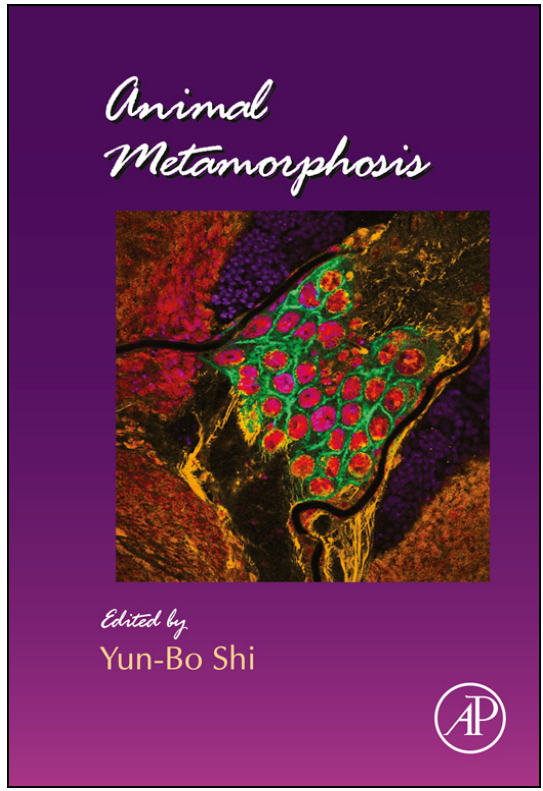

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CHAPTER TWELVE

Developmental Programs and Endocrine Disruption in Frog Metamorphosis: The Perspective from Microarray Analysis

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Contents

Abstract

A major goal for understanding the role of thyroid hormone (TH) in development has been to identify genes regulated by TH in different tissues during frog metamorphosis. The exquisite dependence of metamorphosis on TH also provides a model to study TH endocrine disruption. To identify such TH-regulated genes and select biomarkers for TH endocrine disruption, global gene expression analyses in tadpoles using microarrays have been done in 21 studies, involving five frog species, seven organs, and four endocrine disrupting chemicals. As expected, each organ has a unique set of genes

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associated with its tissue-specific metamorphic outcome, and functions ascribed to many of these genes correspond to histological changes induced by TH. Also, the large number of transcription factors identified in microarrays is consistent with the molecular mechanisms of TH action. On the other hand, microarray analysis has also revealed interesting findings not predicted from previous morphological or molecular studies. Furthermore, endocrine disruption studies identified candidate biomarkers for TH disruption, and the mechanisms of action of several endocrine disrupting chemicals have been examined. The microarray studies described here have produced a wealth of data on gene expression that requires further functional studies to elucidate the roles of these genes in development and endocrine disruption.

1. INTRODUCTION

All vertebrates require thyroid hormone (TH) for normal development, especially for the central nervous system (Howdeshell, 2002). The actions of hormones on development are particularly striking in frogs, where metamorphosis represents one of the most remarkable sets of hormone-dependent changes known among chordates (Just, Kraus-Just, & Check, 1981). The role of hormones in development is more easily studied in free-living tadpoles compared to uterus-enclosed mammalian embryos/fetuses because interpretations of hormonal manipulations in developing mammals are confounded by effects on the mother (Tata, 1993). Thus, the exquisite dependence of frog metamorphosis on TH and ease of analysis compared to mammals lends itself not only to studies of normal hormone-dependent development but also to studies of disturbed development due to endocrine disrupting chemicals.

Metamorphosis transforms the tadpole from one morphotype to another, involving extensive changes in morphology, biochemistry, and physiology (Dodd & Dodd, 1976). For example, tadpoles possess tails for swimming, their intestine is adapted for an herbivorous diet, and brain and skin are larval in character. During metamorphosis, the tail is completely resorbed, intestine, brain, and skin undergo extensive remodeling, and limbs grow out. In addition, the liver changes from ammonotelic to ureotelic metabolism of nitrogenous waste, and blood cells transform from larval to adult versions. TH is responsible for initiating all of these events of metamorphosis. If tadpoles are treated via surgical or chemical thyroidectomy such that they cannot make TH, they grow larger in size as a tadpole but do not undergo metamorphic changes. Conversely, TH added to the rearing water of premetamorphic tadpoles initiates all of the metamorphic changes that would have occurred later.

TH acts to accomplish these metamorphic changes predominantly if not exclusively via the nuclear receptors TRα and TRβ (Das et al., 2010). TRs are ligand-activated transcription factors that alter expression levels of genes responsible for metamorphosis (Buchholz, Paul, Fu, & Shi, 2006). Using transgenic animals, overexpression of a dominant negative form of TR that cannot bind TH inhibits metamorphosis (Buchholz, Hsia, Fu, & Shi, 2003). On the other hand, overexpression of a constitutively active TR (that does not require TH to induce T3-response genes) initiates metamorphic transformation in the absence of TH (Buchholz, Tomita, Fu, Paul, & Shi, 2004). TRs regulate gene expression by binding thyroid response elements (TREs) in promoter regions of TH-response genes. TRs have at least two modes to regulate genes, in that TH-response genes can have positive or negative TREs. On positive TREs, the presence of TH induces gene expression, whereas TH represses expression of genes containing negative TREs. Some TH-response genes with TREs have been identified as transcription factors that have gene targets of their own. Thus, TH induces TH-direct response genes, some of which subsequently regulate other genes, all of which comprises a gene regulation cascade, ultimately leading to metamorphosis.

Since the identification of TRs as nuclear receptors, more than 75 years after the discovery that TH induces frog metamorphosis (Gudernatsch, 1912; Yaoita, Shi, & Brown, 1990), a major research direction has been to identify the genes and pathways underlying TH-dependent metamorphosis. A unifying question for studies on frog metamorphosis is how a single molecule, TH, can trigger such varied developmental outcomes among tissues of the tadpole. The modern paradigm for answering this question centers around the molecular mechanisms underlying how different tissues can have TH-induced tissue-specific gene regulation cascades. A critical component to answering this question is to identify genes comprising these gene regulation cascades in different tissues.

At first, genes induced by TH were identified one by one based on their biochemical activities. For example, tail regression involved protease digestion of intra- and extracellular components, such as collagen, and thus collagenases and cathepsins were found (Yoshizato, 1996). The biochemistry of the liver undergoes metamorphic changes in nitrogen metabolism, such that urea cycle enzymes were found to be TH-regulated (Atkinson, Helbing, & Chen, 1996). Furthermore, distinct hemoglobins were found between tadpoles and frogs, and the adult hemoglobin genes were induced by TH (Weber, 1996). In a radically new approach, subtractive hybridization was employed to identify differentially regulated genes where *a priori* knowledge

of protein activity was not required (Wang & Brown, 1991). These screens found between 14 and 34 genes in tail, hind limb, intestine, and brain (Buckbinder & Brown, 1992; Denver, Pavgi, & Shi, 1997; Shi & Brown, 1993; Wang & Brown, 1993). Massive sequencing efforts on ESTs, cDNA libraries, and genomic DNA in frogs led to increasing numbers of genes known, ultimately making virtually all genes known. Microarray technology allows the expression of many or all genes to be examined simultaneously in a single experiment (Fig. 12.1). The first microarray for frog

Array platforms

	cDNA/PCR product array	Agilent oligoarray	Affymetrix GeneChip
Probe source	PCR products	Oligosynthesis	Oligosynthesis
Probe length	200–600 base pairs	60 base pairs	25 base pairs
Chip production	Custom-built, spotted on membrane or slide	Commercial, *in situ* synthesis on slide	Commercial, *in situ* synthesis on slide
No. of transcripts	~400–1000 selected	~21,500, *X. laevis*	~14,400, *X. laevis* ~29,900, *X. laevis* 2.0 ~51,000, *X. tropicalis*

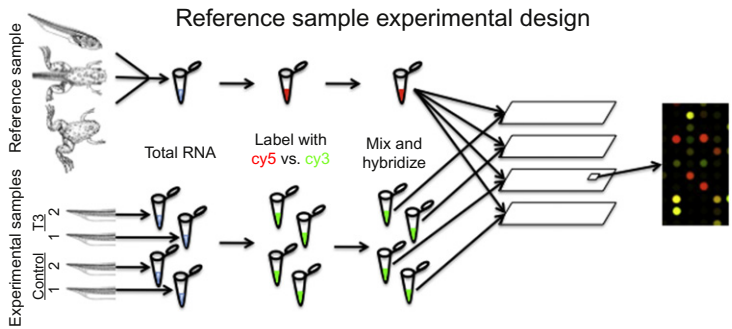

Figure 12.1 Types of microarray platforms and example microarray experimental design. Top panel indicates characteristics of the microarray platforms used in studies of frog metamorphosis. Lower panel depicts an experimental design using a reference sample, commonly used with the Agilent platform. The reference sample is a single preparation of RNA from a mixture of tissues and stages and labeled with one color (e.g., the red fluorescent dye, cy5). The experimental samples, including treatment and control samples, are labeled with a different color (e.g., the green fluorescent dye, cy3). Each experimental sample is mixed with a portion of the reference sample and hybridized to the microarray slide, resulting in each spot on the array with levels of red and green fluorescence corresponding to the level of expression of the RNA in the reference versus experimental sample. Expression differences among experimental samples are determined based on expression levels relative to the reference sample. The other platforms commonly use a one-color design and compare intensity values directly among samples.

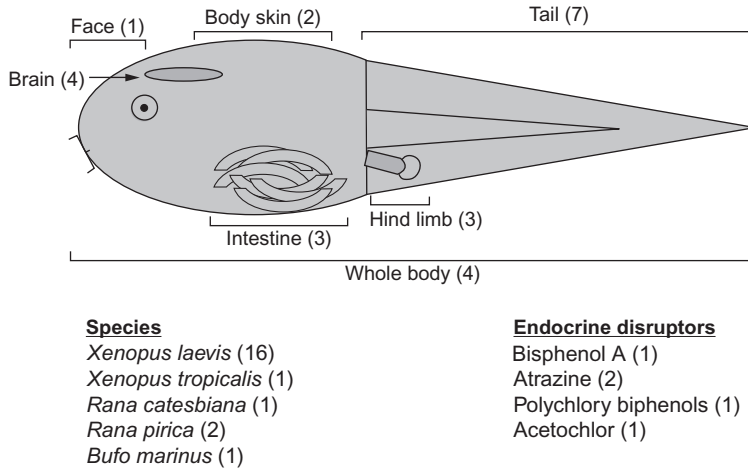

Figure 12.2 Summary diagram of tadpole microarray studies. A total of 21 studies have been carried out on tadpoles using the five species and seven body regions indicated. The numbers in parentheses indicate the number of studies using the associated body region, species, or endocrine disrupting chemical.

metamorphosis was composed of 420 cDNAs, chosen from available sequences in GenBank in 2001 whose products were believed to be important in frog embryogenesis and metamorphosis (Crump, Werry, Veldhoen, Van Aggelen, & Helbing, 2002). With advances in microarray technology, genome-wide analysis of gene expression for more than 20,000 genes during metamorphosis became possible (Das et al., 2006).

The use of microarray technology in studies of frog metamorphosis is the subject of this review (see Fig. 12.2 and Table 12.1 for summaries of these studies). Two main goals have motivated microarray analysis in frog metamorphosis, namely, developmental genetic analysis of the TH gene regulation cascade and biomarker discovery and mechanism of action for endocrine disruption studies. In the first case, researchers want to identify all gene expression changes associated with a particular developmental stage or experimental treatment as a means toward understanding underlying pathways and mechanisms of tissue-specific TH-dependent metamorphosis. We discuss progress made using microarrays to understand developmental programs of tail, brain, hind limb, intestine, and skin. In the second case, researchers wish to identify a gene or genes robustly associated with TH signaling to serve as a reliable gauge of TH endocrine disruption. Also, several studies have used microarrays to examine the mechanism of action of TH disrupting chemicals, including bisphenol A, atrazine, polychlorinated biphenols, and acetochlor. Additional

Table 12.1 Summary of tadpole microarray studies (21 publications)

Tissue/species	Stages[a], conditions	No. of genes	Genes/pathways (upregulated, *downregulated*)	Platform	References
Whole body/ *X. laevis*	NF46, 14 h, ±100 nM T3, ±cycloheximide	188 up, 249 down in T3 + CHX versus control	Cell communication, transcription, protein degradation-dependent signaling, cell cycle, *cell differentiation*, *cytoskeletal biogenesis*, *cell division*	Agilent oligoarray, 21,495 Unigene clusters	Das et al. (2009)
Whole body/ *X. laevis*	400 ppb atrazine from NF43–NF62	44 in males, 44 in females	Ketone body metabolism, urea cycle, amino acid metabolism, *glycolysis/gluconeogenesis*, *fatty acid elongation*	Affymetrix, 14,400 elements	Langerveld et al. (2009), Zaya et al. (2011)
Whole body/ *Bufo marinus*	G29/30, 33/34, 38/40, 40–41, 42/43, 46	871 total; 20 up, 17 down in G46 versus G42	Induced during metamorphosis: hemoglobin α, β, harderin-like, apolipoprotein A1, γ-crystallin, serum albumin, gastrokine, trefoil factor, olfactomedin	cDNA array—5170 random cDNAs from toadlet library	Halliday et al. (2008)
Tail/*X. laevis*	NF47, 52, 56, 58, 60, 62	77 genes, 13 clusters	Transcription, metabolism, apoptosis, signal transduction, transport/binding	MAGEX cDNA[b], 420 cDNAs	Veldhoen et al. (2002)
Tail/*Rana catesbiana*	G31–33 tail tips, 10 nM T3, 0, 1, 2 days	92 genes	*PCNA*, K-ras, caspase 6, Na/dicarboxylate transporter, transcription, intracellular signaling, cell fate determination	MAGEX cDNA[b], 401 cDNAs	Veldhoen et al. (2006)

Tail/*X. laevis*	NF54, 100 nM T3, 0, 1, 2 days	1500 up, 700 down	Intracellular signaling, hydrolase activity, peptidase activity, cell death, *energy pathways*, *glycolysis*, *TCA cycle*, *mitochondrion*, *electron transport*	Agilent oligoarray, 21,654 Unigene clusters	Das et al. (2006)
Tail/*X. laevis*	NF54, 0, 2 days, T3[d], T4, MMI, PTU, PER	1–11 across treatments, time points	Metallothionein as biomarker for MMI	MAGEX cDNA[b], 434 cDNAs	Helbing et al. (2007)
Tail/*X. laevis*	NF54, 20 nM T3, 6, 48 h	6 h–177 up, 125 down; 48 h—432 up, 421 down	Genes at 6- and 48-h time points mostly not overlapping, enriched GO categories in metabolic processes; TRβ, deiodinase II, CRH-binding protein, fibroblast-activating protein α as biomarkers for TH	Affymetrix, 14,400 elements	Searcy et al. (2012)
Tail/*X. tropicalis*	NF54, 10 nM T3, 100 nM CORT, CORT + T3, 18 h	TH only—1636, cort only—573, CORTTH only—1208	TH only: proteases, apoptosis, transport, *amino acid metabolism*, CORT only: mitochondrion, intracellular localization and transport, CORTTH only: protein localization and transport	Affymetrix, 59,000 elements	Kulkarni and Buchholz (2012)
Tail/*X. laevis*	NF52/54, 100 nM T3, 10 nM acetochlor, T3 + AC, 48 h	26 genes in T3 versus T3 + AC	T3-induced genes further increased by acetochlor (e.g., gene 12, TH/bZIP, collagenase 3, KLF9, deiodinase III), non-T3-response genes induced by acetochlor only in the presence	MAGEX cDNA, 420 cDNAs	Crump et al. (2002)

Continued

Table 12.1 Summary of tadpole microarray studies (21 publications)—cont'd

Tissue/species	Stages, conditions	No. of genes	Genes/pathways (upregulated, *downregulated*)	Platform	References
			of T3 (e.g., Mad2, frizzled 7, caspase-9, nuclear factor I-C1, vitellogenin receptor, natriuretic peptide receptor type C), genes whose T3 repression was inhibited by acetochlor (e.g., 51-kDa cytokeratin type I, Genes 17, 18, 19, glutamine synthetase, Gli2)		
Intestine/*X. laevis*	NF53, 10 nM T3, 0, 1, 3, 6 days	Up—255, 710, 1040; down—280, 552, 585	Early genes—proteolysis, *cell cycle*; late genes—transcription, development, ECM, *glucose metabolism*, *transport*, *digestion*, *mitochondrion*	Agilent oligoarray, 21,495 Unigene clusters	Buchholz et al. (2007)
Intestine/*X. laevis*	NF53, 58, 61, 66	Up—463, 2613, 981; down—280, 552, 585	In chronological order: immune response, *catabolism*; transcription, signal transduction, cell–cell signaling, *metabolic processes*, *mitochondrion*; organismal process, development, *catalytic activity*, *RNA processing*	Agilent oligoarray, 21,495 Unigene clusters	Heimeier et al., (2010)
Intestine/*X. laevis*	NF54, 2 nM T3, 10 μM BPA, T3 + BPA, 4 days	T3—1051 up, 728 down; BPA—507 up, 290 down; T + B—1032 up, 738 down	Attenuated regulation of 58% of genes regulated by T3 occurs in the presence of BPA and 57% of the top 100 T3-regulated genes	Agilent oligoarray, 21,495 Unigene clusters	Heimeier et al. (2009)

Hind limb/ *X. laevis*	NF54, 100 nM T3, 0, 1, 2 days	2400 up, 1200 down	Cell cycle, MCM genes, RNA/DNA metabolism, electron transport	Agilent oligoarray, 21,654 elements	Das et al. (2006)
Hind limb/ *X. laevis*	Dox-inducible pCAR:TRDN[c]	2 weeks—42 up, 67 down; 6 weeks—1401 up, 1915 down	Nonmuscle genes unchanged, calsequestrin involved in transgenic muscle-wasting phenotype	Agilent oligoarray, 21,654 Unigene clusters	Cai et al. (2007)
Hind limb/ *X. laevis*	NF54, 0, 1, 4 days, T3[d], T4, MMI, PTU, PER	10–14 across treatments, time points	*RNA helicase II/Gu* as biomarkers for T4, metallothionein as biomarker for MMI, importin as biomarker for MMI and PER	MAGEX cDNA[b], 434 cDNAs	Helbing et al. (2007)
Brain/ *X. laevis*	NF54, 100 nM T3, 0, 1, 2 days	2000 up, 1000 down	Cell cycle, MCM genes, RNA/DNA metabolism, electron transport, Notch, Otx2	Agilent oligoarray, 21,654 Unigene clusters	Das et al. (2006)
Brain/*X. laevis*	NF54, 0, 1, 2, 4 days, T3[d], T4, MMI, PTU, PER	9–66 across treatments, time points	T3 versus T4 shows different expression profiles; MMI, PTU, PER-altered gene regulation by 24 h; cyclin H as biomarker for 24 h T3, *ferrochelatase* as biomarker for 48 h T4	MAGEX cDNA[b], 434 cDNAs	Helbing et al. (2007)

Continued

Table 12.1 Summary of tadpole microarray studies (21 publications)—cont'd

Tissue/species	Stages, conditions	No. of genes	Genes/pathways (upregulated, *downregulated*)	Platform	References
Brain/*X. laevis*	NF53/4, ±1 nM T3, 500 nM PCB, 4 days	526 T3 genes affected by PCB	Enriched GO terms: growth, brain development, cell proliferation, cell adhesion, neuron differentiation, apoptosis	Agilent oligoarray, 43,803 elements	Ishihara et al. (2011)
Hind brain/ *X. laevis*	NF53, recover from cut spine, ±1 mM MMI, 3, 7, 21 days	3 days—202, 7 days—331, 21 days—275, 50% up and down	72 annotated induced genes associated with recovery: transcription factors, membrane proteins, intracellular signaling, coregulators. Top 4 genes: SOCS2, XER81, neurotensin, MORC3	Affymetrix, 29,900 elements	Gibbs et al. (2011)
Body skin/ *X. laevis*	NF54/55, 5 nM T3; 0, 1, 3, 5, 7 days	240 up, 161 down	Genes: stromelysin-3, collagenase, TH/bZIP, adult keratin, *larval keratin*, *Genes 17–20*. Functional groups: transcription, proteolysis at 1 day, cell cycle at 3 days, defense response at 7 days	Affymetrix, 14,400 elements	Suzuki et al. (2009)

Body skin/*Rana pirica*	10 days ± predator from G25	8 up, 11 down	Bullous pemphigoid antigen, uromodulin, aldehyde dehydrogenase, *carboxypeptidase B*, *trypsinogen elastase I and II*, *fibrinogen*	297 PCR products from subtractive hybridization	Mori et al. (2005)
Face and brain/*Rana pirica*	0, 6 h, 4 days, 8 days ± predator from G25	Not reported	Pirica (uromodulin-like gene) associated with predator-induced bulgy morphology, *type I and II keratins*	1020 PCR products from subtractive hybridization	Mori et al. (2009)

[a]NF is Nieuwkoop and Faber staging for *Xenopus*, and G is Gosner staging for other tadpoles.
[b]MAGEX is multispecies array for gene expression, with cDNA sequences representing approximately 90% *X. laevis* and 10% *R. catesbiana* selected for involvement in embryogenesis and metamorphosis.
[c]pCAR:TRDN is dominant negative TR expressed only in muscle cells only in the presence of doxycycline. NF53 tadpoles were treated for 2 or 6 weeks on Dox and harvested at NF56 and 61, respectively. Dox-treated samples were compared to nontransgenic NF56 and NF66 limbs.
[d]0.5–8 nM T3, 10–40 nM T4, 100 mg/L methimazole (MMI), 20 mg/L 6-propylthiouracil (PTU), and 4 mg/L sodium perchlorate (PER).

uses of microarray during frog metamorphosis have included identifying genes underlying phenotypic plasticity of predator-induced polyphenism (*Rana pirica*), finding genes associated with spinal cord regeneration in tadpoles, and looking for possible targets for pest control in *Bufo marinus*.

2. DEVELOPMENTAL PROGRAMS

2.1. Tail

The tail, being large and easily accessible, was the first organ to which microarray techniques were applied (Crump et al., 2002; Veldhoen, Crump, Werry, & Helbing, 2002). Much was already known about histological and biochemical effects of TH, where apoptosis and proteolysis play a predominant role in tail resorption (Yoshizato, 1996). The notochord and tail fin have collagen degraded by proteinases from fibroblasts. The muscle cells and tail fin epithelium die by apoptosis. Both *Xenopus laevis* and *Rana catesbiana* have been analyzed using a cDNA array with over 400 cDNAs. Several global expression analyses have also been carried out with oligoarrays representing many thousands of transcripts (Table 12.1).

Two tail gene expression programs were identified (Das et al., 2006). The tail hydrolytic program (tail fibroblasts switch from synthetic to hydrolytic genes) and muscle program (upregulation of proteases different from those in fibroblasts). As expected, genes involved in protein degradation were upregulated, such as matrix metalloproteases, lysosomal cathepsins, hyaluronidase, and therefore match histological and biochemical events of tail resorption. Also, cell death genes were upregulated, for example, caspase-6, and cell proliferation genes, for example, PCNA, were downregulated. Also as expected, a major program downregulated in tail fibroblasts was the synthesis of several kinds of collagen and extra cellular matrix proteins. Unexpectedly, some structural proteins were upregulated in the face of tissue resorption, including fibronectin, tubulin, actin, myosin heavy chain 3, and a keratin. The explanation for increases in these structural proteins awaits further investigation. Perhaps less obvious but predicted because TH acts through nuclear receptors is that genes involved in transcriptional regulation experience altered TH-induced expression levels, such as TRβ, TH/bZIP, KLF9, and SOX4. One study compared gene expression changes between 48 h of TH treatment and tails at metamorphic climax (Das et al., 2006). One-third of the genes on the array that were involved in muscle contraction were also downregulated at climax. Interestingly, genes most upregulated at climax and 48 h were largely overlapping, but genes most

downregulated at climax and 48 h were very different from each other. The basis of this observation is not understood but may relate to different hormone environments.

Transcriptional repression of most genes encoding the steps leading to glycolysis, electron transport chain (including most of the genes that encode the ATP synthase complex), and tricarboxylic acid cycle genes are downregulated in tadpole tail muscle, which is consistent with cell death and tail resorption. These major metabolic pathways are shut off in all of the tail muscle at climax when the muscle fibers appear to be healthy and before the upregulation of the proteolytic enzymes and the morphological evidence of dying fibers. This global downregulation is followed by the more localized upregulation pattern that coincides with the activation of caspase-3. The upregulation of proteins involved in transport (e.g., Na^+/dicarboxylate transporter) and proteins of the ATP Na/K pump are perhaps required to support altered metabolism and apoptosis, but elucidating their actual role requires functional studies.

Many regulated genes with various biological functions did not have predicted roles in metamorphosis, because their biological activities did not match known physiological or morphological or biochemical phenomena. One of the largest groups of upregulated tail genes is signal transduction, including MAPK phosphatase, TGF-β pathway, GTPase-mediated signaling, and Rho-mediated signaling. The signaling genes that were tested by *in situ* hybridization were expressed in fibroblasts (Das et al., 2006). A role for most of these signaling pathways in metamorphosis is not known, but cyclin C/cdk8 phosphorylation was examined (Skirrow, Veldhoen, Domanski, & Helbing, 2008). One of the most highly upregulated genes in the tail is corticotropin releasing hormone binding protein, whose function was elegantly tracked down as contributing to regulation of timing of tail resorption (Boorse, Kholdani, Seasholtz, & Denver, 2006). The roles of many other genes await similar analysis.

TH action during metamorphosis is modulated by other hormones, principally corticosterone (CORT), the stress hormone in frogs. CORT synergizes with TH via many mechanisms, including at the level of gene regulation, to accelerate metamorphic processes induced by TH (Bonett, Hoopfer, & Denver, 2010). Two genes have been identified as induced by TH and CORT and TRβ and KLF9. Both of these genes are induced in synergy and/or by either hormone alone, depending on tissue. A microarray analysis was performed to identify more regulated genes and more gene regulation interaction patterns by these two hormones (Kulkarni & Buchholz, 2012). Premetamorphic *X. tropicalis* tadpole tails were harvested 18 h after treatment

in vivo with 100 nM CORT, 10 nM T3, or CORT + T3. Using Venn diagrams, genes were segregated into clusters based on up- and downregulation by one or both TH, CORT, and CORTTH in all combinations. Of the thousands of genes regulated, the predominant patterns were genes regulated by TH only and in synergy with TH and CORT. Some genes were regulated by CORT uninfluenced by TH. Most known tail TH-response genes were recovered in this analysis as TH-only genes, but some were also regulated in the CORT and CORTTH treatments. A set of genes was regulated by TH in the absence of CORT but in the presence of CORT, genes expression returned back to control. Similarly, a set of genes regulated by CORT had this regulation blocked by TH. The roles of these genes in metamorphosis are not well understood, but for detailed analysis and discussion please refer to Kulkarni and Buchholz 2012.

2.2. Brain

The first histological effect of TH on the tadpole brain is to initiate cell proliferation within 1–2 days after TH treatment, mostly in the cells lining the ventricles (Cai & Brown, 2004; Denver, Hu, Scanlan, & Furlow, 2009). Subsequent remodeling events involve elimination of neurons innervating degenerating larval structures (e.g. Malthner neurons that innervate the tail) and rewiring of neurons in remodeled organs (e.g., development of binocular vision) (Kollros, 1981). Details about the fate of the newly replicated cells in the brain are not well established.

To identify genes comprising the brain metamorphic program, whole brains from premetamorphic tadpoles of *X. laevis* treated with 100 nM TH for 24 and 48 h were used in a microarray analysis (Das et al., 2006). The tadpoles were also pretreated in 1 mM methimazole (MMI) for 1 week to reduce the effect of endogenous levels of TH. The brain program includes many expected categories corresponding to known histological events, such as cell cycle, protein folding, RNA and DNA metabolism, and translation. The most dramatically TH-induced genes are the members of the minichromosome maintenance (MCM) complex. Five of the six MCM genes on the *X. laevis* array are upregulated after 48 h of TH induction. Induction of these newly identified TH-responsive cell cycle-related genes in the brain is limited to the cells that line the ventricles, the same cells that are stimulated by TH to replicate. Most of these genes that were upregulated within 48 h in the brain were also upregulated in the limb as well (see Section 2.3 and Fig. 12.3).

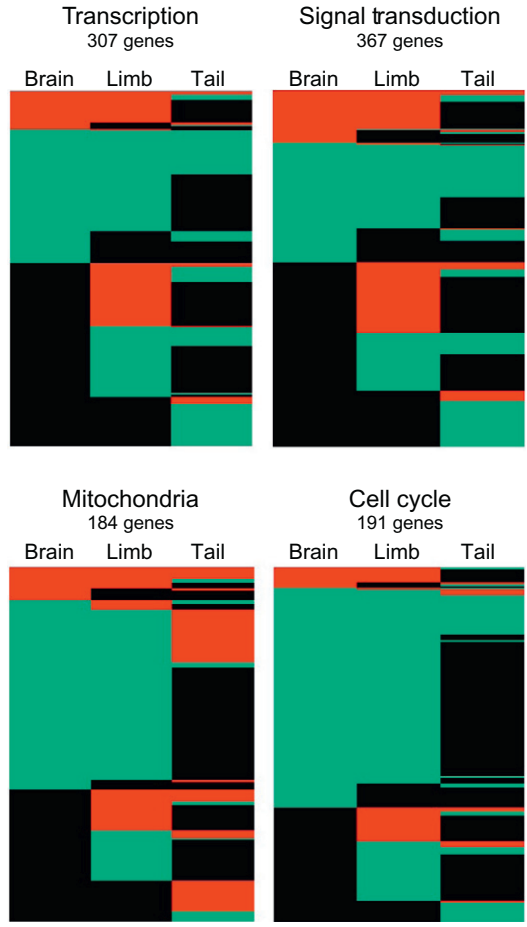

Figure 12.3 Comparison of altered gene expression among brain, limb, and tail. From Das et al. (2006), significantly regulated genes in brain, limb, or tail after 48 h of 100 nM T3 were grouped by gene ontology categories. Here, we chose to display genes from transcription, signal transduction, mitochondria, and cell cycle. Genes up- or down-regulated or no change were assigned green, red, or black, respectively, then sorted by color starting with brain, then limb, and then tail. Among tissues, the gene regulation patterns for each GO category indicate that brain and limb are more similar to each other than to tail, matching their response to TH after 2 days, that is, proliferation versus apoptosis. Among GO categories, gene regulation patterns were similar between transcription and signal transduction and between mitochondria and cell cycle. For example, gene expression patterns among tissues are much more similar to each other in the transcription and signal transduction categories compared to the mitochondria and cell cycle categories.

Two genes were identified as possible candidates for neural-specific development, Notch and Otx2 (Das et al., 2006). Notch activation by TH occurs in the same replicating cells that line the brain and spinal cord ventricle, and the homeobox protein Otx2 has been implicated in mouse brain development. Because brain remodeling requires 1–2 weeks, 48 h of TH treatmentmay not be the appropriate time point to capture altered expression of genes regulating adult brain differentiation and thus more studies are needed to identify such genes.

2.3. Hind limb

The first response to TH in the limb is cell proliferation, which begins within 1–2 days after TH treatment (Brown & Cai, 2007). Subsequently, limb differentiation also occurs where muscle, nerves, and skeletal elements form coordinately. The predominant TH-induced morphological change in the limb is elongation of the elements, as limb patterning can occur in the absence of TH. TH-induced gene expression profiles were carried out on hind limbs of premetamorphic tadpoles treated with 100 nM TH for 14, 24, 48 h (Das et al., 2006). Microarray studies targeting the beginning of the gene regulation cascade in the hind limb were compared to initial steps of the cascade in tail and brain to gain insight into how different tissues can respond differently to TH.

As in the brain, the hind limb program during the first 48 h of TH treatment reflects the intense proliferation that occurs then. The TH-induced limb program shares many genes and virtually all functional categories with the early brain program (Fig. 12.3). Specifically, more than 92% of 955 genes that are upregulated in the brain are also upregulated in the limb after 48 h of TH treatment. Similarly, more than 88% of 199 genes that are downregulated in brain are also downregulated in the hind limb. Genes involved in every step of the cell cycle are TH-regulated and the two programs are remarkably similar containing many cell cycle-related genes. Also, there is high representation of enriched gene products that reside in the nucleolus, which are related to this increase in translation-related proteins. Half of the genes in the array that encode protein-folding proteins are upregulated in the hind limb including chaperone proteins. Many of the components that are involved in the ubiquitination and proteosome pathways are also upregulated, especially in the limb program.

In every functional category, there are more genes upregulated in the hind limb than in the brain and the extent that they are differentially

regulated is usually greater. This difference between limb and brain may be attributable to the higher proportion of cells initially stimulated to begin proliferation. Indeed, as examined by *in situ* hybridization in the limb, TH induces all cell types to upregulate the cell cycle genes, compared to only the ventricle boundaries in the brain. Comparing tail, brain, and hind limb, many transcription factors are upregulated in all three tissues, such as KLF9, c-myc, and NFI-X2. Similarly, chromatin remodelers, such as BAF53, BAZ1B, CHD4, DNMT1, HDAC1, 2, despite their different fates, are also commonly upregulated among tissues. In addition, corepressors and coactivators such as EZH2, HES1, NCoR1, NCoR2, and TRIP3 are also commonly upregulated among tissues. Nevertheless, although there are many genes similarly regulated in all three programs (tail, limb, and brain), the tail (death) program is very different from the limb and brain (growth) programs. In order to identify candidate genes that may determine tissue-specific T3-responses, Das et al. (2006) identified 36 differentially regulated genes from the hind limb that regulate transcription at the earliest time point studied, that is, 14 h of TH treatment. Several of these genes are upregulated in the limb and brain but not in the tail and might control the opposite expression patterns in these two programs, including CEBPD, HBP1, HDAC9, HES1, MYOD1, TRMM55, and ZFP36. In addition to differentially expressed transcriptional regulators and chromatin modifiers, different preexisting chromatin states and presence of tissue-specific coactivators and/or corepressors likely interact with TH-response genes in a tissue-specific manner to explain different gene regulation cascades among tissues.

Downstream of the TH-induced gene regulation cascade, genes in several key functional categories are regulated in the opposite manner in tail versus limb programs. Many of the genes that encode energy pathway proteins that are localized in the mitochondria (e.g., the genes in the mitochondrial electron transport chain) are downregulated in tail muscle but upregulated during limb growth. Also, 8 out of 17 genes encoding tricarboxylic acid cycle enzymes are upregulated in the limb and 6 of these are downregulated in the tail. Additionally, half of the 32 genes in the cytoplasm-localized glycolytic pathway are downregulated in tails at metamorphic climax, and four of those are upregulated in the limb.

In a clever use of microarray analysis, Brown and colleagues investigated the developmental basis of a limb muscle-wasting phenotype (Cai, Das, & Brown, 2007). When a dominant negative version of the TH receptor (TRDN) was inducibly expressed in limb muscle cells in transgenic frogs undergoing metamorphosis, limb muscle fibers disintegrated but the muscle

cells did not die. Microarray was used to identify candidate genes responsible for this TRDN-induced, rhabdomyolysis-like phenotype. They compared nontransgenic prometamorphic (NF55) limbs and frog limbs after 2 and 6 weeks of transgene induction from NF55. Because hind limb is very sensitive to TH, NF55 limbs have experienced significant TH-induced developmental changes. Candidate genes were those induced or repressed in TRDN-expressing transgenic animals relative to NF55 controls and opposite to expression changes in the nontransgenic frog limb. They found 24 candidate genes with altered regulation after 2 weeks of induction, most of which had known muscle expression. Because the muscle lysis phenotype became visible within 2 days, highly regulated genes were analyzed by *in situ* hybridization, and calsequestrin 1 was found to have downregulation within 1 day after induction of the TRDN transgene. Roles for the five other candidate genes tested, which were downregulated by 4 days, are also possible. The other 18 candidate genes await similar analysis. Another pattern observed from this analysis was that a number of genes downregulated in tail just before tail resorption were repressed in limb muscle expressing the TRDN transgene. Thus, tail degeneration requires T3 induction, and limb degeneration requires the blockade of T3 induction.

2.4. Intestine

The intestine remodels extensively during metamorphosis going from a long, coiled herbivorous gut with a single infolding to a short, carnivorous gut with a villus-trough structure in cross section (Shi & Ishizuya-Oka, 2001). The three main tissue types, epithelium, connective tissue (mostly fibroblasts), and muscle, have distinct responses to TH. During transformation, most cells of the larval epithelium die by apoptosis, and later adult epithelial cells appear and proliferate to form the adult absorptive surface. Fibroblasts and muscle cells also proliferate and their layers become thicker. The same remodeling events are believed to occur whether induced by TH or allowed to progress naturally.

To understand gene programs involved in remodeling the intestine, two microarray analyses were carried out, one on natural metamorphosis and one on TH-induced metamorphosis (Buchholz, Heimeier, Das, Washington, & Shi, 2007; Heimeier, Das, Buchholz, Fiorentino, & Shi, 2010). For natural metamorphosis, intestines were collected at different stages, namely premetamorphosis (NF53, prior to endogenous TH in circulation), prometamorphosis (NF58, beginning of larval epithelial apoptosis), climax

(NF61, beginning of adult epithelial proliferation), and end of metamorphosis (NF66, tail resorption). For induced metamorphosis, the guts from premetamorphic tadpoles treated with 10 nM TH for 0, 1, 3, and 6 days were analyzed. The intestine of tadpoles treated with TH for 3–6 days had extensive cell death and adult cell proliferation but no adult epithelial differentiation, and thus resembled stages at climax of natural metamorphosis (NF61). Despite this morphological similarity, only 20.9% and 33% of the genes upregulated at prometamorphosis (stage 58) overlapped with genes upregulated after 3 and 6 days of T3 treatment, respectively. A similar percentage overlap was observed for downregulated genes. Thus, even though similar histological changes occur during induced and spontaneous metamorphosis, the majority of gene expression changes between natural and induced intestine remodeling are not congruent, likely due to different levels of TH and/or presence of additional hormones during natural metamorphosis.

The functional activities of many genes altered during early metamorphic stages or early TH treatment time points are consistent with the histological epithelial tissue degeneration. Genes transiently up- and downregulated have roles consistent with larval epithelial degeneration, the predominant early event in TH-induced remodeling. The proteolysis gene ontology (GO) category had significantly regulated genes, such as stromelysin-3 in larval cell apoptosis and collagenase 3 in tissue breakdown. Also, genes associated with the cell cycle were significantly downregulated. After the initial wave of apoptosis, adult cells proliferate and differentiate, accompanied by the proliferation of connective tissue and muscle cells, which would presumably include extensive changes in gene regulation. Indeed, a significant number of transcription factors have altered expression at later time points or developmental stages, as well as development genes and extracellular matrix genes. In late stages of remodeling, genes for cell proliferation, signal transduction, and cell–cell signaling are significantly upregulated. Therefore, the two waves of morphological remodeling, namely cell death then cell proliferation and differentiation, are reflected by corresponding waves of gene expression. One of the pathways strongly upregulated during this period is TGF-β signaling including TGF-β ligands (e.g., BMP-1, -2, -3, -4, -7), type I and II receptors, and SMADs. As the tadpole progressed through prometamorphosis, four genes in this pathway were upregulated. By climax, 15 genes were upregulated, and by stage 66, the number of genes upregulated compared to premetamorphosis was only 5, and one gene was downregulated. TGF-β pathway genes were also identified after 6 days of TH treatment. Many of these genes may be indirect response genes because another

induced signaling molecule, sonic hedgehog, is required for BMP-4 induction. Functional studies have supported these results suggesting that upregulation of the TGF-β pathway is important for the remodeling taking place at the climax of metamorphosis (Ishizuya-Oka, Hasebe, Shimizu, Suzuki, & Ueda, 2006).

As with other tissues, downregulated genes made up approximately half of the genes regulated by TH. Strongly downregulated GO categories of genes were glucose and fatty acid metabolism and the electron transport chain. Metabolic pathways such as glycolysis, digestion, and the complexes that transfer electrons and synthesize ATP in the mitochondrial inner membrane all appear to shut down at metamorphic climax. Such downregulation of genes, nearly 30, involved with energy consumption was temporary during the remodeling period associated with cell death and lack of proliferation. Later developmental stages saw the expression levels of these genes return to those found in the tadpole to achieve a functional absorptive organ, including intestinal fatty acid binding protein allowing nutrient absorption.

An additional analysis from the natural metamorphosis data set was the identification of larval-specific and adult-specific genes. In all, 17 larval- and 52 adult-specific genes were identified, 11 and 30 of which could be associated with human homologs. The larval-specific genes varied in their potential functions and included GO categories associated with catalytic activity and RNA processing. The larval-specific genes such as UDP-glucose dehydrogenase and solute carrier 22A6 may play a role in signal transduction and cell migration, while mucin, cytochrome P450, thioredoxin reductase, keratin 8, and lactotransferrin are described as potential molecular markers for colon, breast, and other cancers, suggesting that inappropriate expression of larval-specific genes in adult intestine may cause or be indicative of cancer formation. Many adult-specific genes are associated with digestion. For example, dietary enzymes, including the serine proteinases PRSS2 and PRSS3, are significantly increased in the adult intestine. Trypsin is essential for food digestion, but is also involved in other physiological and pathological processes, such as inflammation and tumor invasion. Two other adult-specific genes evaluated, ELA3 and NFI-X2, were predominantly expressed in the epithelium. These genes may provide good adult-specific markers to evaluate adult epithelial regeneration.

Similar processes are occurring in the tail and intestine at the beginning of remodeling, where 100% of the cells in the tail and the vast majority of cells in the intestine, the larval epithelial cells, are destined to die. The early response to TH in the limb is proliferation and the same is true for the brain, even though, like the intestine, the brain undergoes extensive remodeling. The difference between early responses between brain and intestine is that

tissue degeneration is not as extensive in the brain. Comparison of gene expression data across these tissues indicates that tissues with similar cellular responses to TH have the most overlap in TH-induced genes. Thus, the initial proliferative responses of the limb and brain to TH explain their similar sets of regulated genes, which share little overlap with genes similarly induced in tail and intestine associated with cell death occurring in the initial response to TH. Despite the association between morphology and altered gene expression among tissues, a core set of 59 genes similarly expressed among all these tissues was identified. How these similarly expressed genes are involved in the mechanisms of gene regulation underlying tissue-specific remodeling has yet to be determined.

2.5. Skin

The larval skin is composed of three main cell types, apical cells, skein cell, and basal cells, organized in two layers (Suzuki, Utoh, Kotani, Obara, & Yoshizato, 2002). During metamorphosis or precocious exposure to TH, basal cells differentiate into adult-type skin stem cells and replace the apoptotic apical and skein cells with cornified, multilayered adult skin with mucus, and serous glands, the latter producing antimicrobial peptides among other proteins.

Global gene expression during TH-induced metamorphosis in body skin was analyzed after 0 versus 1, 3, 5, and 7 days of 5 nM T3 (Suzuki et al., 2009). They found 240 upregulated genes and 161 downregulated genes organized into nine functional groups. Within the first 24 h, the T3-response genes, TRβ, TH/bZIP, KLF9, and ST3, were induced in the skin, as observed in all other organs from previous studies. Transcription and proteolysis were also functional categories recognized by 24 h, followed by cell cycle genes by 3 days, and then defense genes (including antimicrobial peptides) by 7 days. Adult keratin genes and antimicrobial peptide genes were among the most upregulated genes, and genes 17–20 (apical cell-specific genes identified by subtractive hybridization; Wang & Brown, 1991) were among the most downregulated. A major change in gene expression unanticipated by histology was the gradual down regulation of immune response genes (mostly MHC genes). Explanation for this change awaits further work.

2.6. Direct response genes

Because TH induces all metamorphic changes and its effects are mediated through transcriptional regulation by TRs, the identification and characterization of the TH early, direct target genes are of critical importance in elucidating mechanisms underlying the tissue-specific responses to TH.

To identify these genes, two chemicals, cycloheximide and anisomycin, that inhibit protein translation were included in an experiment directed toward identifying early, direct target genes (Das, Heimeier, Buchholz, & Shi, 2009). Specifically, stage 46 *X. laevis* tadpoles were treated with vehicle, 100 nM TH, inhibitors, and 100 nM TH + inhibitors for 14 h, and RNA from whole tadpoles was used in the microarray. This analysis identified 188 and 249 inhibitor-resistant up- and downregulated genes, respectively (i.e., TH + inhibitor vs. vehicle). For TH only, 311 and 162 genes were up- and downregulated. Because TH alone treatment is expected to have a combination of direct response genes and late-response genes, one might expect the TH + inhibitor to be a subset of the TH-only genes. Surprisingly, less than half of the genes were in common between these two treatments. Although most inhibitor-resistant TH-response genes were not in the TH only set of genes, they are believed to be direct TH-response genes. The inhibitors stabilized many mRNA transcripts and likely increased the ability of these genes to be detected by the microarray compared to TH treatment alone.

GO analysis showed that categories related to metabolism/catabolism, cell proliferation (cell cycle/DNA replication), and metallopeptidase activity were found to be common between the TH and TH + inhibitor groups, suggesting that the genes in these categories are involved in the early events of metamorphosis. In particular, increased metallopeptidase activity suggests that the remodeling of the extracellular matrix and other protein degradation events are important early signaling events induced by TH during metamorphosis. In fact, one of the first TH-direct response genes found during metamorphosis was stromelysin-3 (MMP-11), which is important for TH-induced cell death during metamorphosis (Fu et al., 2005). In addition, GO categories like cell communication, steroid hormone receptor activity, and transcription factor activity were found to be significant only in the TH + inhibitor group, indicating that these activities are also involved in early events of metamorphosis. Some of these transcription factors have been reported previously as direct targets of TH, such as TRβ and TH/bZIP. This may not be surprising as it is expected that transcriptional regulation should be an important initial step of metamorphic events.

Interestingly, DNA replication genes, apoptosis, and ribosomal biogenesis and assembly genes were highly enriched in TH upregulated genes but not significantly enriched in the inhibitor-resistant upregulated genes. These results suggest that, although cell cycle progression was induced by both TH and TH + inhibitors, some late-response genes, sensitive to inhibitors, are required for the cells to progress to DNA replication. These data suggest that, in the absence of protein synthesis, TH may induce cell cycle changes.

The direct target genes by themselves, however, are not sufficient for DNA replication but rather prepare the cells to enter the S-phase. This is strongly supported by the enrichment of G1/S-phase transition genes but the absence of DNA replication genes in the microarray analysis. Likewise, the absence of genes in GO categories related to ribosomal biogenesis and assembly in the inhibitor-resistant TH-response genes but their presence in the genes induced by TH alone suggest the possibility that downstream genes are required to sustain cell proliferation. In addition, genes involved in apoptosis are also not significant in TH + inhibitor-treated animals, indicating that these genes are upregulated downstream of direct response transcription factors.

In contrast to the number of upregulated GO categories shared by TH and TH + inhibitors versus control, little overlap was found among the significantly enriched GO categories between the genes downregulated by TH and the genes downregulated by TH + inhibitors. Interestingly, a number of categories involved in cellular processes, such as cell differentiation and cytoskeletal changes, were significantly enriched in genes downregulated by TH in the presence of inhibitors, whereas a number of categories at the organismal level, such as organ development and multicellular organismal processes, were significantly enriched in genes downregulated by TH alone. Such changes suggest that downregulation of cellular processes occurs prior to the changes at the organ levels and that the latter requires late/indirect TH-response genes.

Interaction of the direct TH-induced transcription factors and signaling pathways with the premetamorphic set of transcription factors undoubtedly contribute to tissue specificity of the TH response. Despite the presence of TREs in their promoters, some direct TH-response genes are not expressed in all tissues, where ST3 is restricted to fibroblasts and sonic hedgehog to intestinal epithelium. Thus, the complement of transcription factors as well as epigenetically modified DNA and histones determine how a tissue responds to TH. Use of microarrays to identify TH-regulated genes is a critical step to understanding causes and consequences or tissue-specific responses to TH, but further studies involving these genes are required to determine how different tissues respond to the same hormone differently.

3. ENDOCRINE DISRUPTION

3.1. Biomarkers for TH disruption

A major goal of endocrine disruption research is to identify molecular biomarkers for detection of factors that may inhibit, mimic, or accentuate hormone action. Because TH-induced morphological changes in tadpoles are not observed before 3 days, molecular changes may serve as rapid and

sensitive endpoints. Thus, microarray analysis is well suited to enable selection of one or a few genes from the entire set of TH-response genes that are particularly appropriate for detecting TH disruption. Additionally, microarray analysis can be used to identify biomarker genes for detection of the presence of a particular endocrine disruptor by identifying genes uniquely induced by it.

One of the first microarray analyses for biomarker development used the indigenous species, *R. catesbiana* (Veldhoen et al., 2006). Using the MAGEX cDNA array with 401 cDNAs, they identified K-ras (Kirsten rat sarcoma) and PCNA (proliferating cell nuclear antigen) as being strongly up- and downregulated by TH, respectively. These genes were similarly TH regulated in *X. laevis* and mammals, as well. The microarray results for these two genes, as well as for TRα and TRβ, were further analyzed in *R. catesbiana*, where robust changes in gene expression of TRβ, K-ras, and PCNA were identified in 6, 24, and 48 h, respectively. This study showcased the utility of using heterologous cDNA arrays compared to oligonucleotide arrays for analysis of nonmodel species. Ninety percent of the cDNAs on the MAGEX array are from *X. laevis* sequences, and the other 10% are from *R. catesbiana*. Because cDNA arrays have extended sequence with which the sample cDNAs can hybridize, it is robust to cross-species sequence divergence (Helbing, Maher, Han, Gunderson, & Borheras, 2010). In contrast, short oligoarrays (25–60 nucleotides) will not as readily hybridize with divergent sequences. Furthermore, sequences from EST libraries used to make global gene expression microarray assays often have 3′UTRs, which are typically highly divergent even among closely related species, thereby making these sequences unsuitable for nonmodel taxa. cDNA arrays have the further advantage that individual cDNAs can be selected for actual or possible relevance to endocrine disruption.

Robustly induced TH-response genes, such as TRα, TRβ, and KLF9, were identified prior to microarray studies that were sufficient to detect the action of TH agonists. However, their transcripts were not significantly changed in the presence to TH synthesis inhibitors, such as MMI, propylthiouracil (PTU), and perchlorate (PER). To identify useful genes to detect disruption caused by these TH synthesis inhibitors, Helbing et al. (2007) compared results from a 14-day morphological exposure assay to short-term (0–4 day) gene expression profiles of brain transcripts after T4, T3, MMI, PTU, and PER treatments (Helbing et al., 2007) (T4 and T3 are the precursor and active versions of TH). As expected, genes involved in transcription, signal transduction, and hormone regulation were significantly

regulated by TH. Surprisingly, there was little overlap in genes regulated by T3 and T4, even though they have similar morphological outcomes. This finding cannot be accounted for by differences in timing of T4 to T3 conversion by deiodinases and awaits an explanation. For the inhibitors, similar numbers of genes in similar categories were identified, and association analysis of genes regulated by MMI, PTU, and PER suggested they have similar gene regulation changes. Thus, the inhibitors may have similar modes of action on brain gene regulation. However, the rapid responses, that is, within 1–2 days, suggests that the mode of action may be a direct effect on brain tissue, rather than blockade of TH synthesis with attendant delay in running out of stores of hormone in the thyroid gland.

A complimentary analysis to the previous MAGEX brain study using the same animals was carried out using hind limb and tail (Helbing et al., 2007). As with the brain, the strategy was to find candidate genes using microarray that could then be used in quantitative comparisons using qPCR. Fewer genes overall were identified in these tissues compared to the brain. As in the brain, T4 and T3 had nonoverlapping sets of transcripts. Also, the sets of transcripts significantly regulated by MMI, PTU, and PER were very different from each other. Some genes were identified as potential biomarkers, such as RNA helicase II/Gu or DAD1 (defender against death 1). However, their use as biomarkers is dependent on the tissue and time point after exposure. These studies revealed the important issue that tissue and exposure duration need to be carefully determined to identify biomarkers with high and robust predictive value.

In a recent microarray analysis, a meta-analysis approach was used to identify robust TH-responsive genes as determined by detection across several array platforms (Searcy et al., 2012). They used an Affymetrix array with tail tissue treated for 6 and 48 h, with 20 nM TH. Many of the regulated genes included those previously identified, such as transcription factors and proteases. However, when comparing across platforms, they found 30% or less overlap of TH-regulated genes, which may be explained by genes not represented on one of the arrays and the different TH doses used. On the other hand, there was a high proportion of overlap with TH-induced tail genes identified by subtractive hybridization. They reasoned that overlap among platforms would represent reliable and robust genes for use as biomarkers for TH disruption. As a proof of principle, they examined the effect of wastewater effluent (WWE) on four TH-response genes, TRβ, D2, CRHBP, FAPα. WWE augmented the induction of these transcripts in the presence of TH, and transcripts of CRHBP and FAPα were increased with WWE even without TH.

3.2. Effects/mechanism of EDC action

3.2.1 BPA

BPA has well-known estrogenic effects, but BPA also affects brain differentiation, a process not known to be strongly influenced by estrogen (vom Saal & Hughes, 2005). TH, on the other hand, is critical for proper brain development, opening the possibility that BPA may be a TH endocrine disruptor as well. Indeed, BPA inhibited TH-induced external morphological changes as well as intestinal remodeling (Heimeier, Das, Buchholz, & Shi, 2009). The effect of BPA on established TH-response genes was to partially block induced changes. To examine effects of BPA on transcription across all genes, tadpoles were treated with 2 nM TH, 10 μM BPA, or both together for 4 days and then a microarray analysis was carried out on the intestine. Genes regulated by TH and TH + BPA compared to controls were relatively similar, despite the general inhibition of BPA on metamorphic transformation. However, because BPA only partially inhibited TH-induced changes in gene regulation, it is possible that BPA attenuates but does not abolish TH-response gene induction. Thus, when comparing gene sets from TH versus TH + BPA, BPA inhibited regulation either up or down in 501 genes, more than 50% of which were TH-response genes. Because BPA reduced most TH-induced gene regulation, BPA likely blocks TH-dependent metamorphosis via inhibition of most if not all molecular and developmental pathways required for metamorphosis.

3.2.2 Atrazine

Atrazine has negative effects on growth, metabolism, immune function, and sexual differentiation in fish and frogs (Hayes, 2005). However, the molecular mechanisms underlying these organismal phenotypes are not well understood. To better understand how atrazine interacts with the organism at the level of gene expression, Ide and colleagues carried out a microarray analysis of tadpoles chronically exposed to 400 ppb atrazine from hatching to climax of metamorphosis (Langerveld, Celestine, Zaya, Mihalko, & Ide, 2009). This atrazine treatment throughout the larval period affected growth, development, and survival. Fat bodies were also reduced in both male and female tadpoles. Microarray analysis from whole bodies identified changes in males (44 genes) and females (77 genes) at metamorphic climax, with less than 50% overlap among sexes. The genes identified were involved in growth and metabolism genes, proteolysis, fibrinogen complex formation, and immune system function. Reproductive function genes were not

observed, but gonads constitute a small proportion of whole-body RNA. A reanalysis of these microarray data looking for up- or downregulated metabolic pathways found genes involved in increased fat usage and protein degradation and decreased storage of fat, protein synthesis, and glucose conversion to energy (Zaya, Amini, Whitaker, & Ide, 2011). These changes correlate with morphological effects of atrazine, and could be due to a direct effect of atrazine in these genes at the transcriptional level. Alternatively, the authors suggested that atrazine may reduce food consumption thus indirectly leading to altered gene regulation of metabolism genes. Detoxification of atrazine is an energy intensive process and may place high demand for energy, perhaps an explanation for the etiology of atrazine's effects on slowed growth and development. Reduced survivorship may be due to deleterious effects on immune function perhaps leading to increased susceptibility to infection. However, the cause of death was not determined. The role of altered blood cell function in atrazine-treated tadpoles is not clear and represents another example of microarray analysis revealing cryptic changes not obvious from gross morphological or histological analysis. Different effects of atrazine observed between the sexes are not well understood.

3.2.3 PCBs

PCBs affect neural development, thereby implicating a potential role in TH endocrine disruption based on TH's role in development of the nervous system (Jacobson & Jacobson, 1997). Tadpole metamorphosis is used as a model system to understand the role of PCBs on development to avoid the difficulties of studying endocrine disruption in uterus-enclosed fetuses in mammals. In tadpoles, hydroxylated polychlorinated biphenyls (OH-PCBs) significantly delayed TH-induced metamorphosis (Ishihara, Makita, & Yamauchi, 2011). Microarray on brain tissue showed that 526 TH-response genes (25% of the 2139 T3-regulated genes) had altered TH responses in the presence of 4-OH-PCB-106 and/or -159. GO categories significantly associated with these genes were growth, brain development, cell proliferation, cell adhesion, neuron differentiation, and apoptosis, providing molecular underpinning of the presumed effects of PCBs on the tadpole brain. The fact that most TH-response gene regulation was unaffected suggests that PCBs may not act directly through TRs or that PCBs influence TRs only in a subset of TH-response genes. The authors suggested the possibility that the 526 genes could be a source of biomarkers for PCB disruption of T3-dependent brain development but did not characterize any.

3.2.4 Acetochlor

The pre-emergent herbicide acetochlor accelerated TH-induced metamorphosis in ranid species and *X. laevis* (Crump et al., 2002). In the absence of TH, acetochlor had no significant effect on body area (reduced body area is a measure of metamorphosis due to gill resorption). Gene expression induced by TH was altered in the presence of acetochlor in a manner corresponding to acetochlor's effect on morphological transformation. Acetochlor increased expression of genes that were (1) induced by TH, (2) not affected by TH, and (3) repressed by TH. Another group of genes induced by TH were unaffected by the addition of acetochlor. Given these patterns of gene expression that not all TH-response genes were affected, a direct agonist or antagonist action of acetochlor on TR is unlikely, though promoter-specific or tissue-specific affects allowing direct contact with TR is not ruled out. However, it is more likely that acetochlor, like BPA, affects a subset of TR-containing gene regulatory complexes explaining the complex effects of the endocrine disruptor on TH-response gene expression.

4. SPECIAL TOPICS

4.1. Phenotypic plasticity

Microarray analysis was used to examine the molecular basis of *R. pirica* plasticity to salamander larvae predators (Mori et al., 2005, 2009). Upon exposure to caged salamander larvae, tadpoles get a bulgy fluid-filled body with a deeper tail, and this phenotype goes away with predator removal. To examine the endpoint of this phenotypic plasticity, namely, the altered skin morphology, subtractive hybridization between control and body skin was performed, where 297 differentially expressed transcripts were identified. In an unusual use of microarray, these transcripts were then used as targets on a cDNA array to confirm the subtractive hybridization results. Downregulated genes included carboxypeptidase B, trypsinogen, elastase, fibrinogen, which may have the effect of increasing rate of fibrinolysis allowing skin to stretch and become bulgy. Upregulated genes included bullous pemphigoid antigen, uromodulin, aldehyde dehydrogenase most of which have unclear relation to the bulgy phenotype. The researchers then repeated the experiment and obtained larger number of subtracted clones, which were again used in a microarray validation analysis (Mori et al., 2009). This expanded set of genes identified downregulation of some keratins, and upregulation of a novel uromodulin-like protein in patterns expected for genes involved in the bulgy phenotype. Keratins are downregulated in

the bulgy phenotype whose expression comes back upon removal of the predator. Uromodulin has the opposite expression pattern. *In situ* hybridization showed these genes are expressed in epidermis. Brain genes were identified by subtractive hybridization, but the proportion of brain tissue in the RNA sample was too low for positive signals in the microarray analysis. Uromodulin is an abundant protein in mammalian and frog kidneys, but its role is unclear, although alteration of water permeability of tadpole skin may be a possibility. Keratins function to retain cell shape, and their downregulation may allow cell deformation upon water retention in body skin. These molecular associations of uromodulin and keratins with the bulgy phenotype provide potential molecular explanations of the bulgy phenotype. An interesting area of future research is to examine the control upstream of these genes, for example, the steps from predator presence to regulation of these genes remains unanswered.

4.2. Biocontrol

Another goal for a microarray study was to identify genes upregulated during metamorphosis that could be used in a strategy to control the invasive cane toad, *B. marinus* (Halliday et al., 2008). The authors produced a 5170-spot cDNA array of PCR products from randomly selected clones from a toadlet cDNA library. Whole-body microarray comparisons were made among 9, 18, 28, 30, and 53 days postfertilization (9, 18, 28 dpf are premetamorphic stages, and 30 and 53 are postmetamorphic stages). The authors concentrated on genes upregulated during metamorphosis, and identified nine strongly upregulated genes, namely, serum albumin, hemoglobin α and β, olfactomedin, trefoil factor, gastrokine, harderin-like protein, gamma crystallin, apolipoprotein. The potential utility of these highly upregulated genes for biocontrol requires further research.

4.3. Regeneration

The promise of recovery from spinal cord injury in humans spurs research in animals that have this ability. The ability of tadpoles to regenerate axons across a spinal cord transection is lost during metamorphosis (Gibbs, Chittur, & Szaro, 2011). Thus, microarray analysis lends itself well to finding genes permitting regeneration in tadpoles and losing this ability during metamorphosis. Recovery from spinal cord transection in premetamorphosis was slowed by treatment with MMI and blocked by TH, suggesting that low levels of TH may promote regeneration but are not required and that

TH-induced development goes toward a developmental state not capable of regeneration. To look for genes potentially involved in this process, microarray was done using hindbrain, the source of regenerating axons. The authors focused on genes upregulated by spinal cord injury in the regeneration permissive state (MMI treated) and found 72 annotated genes, including transcription factors, membrane proteins, cell–cell and intracellular signaling, and transcriptional coregulators. The five most regulated genes were SOCS2, XER81, neurotensin, MORC3, and a protein associated with RNA polymerase. All but the last of this list have known functions in promoting neural development, survival, and neurite outgrowth. The larval state appears to be important for regeneration, and characterizing precisely the larval state and which specific genes involved in regeneration will require functional studies on candidate genes.

5. SUMMARY AND CONCLUSIONS

Knowledge of how TH can induce wildly divergent responses among tissues during frog metamorphosis will include identification of the component genes of TH-induced tissue-specific gene regulation cascades. Microarray analyses have contributed most significantly to this effort and have also revealed aspects of TH-induced metamorphosis unanticipated by previous morphological and physiological studies. As expected, each organ has unique TH-regulated genes concordant with its distinct role in the organism, for example, larval and adult keratins in the skin, proteins associated with nutrient absorption in the intestine. On the other hand, some tissues, such as brain, limb, and intestine, have a large set of commonly regulated genes despite having distinct morphological outcomes. These common genes are comprised of cell cycle genes and consistent with cell proliferation as a component of their metamorphic remodeling. In this respect, the correlation between expected functions of genes in cell biology and histological observation were consistent, thereby matching known morphological events with molecular signatures. Similarly, the tail and intestine had overlapping sets of genes, including decreased metabolism and electron transport chain proteins and increased extracellular proteases, consistent with tail regression and apoptosis of larval intestinal epithelium. Visualization of these comparisons among tissues reveals that cellular responses to TH induction, such as mitochondrial and cell cycle regulation, are much more divergent among tissues than changes in transcription and signal transduction (Fig. 12.3).

Not predicted from premicroarray studies was a set of genes upregulated by TH in every tissue examined, such as TRβ, TH/bZIP, KLF9, NFI-X2, all of which are transcription factors. This common set of development genes in organs with different responses to TH, from cell death to cell proliferation, suggests that it is quite likely that a combination of organ-specific TH-regulated early genes, especially downregulated ones, and organ-specific responses to the changes in common TH-regulated genes dictates organ-specific metamorphic changes. Thus, an important area of interest will be to determine the downstream targets of the common early regulated genes.

Additional unexpected findings from the microarray analyses are the total number of TH-response genes involved, well above 1000 genes as components of the TH gene regulation cascade and well over 200 direct response genes. Our bias in understanding the molecular mechanisms of direct induction versus repression of gene expression by TH did not prepare us for the identification of equivalent numbers of up- and downregulated genes. Finally, a newly uncovered complexity to the gene regulation cascade, namely, the web of intra- and extracellular signaling pathways induced by TH, was not initially predicted but undoubtedly contributes to the outcome of tissue-specific responses to TH. These novel and important findings about the number and identity of TH-induced genes and their spatial and temporal changes during tadpole development secures the value of microarray studies on frog metamorphosis.

Not only developmental studies but also studies on endocrine disruption have benefitted from microarray analyses. Based on morphological or physiological endpoints, many chemicals appear to interfere with endocrine physiology. However, as hormonal control of development and homeostasis is complex and as hormone systems can interact with each other, the mechanisms of action of a given chemical are not often obvious. Identifying the global pattern of altered gene regulation of an endocrine disruptor gives insight into how that chemical is acting and interacting with the endocrine system. Another use of microarray in endocrine disruption studies lies in the search for biomarkers of TH endocrine disruption. A useful biomarker requires particular properties of its response to TH, namely it must be a sensitive and robust marker of TH signaling. Global analysis of potential genes allows one to select the most appropriate gene or small set of genes from the large group of TH-response genes that may not all have desirable fold-change levels or expression kinetics. This strategy for identifying suitable genes has also been employed in development studies where larval- versus adult-specific genes have been found for intestinal epithelium and skin and

genes responding to CORT or TH only without influence from the other hormone. Thus, microarray studies are well suited to the goal of identifying biomarkers for developmental as well as endocrine disruption studies.

The wealth of knowledge obtained using microarray experiments has come despite some technical issues associated with the technique. The high variability among workers in terms of genes identified as TH-regulated stems in part from the microarray methods and also in part from different rearing and TH treatment conditions among studies. These interstudy discrepancies indicate that microarray data are not definitive but rather indicative of important genes and pathways. Another issue is that currently many elements on the microarrays have no ascribed function because they are ESTs representing nonconserved 3′UTRs or hypothetical proteins with low or no homology to a mammalian sequence, giving little information about gene function. Improvements in gene annotation and genomics should refine the complement of known response genes.

While much has been learned about gene regulation cascades, much is still left to understand. Noncoding RNAs are now believed to have a major impact on gene regulation and undoubtedly contribute to tissue-specific TH responses. Differential expression of noncoding RNAs due to TH can be analyzed by microarray technology but thus far have not been examined. Another gap in our knowledge is distinguishing cause from effect in the gene regulation cascade. Microarray analysis provides molecular correlations with cellular and histological changes. In many cases cause and effect relationships can be inferred, such as upregulation of proteases with ECM degradation or upregulation of cell cycle genes with cell proliferation. However, it is often difficult to sort out causes versus effect underlying control of the tissue-specific gene regulation cascades. Determining the web of interactions starting with direct response genes to how they interact with already present tissue-specific transcription factors and TH-indirect response genes may benefit from additional microarray analyses using transgenic-inducible overexpression of TH-direct response transcription factors but likely other research approaches as well. Another important consideration is that the relationship between mRNA levels, as detected by microarray analysis, and the cognate protein levels for most genes is not currently known. The relationship between protein levels and their activity levels altered by posttranslational modifications is also not known. Thus, identifying TH-induced genes is a critical first step, but other types of research, including proteomics and functional studies, are required to elucidate protein levels and the downstream effects of protein activity.

The tissues that comprise organs typically undergo their own TH-induced changes independent of other associated tissues, and they have their own tissue-specific TH-response genes, such as sonic hedgehog in the intestinal epithelium and stromelysin-3 in fibroblasts. Also, the same tissue can undergo distinct responses depending on their location in the body, for example, fibroblasts and epidermis in tail versus body. However, tissues within an organ may be of low abundance and critical cells within tissues (such as stem cells) will be rare. In these cases, mRNA from rare cells may not be well represented in the large pool of mRNA from abundant cells and thus gene expression changes may not be detectable from the rare cells using microarray. Special techniques, such as dissecting out particular tissues from an organ or isolating particular cells using laser capture microdissection may allow detection of rare but critical TH-induced gene expression changes. Also, due to the nature of how the microarray works, low abundance, but high fold-change genes may not be detected. For example, TH/bZIP was identified using subtractive hybridization and is a very high fold-change gene, but it is often not detected using microarrays due to its apparent low abundance even when induced. In addition to TH/bZIP, there may be other such potentially critical genes, which may yet be detected by other means, such as transcriptomics or RNA-seq.

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