover, all seven gene probes reflected a decrease in gene expression in the appropriate populations. The decrease in gene expression following AHR activation, while not unprecedented, is generally not considered to be the primary mode by which AHR agonists affect transcriptional regulation (Gasiewicz et al., 2008).

Several possibilities exist as to why seven of the modulated genes from our gene profiling analysis are modulated in a stage specific manner and why all of these genes show a decrease in expression levels. First, and perhaps most obvious is that the AHR is directly involved in active repression of these genes through recognition of AHREs present in the upstream regions of the genes. Indeed, all seven genes had at least one core AHRE consensus sequence (GCGTG), and Pprc1, Tmem97 and Wdr12 also possess extended AHRE consensus sequences (TNGCGTG) in their enhancer regions (data not shown). Alternatively, the AHR may act indirectly on the transcription of these seven genes by inducing repressor protein(s) involved in turning off their expression. Additionally, the TN3 stage of thymocyte development is the developmental stage where TCRβ rearrangement and pre-TCR dependent signals drive T-cell commitment. Thus, there may be factors intrinsic to the TN3-TN4 stages, (including but not limited to the pre-TCR complex for example), which coordinate with AHR dependent signal transduction in order to modulate genes controlling growth and developmental processes.

Indeed, of the seven genes whose expression decreases in the TN3-TN4 stages, five of the genes have all been associated with cell growth and proliferation processes. Of these genes, decreased expression in four of the five is consistent with a block in progression through the cell cycle. Specifically, 5 –methythioadenosine phosphorylase (Mtap) is a critical enzyme required for the salvage pathway for methionine and adenine biosynthesis (Avila et al., 2004). In the absence of Mtap, the precursor product Methylthioadenosine (Mta) accumulates, which has been demonstrated to inhibit cell proliferation in some cellular systems (Avila et al., 2004). Paradoxically, loss of Mtap has also been associated with several types of tumors, however, excess Mta is thought to be secreted by these abnormal cells (Avila et

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al., 2004). While decreased Mtap expression within TN3-TN4 stage thymocytes could potentially account for the observed decrease in DNA synthesis observed in these cells, the kinetics of its loss would seem to suggest it is not the primary candidate. Moreover, gene expression levels of Mtap do not decline significantly until the 12 hour time point, concurrent with the time when BrdU incorporation is also inhibited. Thus, while Mtap deficiency and Mta accumulation may contribute to the observed cell cycle defects, the kinetics are inconsistent with a causative relationship.

In comparison to Mtap, expression of Pprc1, Tmem97, and Wdr12 are all attenuated at the six hour time point in at least one of the three T-cell committed sub-populations (TN3, TN4A, and TN4B). Of these, Tmem97, (also known as Mac30), and Wdr12 have both been implicated in cellular processes affecting cellular growth and proliferation. Specifically, Wdr12 is a critical member of a three protein nucleolar protein complex known as a PeBoW complex (Rohrmoser et al., 2007). Knock-down experiments demonstrate that disruptions to the formation of the complex result in a failure to make the 28S rRNA, resulting in a p53 dependent cell cycle arrest (Rohrmoser et al., 2007). Tmem97 expression in contrast, is associated with several different human tumors and has been implicated as a possible oncogene (Moparthi et al., 2007). However, Tmem97 has also been implicated in cholesterol/lipid biosynthetic pathways (Wilcox et al., 2007). Thus, an improved understanding of the putative function of Tmem97 is required before a definitive role in mediating AhR-dependent changes in T cell development can be concluded. Nevertheless, the kinetics and magnitude by which its expression levels are attenuated within the TN3 compartment suggest further study may be warranted.

In addition to identification of potential cell cycle regulatory genes, our analysis revealed modulation of the immune specific genes IL-7R, and TGFβR1. Specifically, elevation of TGFβR1 expression by TCDD in the TN1&TN2, TN3, and TN4A stages may increase the sensitivity of these cells to members of the TGFβ superfamily that signal, at least in part through TGFβR1 including TGFβ1, TGFβ2, bone morphogen proteins and activins. Collectively, TGFβ superfamily proteins are known to both positively and negativley modulate early thymocyte development, however the mechanism by which

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the appropriate balance is maintained is an area of active investigation (Licona-Limon and Soldevila, 2007). Thus a model system where the receptor expression levels are affected by AHR activation during early thymocyte development may be a useful model for dissecting the contribution of TGFβ superfamily proteins during thymocyte development. In addition, TGFβ is well established as an AHR dependent target in many other studies suggesting an important AHR dependent regulatory loop with potential impacts during thymocyte development. Notably, the absence of a significant induction of TGFβ at six and twelve hours after TCDD exposure in the present study however, suggests that TGFβ production is cell, tissue and/or time dependent.

In contrast to TGFβR1, IL-7R expression is attenuated by TCDD during the TN1&2 and TN3 stages of development potentially decreasing the sensitivity of these cells to IL-7. IL-7 is normally associated with cell survival and maturation just prior to TCRβ selection (Petrie and Zuniga-Pflucker, 2007). Thus, the attenuation of IL-7 receptor levels by TCDD suggested by the current study may result in fewer cells receiving the appropriate maturational signals required for pre-TCR dependent cell proliferation during the TN3 to TN4 transition. Taken together, the subtle effects of AHR modulation on IL7R and TGFβR1 levels observed in our study may offer clues as to how the AHR potentially tunes early thymocyte development in response to normal intrinsic microenvironmental signals.

Of course, speculation about the potential role individual AHR dependent gene targets may play in modulating early thymocyte development and cell proliferation fails to acknowledge the broad network of gene targets being modulated that ultimately contribute to the observed phenotype. To gain a broader understanding of the cellular and molecular pathways being modulated by AHR activation, we employed the ingenuity pathway assist. Indeed, the top molecular and cellular functions modulated in the early thymocyte progenitor compartment belong to cell growth and proliferation family with the association of 29 of 108 gene probes. Moreover, genes associated with cancer, hematological, immune and inflammatory diseases were enriched in our gene profiling experiment suggesting that understanding the role of the activated AHR during early thymocyte development may provide insight and potentially novel

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therapeutic approaches for these immune diseases.

Taken together, in this gene profiling experiment we have shown for the first time that within the same immunological tissue, AHR activation by TCDD modulates gene expression differently depending on the stage of thymocyte differentiation. The gene expression fingerprint within T cell committed stages of thymocyte development where TCDD was previously shown to affect cell cycle regulation (TN3, TN4A, and TN4B), is uniquely different than the TN1&2 stages where cell cycle regulation is largely unaffected and cells are still uncommitted to the T cell lineage. The specificity of these gene changes both temporally and in TN3, TN4 supports the notion that the AhR has an intrinsic developmental role at this stage of thymocyte ontogeny. Functional networks associated with cell growth, proliferation and cellular development are clearly modulated by AHR activation within early thymocyte progenitors. This finding is consistent with the rapid and dramatic decrease in cell proliferation we previously observed in these stages of thymocyte development following exposure to TCDD However, more work is needed to dissect how and why proper regulation of the AhR is so important at these thymocyte progenitor stages and how the AHR plays such a critical role in determining the ultimate function of T-cells. Finally, the observed differences in the AHR-modulated gene expression fingerprint revealed within different developmental stages in the thymus may have implications for other tissues where AHR signal transduction appears to be functionally important.

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Footnotes:

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Figure Legends:

Figure 1: Cell sorting gates used for purifying early thymocyte progenitor populations. TN cells were identified by the absence of CD3/CD4/CD8 (upper left panel) and then TN1&2 cells were sorted based on the expression of CD44+CD25+ (bottom left panel). For the TN3, TN4A and TN4B cells, TN cells were further identified based on high expression of CD24 (upper right panel), and then separated based on CD25 levels ($CD25^{hi}$ - TN3; $CD25^{intermediate}$ -TN4A; and $CD25^{lo}$ - TN4B; lower right panel). The frequencies of gated cells are denoted adjacent to each population. Flow cytometric sorting gate is a control sample representative of two independent experiments. There was no difference in cell distribution among the populations with TCDD at either of the two time points.

Figure 2: Heat map of 108 gene probes significantly modulated as determined by ANOVA for treatment X fraction X time. Genes are clustered according to similar expression patterns across the four populations and the gene name abbreviations are shown on the right hand side. Fold intensity scale is shown at the top of the heat map.

Figure 3: Gene expression differences between control and TCDD exclusive to the T-cell committed thymocyte progenitor populations. Seven gene probes were identified in which there was little to no difference between control and TCDD in the TN1&2 subsets, but, a significant difference in TN3, TN4A, and/or TN4B. The difference in probe intensity between control and TCDD is plotted for each of the seven genes at the six and 12-hour time points.

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Figure 4: Fold differences detected by RT^2-PCR are correlated to fold differences detected by microarray analysis for selected genes in each population. RT^2-PCR was performed on four known AHR regulated genes in each population and the fold difference between vehicle and TCDD exposure at six hours was compared with fold differences detected by microarray. Only genes showing a significant treatment ($p \leq 0.05$) effect by RT^2-PCR analysis are compared.

Figure 5: Top implicated biological networks containing genes significantly changed by TCDD exposure. Expression values are overlaid in green (decreased) or red (increased). For conventions and symbols, refer to Ingenuity website legend description (https://analysis.ingenuity.com/pa/info/help/help.htm#legend.htm).

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Table 1: Stage-specific analysis of mRNA expression for developmentally regulated thymocyte genes demonstrates transcriptional purity of the cell sorting and confirms selected microarray data.[a]

[a]Thymocyte progenitors were sorted into individual TN1-TN4 sub-populations, RNA extracted and purified and real-time RT-PCR performed using primers specific for developmentally regulated transcripts within early thymocyte progenitor populations. Relative fold difference was determined by dividing the expression levels of the gene from the enriched or deficient population (listed in the left column) by the expression level of the same gene in the population shown in the top column. Genes selected for RT^2-PCR analysis were: CD44, CD8α, and Ptcra (Pre-TCRα).

	Relative Fold Changes in Selected Progenitor Populations			
Thymocyte Subset:	**TN1&2**	**TN3**	**TN4A**	**TN4B**
TN1&2 enriched genes with reduced relative expression in other subsets	**CD44**	0.03	0.02	0.03
TN1&2 deficient genes with elevated relative expression in other subsets	**PtcrA**	31.0	5.8	5.3
	CD8	-	15.9	23.0
TN3 deficient genes with elevated relative expression in other subsets		**CD8**	12.6	18.3

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Table 2: Gene profiling six to twelve hours of AHR activation in thymocyte progenitors results in 108 significantly modulated gene probes. Results determined by Bonferroni-Holm correction and 3 way ANOVA reporting a significant main effect with treatment, and/or significant interaction of treatment and time. The mean fold induction across all four sorted populations from TCDD exposed mice is shown at six and twelve hours.

Unigene (Avadis)	Gene Symbol	Gene Title	Mean fold change; 6 hours TCDD	Mean fold change across; 12 hours TCDD
			1.2	1.1
Mm.372315	1700097N02Rik	RIKEN cDNA 1700097N02 gene		
Mm.273197	2010002N04Rik	RIKEN cDNA 2010002N04 gene	-1.0	-1.7
	A630007B06Rik		-1.0	-1.5
Mm.131555	/// LOC674488	similar to oocyte-testis gene 1		
Mm.19941	Acpp	acid phosphatase, prostate	1.2	1.4
			1.1	1.4
Mm.291826	Adipor2	adiponectin receptor 2		
Mm.290446	Ahrr	aryl-hydrocarbon receptor repressor	1.1	4.3
Mm.23125	AI425999	expressed sequence AI425999	1.7	1.7
	Ak3l1 ///		-1.0	-1.8
Mm.42040	LOC100047616	adenylate kinase 3 alpha-like 1		
Mm.36006	Ak7	adenylate kinase 7	1.5	1.1

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Mm.398221	Aldh3a2	aldehyde dehydrogenase family 3, subfamily A2	-1.0	-1.5
Mm.291372	Arhgap8	Rho GTPase activating protein 8	-1.2	-3.3
Mm.380514	AW061096	expressed sequence AW061096	-1.0	-1.7
Mm.306720	C4bp	complement component 4 binding protein	1.2	1.5
Mm.10702	Cacybp	calcyclin binding protein	-1.1	-1.1
Mm.25457	Ccno	cyclin O	1.5	2.4
Mm.440604	Ccr9	chemokine (C-C motif) receptor 9	1.4	1.1
Mm.29204	Cd96	CD96 antigen	1.2	-1.7
Mm.1135	Cpa3	carboxypeptidase A3, mast cell	-1.1	-1.5
Mm.930	Ctsl	cathepsin L	-1.2	-2.4
Mm.14089	Cyp1a1	cytochrome P450, family 1, subfamily a, polypeptide 1	1.4	5.2
Mm.214016	Cyp1b1	cytochrome P450, family 1, subfamily b, polypeptide 1	2.6	14.1
Mm.60526	Dmn	desmuslin	1.1	2.4
Mm.461605	E430022K19Rik	RIKEN cDNA E430022K19 gene	3.0	1.3
Mm.379893	EG545216 /// Glcci1 /// LOC100046012	glucocorticoid induced transcript 1	1.5	1.4
Mm.296317	EG624866	predicted gene, EG624866	1.1	1.8
Mm.38929	Esm1 /// LOC632677	endothelial cell-specific molecule 1	-1.3	-1.4
Mm.261859	Exoc3	exocyst complex component 3	-1.5	-1.9
Mm.28095	Flnb	filamin, beta	-1.1	-3.6
Mm.11982	Gas2l3	growth arrest-specific 2 like 3	1.6	1.0

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Mm.210787	Glcci1	glucocorticoid induced transcript 1	1.1	1.5
Mm.245741	Heg1	HEG homolog 1 (zebrafish)	3.2	1.6
Mm.57250	Hic1	hypermethylated in cancer 1	1.0	1.6
Mm.239865	Hspa4	heat shock protein 4	1.0	1.2
Mm.209419	Hspa9	heat shock protein 9	-1.1	-1.0
Mm.106343	Ikzf2	IKAROS family zinc finger 2	1.4	1.1
Mm.731	Il12rb1	interleukin 12 receptor, beta 1	1.0	1.6
Mm.389	Il7r	interleukin 7 receptor	-1.2	-1.1
Mm.58	Itgb7	integrin beta 7	-2.2	-3.1
Mm.228812	Jakmip1	janus kinase and microtubule interacting protein 1	1.4	1.1
Mm.26938	Klf2	Kruppel-like factor 2 (lung)	-1.9	-3.9
Mm.248615	Lgals3	lectin, galactose binding, soluble 3	-1.0	-31.9
Mm.33240	Mpzl2	myelin protein zero-like 2	1.4	1.1
Mm.28500	Mtap	methylthioadenosine phosphorylase	1.0	1.2
Mm.275281	Narg1	NMDA receptor-regulated gene 1	-1.2	-1.1
Mm.170515	Nfkbia	Nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, alpha	1.2	1.0
Mm.220367	Nol5	nucleolar protein 5	1.2	1.2
Mm.402190	Nolc1	nucleolar and coiled-body phosphoprotein 1	1.0	1.3
Mm.2380	Npas2	neuronal PAS domain protein 2	2.3	2.6
Mm.252	Nqo1	NAD(P)H dehydrogenase, quinone 1	1.2	2.1
Mm.423531	Nrp1	neuropilin 1	-1.1	-1.2
Mm.3304	Nsg2	neuron specific gene family member 2	-1.1	-1.5

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Mm.390681	P2ry5	purinergic receptor P2Y, G-protein coupled, 5	1.4	1.9
Mm.259103	Phc2	polyhomeotic-like 2 (Drosophila)	-1.5	-2.4
		phosphatidylinositol 3 kinase, regulatory	-1.1	-2.7
		subunit,		
Mm.253819	Pik3r3	polypeptide 3 (p55)		
Mm.192699	Plcg2	phospholipase C, gamma 2	-1.2	-2.5
Mm.897	Pou2af1	POU domain, class 2, associating factor 1	-1.1	-1.8
		protein phosphatase 1H (PP2C domain	1.1	1.2
Mm.70065	Ppm1h	containing)		
		peroxisome proliferative activated receptor,	-1.2	-1.3
Mm.2415	Pprc1	gamma, coactivator-related 1		
Mm.379451	Pqlc3	PQ loop repeat containing	1.1	2.0
Mm.27705	Prim2	DNA primase, p58 subunit	1.3	1.2
Mm.16766	Prkacb	protein kinase, cAMP dependent, catalytic, beta	1.2	1.2
Mm.381172	Prkg1	protein kinase, cGMP-dependent, type I	1.2	1.9
		PWP2 periodic tryptophan protein homolog	-1.1	-1.4
Mm.103522	Pwp2	(yeast)		
Mm.196846	Rad23b	RAD23b homolog (S. cerevisiae)	1.3	1.1
Mm.42150	Rasgrp1	RAS guanyl releasing protein 1	1.0	1.2
Mm.465324	Rasgrp4	RAS guanyl releasing protein 4	-1.3	-3.4
		regulator of chromosome condensation	1.3	1.6
		(RCC1) and BTB (POZ) domain containing		
Mm.280068	Rcbtb2	protein 2		
Mm.2416	Scin	Scinderin	1.2	4.3
Mm.335112	Scn4b	sodium channel, type IV, beta	1.2	3.2

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Mm.245527	Slc35b4	solute carrier family 35, member B4	-1.2	-1.5
Mm.8575	Sox13	SRY-box containing gene 13	-1.9	-1.2
		ST8 alpha-N-acetyl-neuraminide	1.4	1.8
Mm.260838	St8sia1	alpha-2,8-sialyltransferase 1		
		serine/threonine kinase 39, STE20/SPS1	1.3	4.3
Mm.198414	Stk39	homolog (yeast)		
Mm.197552	Tgfbr1	transforming growth factor, beta receptor I	1.4	1.1
Mm.246767	Thada	thyroid adenoma associated	-1.2	-1.6
Mm.246398	Tiparp	TCDD-inducible poly(ADP-ribose) polymerase	2.2	3.6
Mm.29431	Tmem97	transmembrane protein 97	-1.9	-1.1
Mm.436756	Tmprss4	transmembrane protease, serine 4	-1.2	-3.3
Mm.323595	Tob2	transducer of ERBB2, 2	0.1	-0.25
Mm.153272	Tsc22d1	TSC22 domain family, member 1	-1.3	-1.8
Mm.130793	Upk1b	uroplakin 1B	1.4	2.7
Mm.268000	Vim	vimentin	-1.6	-1.9
Mm.281079	Wdr12	WD repeat domain 12	-1.1	-1.1
Mm.210188	Zcchc2	zinc finger, CCHC domain containing 2	1.3	1.1
Mm.446560		Transcribed locus	1.2	1.2
Mm.441872		Transcribed locus	2.1	1.1

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Table 3: Ingenuity Pathway Assist (IPA) analysis of genes significantly modulated by AHR activation

Top IPA Associated Functional Networks	Score
Endocrine System Development and Function, Lipid Metabolism, Small Molecule Biochemistry	43
Endocrine System Development and Function, Small Molecule Biochemistry, Drug Metabolism	28
Lipid Metabolism, Small Molecule Biochemistry, Cellular Movement	27
Dermatological Diseases and Conditions, Genetic Disorder, Immunological Disease	22
Cell Morphology, Cellular Development, Cellular Growth and Proliferation	20

Top IPA Bio Functions - Diseases and Disorders	P value range	# Genes
Cancer	1.26E-05 - 3.49E-02	35
Hematological Disease	6.08E-05 - 3.49E-02	17
Inflammatory Response	2.28E-04 - 2.49E-02	14
Immunological Disease	3.25E-04 - 3.49E-02	16
Gastrointestinal Disease	1.35E-03 - 3.49E-02	6

Top IPA Bio Functions - Molecular and Cellular Functions	P value range	# Genes
Cellular Growth and Proliferation	3.01E-06 - 3.45E-02	29
Lipid Metabolism	1.53E-05 - 3.11E-02	6
Small Molecule Biochemistry	1.53E-05 - 3.11E-02	15
Drug Metabolism	4.59E-05 - 1.57E-02	3

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Cellular Development	9.03E-05 - 3.45E-02	21

Top IPA Bio Functions - Physiological System Development and Function	P value range	# Genes
Hematological System Development and Function	1.77E-06 - 3.49E-02	21
Tissue Morphology	1.77E-06 - 2.73E-02	16
Tumor Morphology	1.26E-05 - 2.73E-02	10
Endocrine System Development and Function	1.53E-05 - 3.11E-02	3
Respiratory System Development and Function	1.52E-04 - 3.94E-03	2

Top IPA Canonical Pathways	P value	Ratio
Aryl Hydrocarbon Receptor Signaling	1.76E-04	5/155 (0.032)
Xenobiotic Metabolism Signaling	1.91E-04	6/254 (0.024)
Virus Entry via Endocytic Pathways	2.96E-04	4/96 (0.042)
Glucocorticoid Receptor Signaling	3.33E-04	6/278 (0.022)
PPARa/RXRa Activation	3.92E-04	5/185 (0.027)

Figure 1

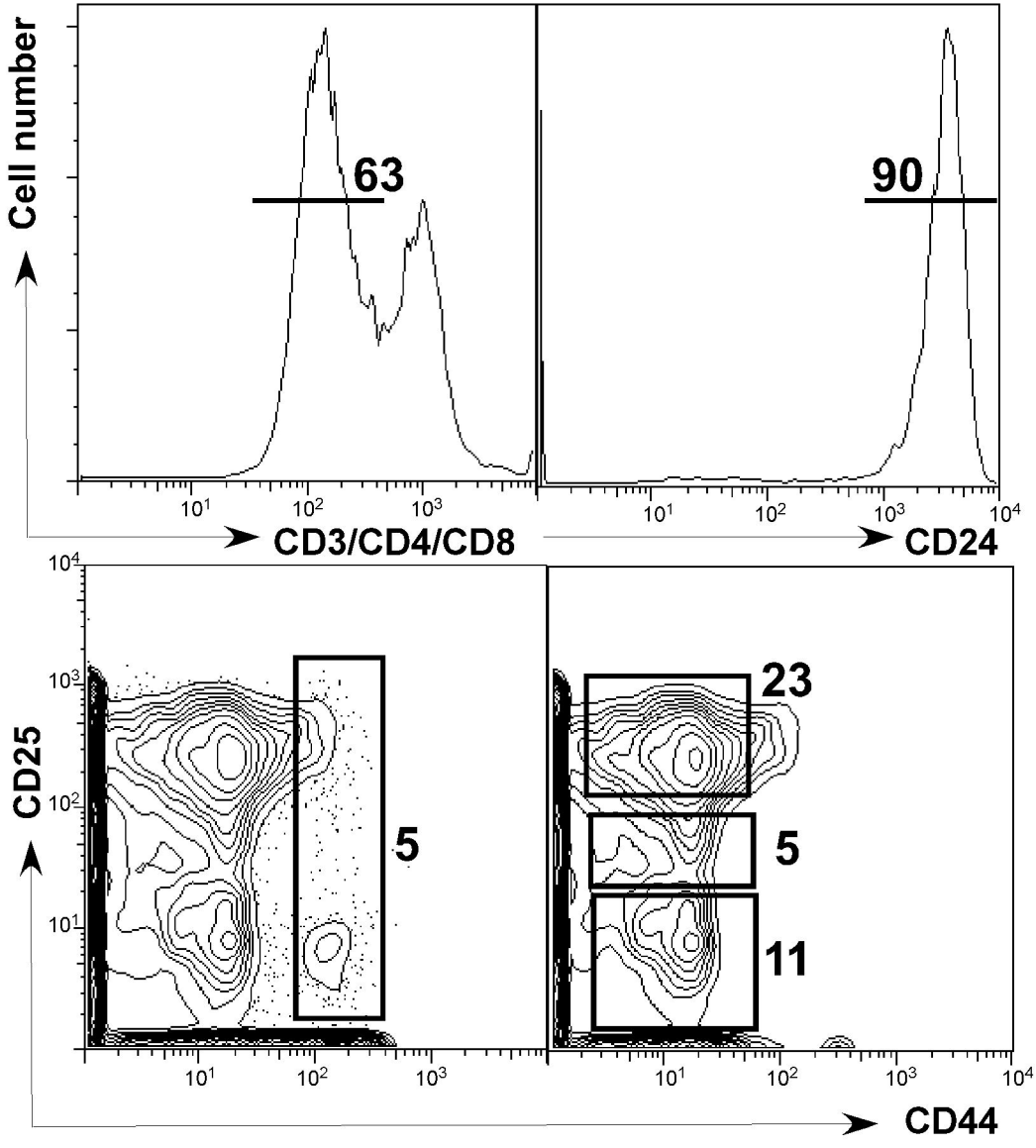
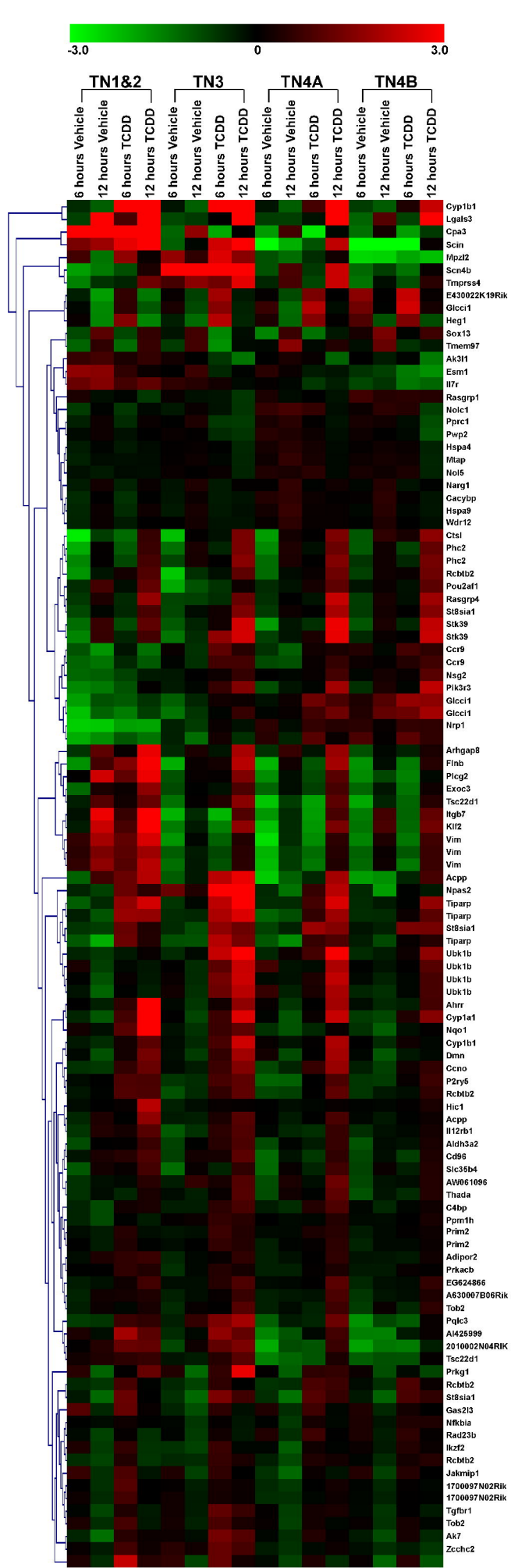

Cell number
63
90
10^1
10^2
10^3
10^4
CD3/CD4/CD8
CD24
CD25
5
23
5
11
CD44

Figure 2


-3.0
0
3.0
TN1&2
TN3
TN4A
TN4B
6 hours Vehicle
12 hours Vehicle
6 hours TCDD
12 hours TCDD
Cyp1b1
Lgals3
Cpa3
Scin
Mpzl2
Scn4b
Tmprss4
E430022K19Rik
Glcci1
Heg1
Sox13
Tmem97
Ak3l1
Esm1
Il7r
Rasgrp1
Nolc1
Pprc1
Pwp2
Hspa4
Mtap
Nol5
Narg1
Cacybp
Hspa9
Wdr12
Ctsl
Phc2
Phc2
Rcbtb2
Pou2af1
Rasgrp4
St8sia1
Stk39
Stk39
Ccr9
Ccr9
Nsg2
Pik3r3
Glcci1
Glcci1
Nrp1
Arhgap8
Flnb
Plcg2
Exoc3
Tsc22d1
Itgb7
Klf2
Vim
Vim
Vim
Acpp
Npas2
Tiparp
Tiparp
St8sia1
Tiparp
Ubk1b
Ubk1b
Ubk1b
Ubk1b
Ahrr
Cyp1a1
Nqo1
Cyp1b1
Dmn
Ccno
P2ry5
Rcbtb2
Hic1
Acpp
Il12rb1
Aldh3a2
Cd96
Slc35b4
AW061096
Thada
C4bp
Ppm1h
Prim2
Prim2
Adipor2
Prkacb
EG624866
A630007B06Rik
Tob2
Pqlc3
AI425899
2010002N04RIK
Tsc22d1
Prkg1
Rcbtb2
St8sia1
Gas2l3
Nfkbia
Rad23b
Ikzf2
Rcbtb2
Jakmip1
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1700097N02Rik
Tgfbr1
Tob2
Ak7
Zcchc2

Figure 3

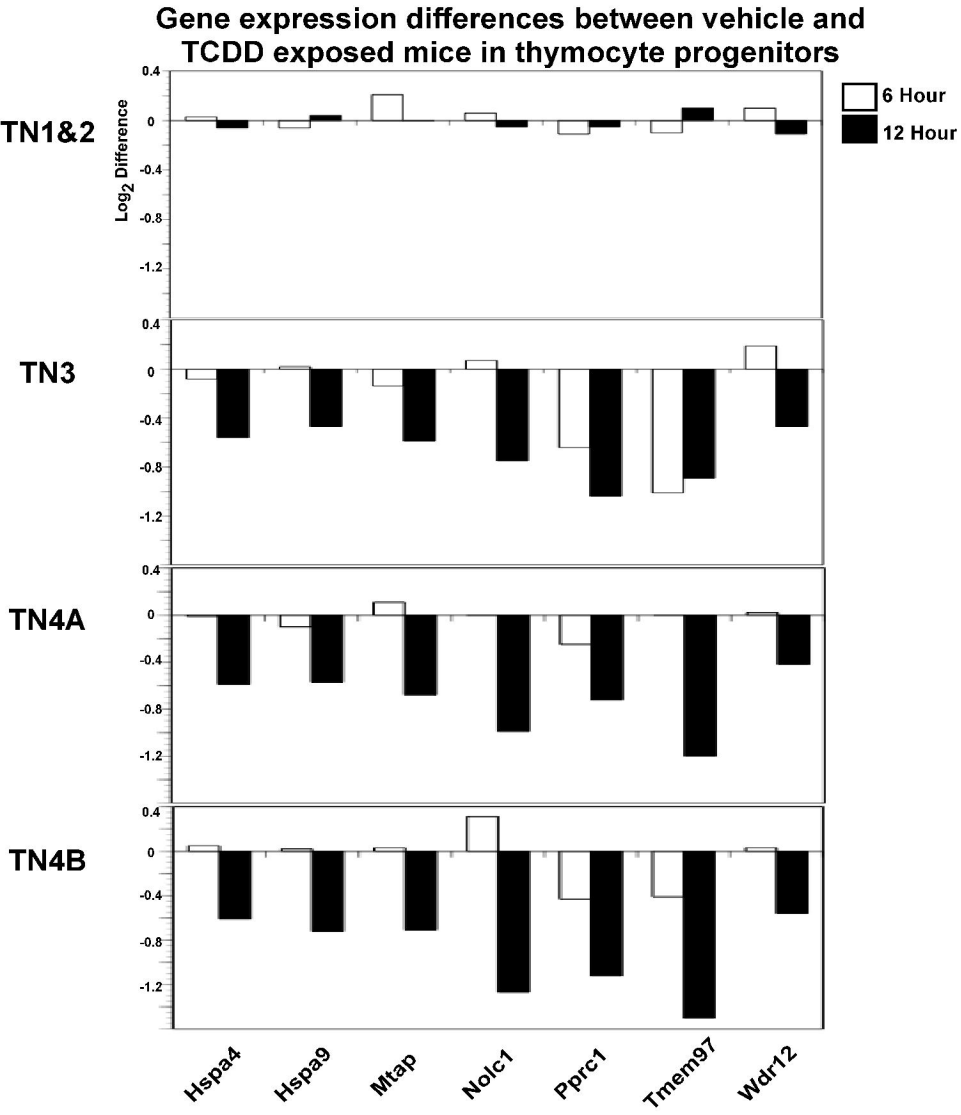

Gene expression differences between vehicle and TCDD exposed mice in thymocyte progenitors
6 Hour
12 Hour
Log2 Difference
TN1&2
TN3
TN4A
TN4B
0.4
0
-0.4
-0.8
-1.2
Hspa4
Hspa9
Mtap
Nolc1
Pprc1
Tmem97
Wdr12

Figure 4

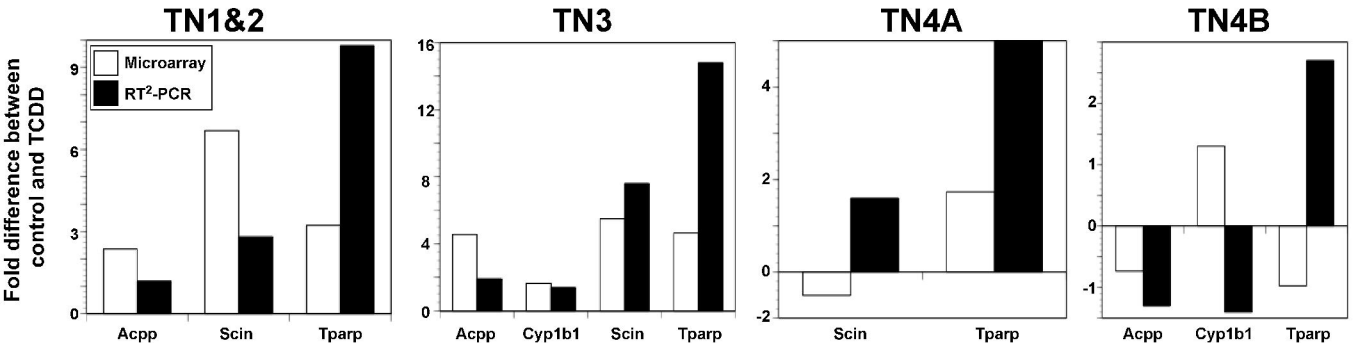
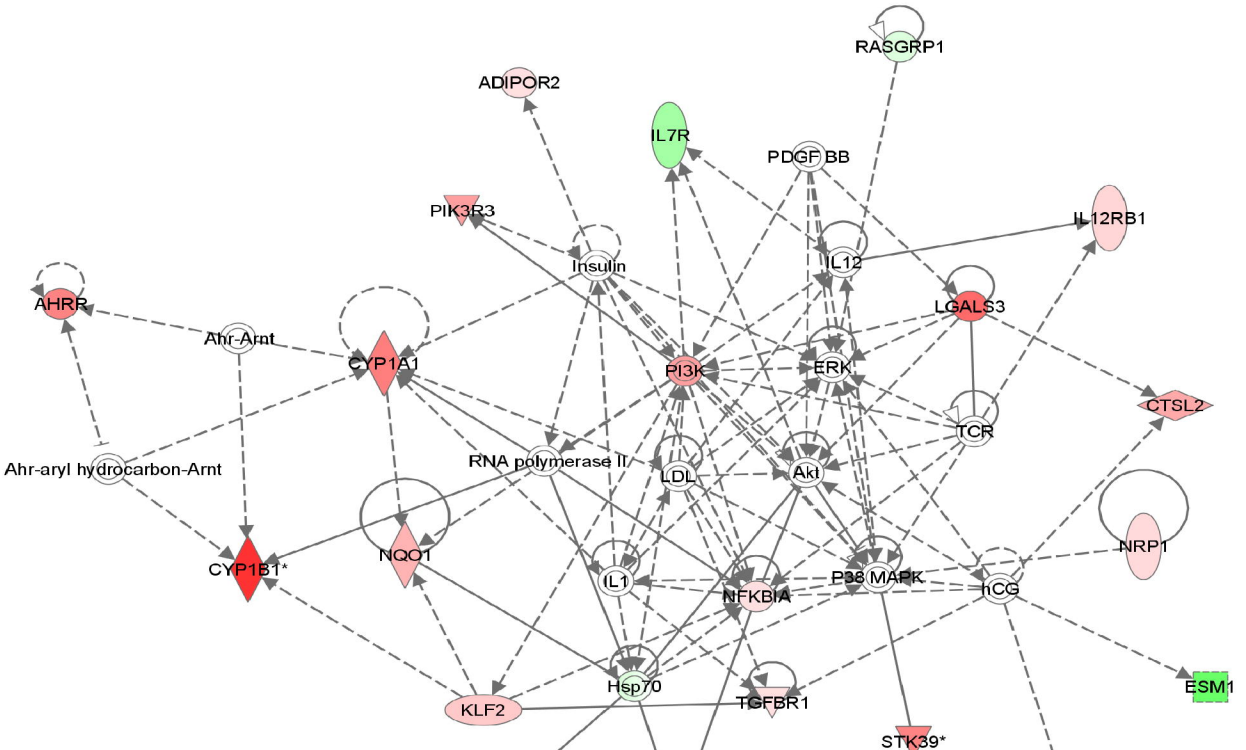

TN1&2
TN3
TN4A
TN4B
Microarray
RT²-PCR
Fold difference between control and TCDD
Acpp
Scin
Tparp
Cyp1b1

Figure 5.

Endocrine System Development and Function, Lipid Metabolism, Small Molecule Biochemistry



Cell Morphology, Cellular Development, Cellular Growth and Proliferation