Steroid Metabolism in Cnidarians: Insights from *Nematostella vectensis*

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Abstract

Cnidarians occupy a key evolutionary position as a sister group to bilaterian animals. While cnidarians contain a diverse complement of steroids, sterols, and other lipid metabolites, relatively little is known of the endogenous steroid metabolism or function in cnidarian tissues. Incubations of cnidarian tissues with steroid substrates have indicated the presence of steroid metabolizing enzymes, particularly enzymes with 17β-hydroxysteroid dehydrogenase (17β-HSD) activity. Through analysis of the genome of the starlet sea anemone, *Nematostella vectensis,* we identified a suite of genes in the short chain dehydrogenase/reductase (SDR) superfamily including homologs of genes that metabolize steroids in other animals. A more detailed analysis of *Hsd17b4* revealed complex evolutionary relationships, apparent intron loss in several taxa, and predominantly adult expression in *N. vectensis*. Due to its ease of culture and available molecular tools *N. vectensis* is an excellent model for investigation of cnidarian steroid metabolism and gene function.

Keywords

Abbreviations
CYP
HSD
AKR

1. Introduction

Steroids regulate fundamental aspects of development and physiology in vertebrates and many invertebrates (Baulieu and Kelly, 1990; Lafont and Mathieu, 2007). Steroid biosynthesis is catalyzed by a suite of enzymes including members of the cytochrome P450 (CYP), short chain dehydrogenase (SDR), and aldo-keto reductase (AKR) superfamilies. Like the CYPs and AKRs, SDRs are evolutionarily ancient and functionally heterogeneous (Belyaeva and Kedishvili, 2006; Kallberg et al., 2002). In addition to steroids, SDRs metabolize diverse substrates including retinoids, alcohols, fatty acids and prostaglandins. SDRs also vary in their tissue distribution, subcellular localization, co-factor preference, and predominant catalytic direction (Persson et al., 2003).

Steroid metabolism and signaling pathways are well-characterized in some taxa (e.g., vertebrates and insects) but relatively uncharacterized in others. Comparative studies of steroid metabolic enzymes and receptors have already provided insight into the evolution of endocrine signaling; however, these patterns are still incompletely known. Cnidarians (e.g., sea anemones, corals, hydra, and jellyfish) are intermediate in complexity between sponges and bilaterian animals, and thus occupy a key evolutionary position. As "basal" metazoans, cnidarians are equally related to the protostomes (e.g., insects, crustaceans, molluscs, nematodes) and deuterostomes (e.g., vertebrates, tunicates, echinoderms). Despite their evolutionary position as a sister group to the bilaterian animals, recent analyses of cnidarian genes and genomes have revealed that cnidarians have fairly complex genomes. In some cases, cnidarians have retained orthologs of deuterostome genes that have apparently been lost from some protostome lineages (Putnam et al., 2007; Sullivan and Finnerty, 2007). Therefore, characterizing steroid metabolism and action in cnidarians can provide considerable insight into the diversification and potential divergence of steroid metabolism and steroid signaling pathways in "higher" animals. Comparisons of steroid metabolism between cnidarians and bilaterian animals can also provide insight into the likely metabolic capability of an inferred cnidarian-bilaterian ancestor.

The recently completed genome (Putnam et al., 2007) of the starlet sea anemone, *Nematostella vectensis,* provides an unprecedented opportunity to investigate steroid metabolism in a cnidarian. In this paper, we first review the state of knowledge regarding cnidarian steroid metabolism. We then identify a suite of SDRs in the *N. vectensis* genome and describe their phylogenetic relationships, with a more detailed examination of *Hsd17b4*. We have chosen *Hsd17b4* because of its conservation (and lessons from functional evolution) known into other species and its role in pathogenesis of peroxisomal deficiencies (Breitling et al., 2001; van Grunsven et al., 1998). Finally, we discuss these enzymes in a functional and evolutionary context and suggest directions for future work*.*

2. Steroid Metabolism in Cnidarians

Diverse and often novel lipid metabolites have been identified in cnidarians including prostaglandins (Koljak et al., 2001), steroids and sterols (Fleury et al., 2008; Qi et al., 2008), and other terpenoids (Eterovic et al., 1993; Frenz-Ross et al., 2008)*.* Many of these compounds have been discovered through prospecting for natural products with biomedical applications, particularly in soft-bodied anthozoans (e.g., zoanthids, gorgonians and alycyonaceans). These animals are benthic as adults and lack the physical defense of a mineralized skeleton, so secondary metabolites often serve as chemical defenses against predation, fouling, or infection (Paul et al., 2007; Paul and Puglisi, 2004). While some metabolites have been experimentally demonstrated to play a defensive role, the functions of others are unknown, and some compounds are likely to be physiologically important within the animals. The

synthetic pathways and enzymes have not yet been identified for most cnidarian lipid metabolites. As a notable exception, cyclooxygenase genes responsible for coral prostaglandin synthesis have been identified and functionally characterized (Koljak et al., 2001). Cnidarians have not been shown to synthesize sterols *de novo*; thus the diverse steroid and sterol metabolites are derived from either (1) synthesis by dinoflagellate symbionts or associated microbes, (2) dietary sources, or (3) metabolites of compounds derived from the previous two sources (Kerr and Baker, 1991; Nelson et al., 2000; Withers et al., 1982).

Studies of steroid metabolism in cnidarians have often been driven by the general hypotheses that steroid signaling pathways are deeply conserved; thus, several studies have tested whether cnidarian homogenates can synthesize vertebrate-type steroid hormones from labeled substrates. Vertebrate-type sex steroids have been identified in cnidarian tissues using immunological methods and shown to vary during the reproductive cycle, although the source and function of these steroids have not been identified (Pernet and Anctil, 2002; Slattery et al., 1999; Tarrant et al., 1999; Twan et al., 2003). Work by Gassman (1992) indicated that scleractinian corals ("hard" corals that are primary builders of tropical reefs) can synthesize testosterone and androstenedione from progesterone. Studies with alcyonacean corals ("soft" corals) from the Antarctic by Slattery and colleagues (1997) identified 5α-pregnane-3,20-dione, 5α-pregnane-3β-ol-20-one and an esterified progestin as the primary metabolites following incubations with labeled progesterone, indicating the presence of a 5α-reductase, 3β-HSD and an acyl transferase. Incubations of scleractinian and alcyonacean homogenates with labeled estrogens or androgens have also indicated the presence of 17β-HSDs (Slattery et al., 1997; Tarrant et al., 2003). Twan et al. (2003; 2006) have inferred the presence of an aromatase-like enzyme from release of tritiated water following incubations of *Euphyllia ancora* coral tissue with 1β-^{3}H-androstenedione. Other studies have failed to detect aromatase activity in cnidarian homogenates following incubations with labeled androgens (Gassman, 1992; Slattery et al., 1997; Tarrant et al., 2003).

In vertebrates, steroidal estrogens are synthesized from androgens by CYP family 19 genes (CYP19s), also known as aromatases (Miller, 1988). CYP19s with aromatase activity have been identified in all vertebrate classes as well as the invertebrate cephalochordate (amphioxus) *Branchiostoma floridae* (Callard et al., 1984; Castro et al., 2005; Mizuta et al., 2008; Mizuta and Kubokawa, 2007). Aromatase activity has also been detected in incubations of radiolabeled androgens with mollusc tissues (Le Curieux-Belfond et al., 2001; Matsumoto et al., 1997), although the responsible enzyme has not yet been cloned. CYP19 homologs have not yet been identified in any other invertebrate taxa (Castro et al., 2005; Goldstone et al., 2006), including the cnidarian *N. vectensis* (Goldstone, in press), although some other enzyme may serve a similar convergent function. In vertebrates, estrogens primarily function through binding to estrogen receptors (ERs), members of the nuclear receptor superfamily that regulate transcription of target genes upon activation by estrogenic ligands (Beato et al., 1995). ER homologs have been identified in molluscs, but the mollusc ER does not bind estrogen and is constitutively active in cell-based assays (Keay et al., 2006). ERs have not been identified in the sequenced genomes of echinoderms, insects, nematodes, or *N. vectensis* (Bertrand et al., 2004; Enmark and Gustafsson, 2001; Goldstone et al., 2006; Howard-Ashby et al., 2006, Reitzel and Tarrant, submitted; Sluder et al., 1999); however, exogenous estrogens have been shown to affect reproduction in *C. elegans* and corals (Custodia et al., 2001; Tarrant et al., 2004). Thus, estrogens may bind to another nuclear receptor in these organisms or function through a different pathway.

In contrast to the phylogenetically restricted distribution of aromatase activity and CYP19 genes, 17β-HSD activity is commonly detected in incubations with invertebrate tissues including echinoderms (Hines et al., 1994; Watts et al., 1994), crustaceans (Swevers et al., 1991), molluscs (Matsumoto et al., 1997), and cnidarians (Slattery et al., 1997; Tarrant et al., 2003). 17β-HSDs may be members of either the SDR or the AKR superfamily and catalyze the reversible oxidation or reduction of substrates, including 17-ketosteroids (reviewed by Mindnich et al., 2004). In vertebrates 17β-HSDs interconvert relatively active and inactive forms of estrogens and androgens, and thus play a pivotal role in regulating the available pool of biologically active steroids (reviewed by Baker, 2001; Peltoketo et al., 1999). In vertebrates, at least fourteen types of enzymes have been identified with 17β-HSD activity (Moeller and Adamski, 2008). These enzymes vary considerably in their affinity for substrates and cofactors, their subcellular location, and the predominant catalytic direction within intact cells (Moeller and Adamski, 2006; Peltoketo et al., 1999). Homologs of *HSD17Bn* genes have been identified in many invertebrates and fungi, although many of these genes await functional characterization (Belyaeva and Kedishvili, 2006; Breitling et al., 2001; Kallberg et al., 2002). A notable exception is the 17β-HSD which has been cloned and functionally characterized from the fungus *Cochiobolus lunatus* (Lanišnik Rižner et al., 2001).

Kinetic methods have been applied to characterize 17β-HSD activity in scleractinian corals (Blomquist et al., 2006). Some coral species contain dinoflagellate symbionts (zooxanthallae) within their cells; other species naturally lack symbionts. Both coral homogenates (from species containing and lacking zooxanthellae) and zooxanthellae extracts exhibited 17β-HSD activity with steroid substrates. The activity in coral homogenates was predominantly oxidative with a preference for estradiol over testosterone and $NADP^+$ over NAD^+. The ratios of substrate and cofactor preference were highly variable, suggesting the presence of several enzymes in varying proportions. Flavonoid phytochemicals strongly inhibited conversion of estrogens, and the endogenous substrate is unknown. The unusual properties of the coral enzymes (particularly the preference for $NADP^+$ in oxidative reactions), the ecological importance of corals to tropical reefs, and the evolutionary significance of cnidarians form a compelling argument for additional study of steroid metabolism in cnidarians.

3. Materials and Methods

3.1 Phylogenetic Analysis

We queried *N. vectensis* gene models and the assembled genome through the Joint Genome Institute (JGI) database (version 1, http://genome.jgi-psf.org/Nemve1/Nemve1.home.html). To generate an initial list of *N. vectensis* SDRs, a subset of human SDRs (after Belyaeva and Kedishvili, 2006) were compared against the *N. vectensis* genome with the BLASTp algorithm. Redundant gene models were eliminated from the initial list according to two criteria: 1) overlapping predictions from the same genomic location or 2) gene models with identical sequence. After this reduction, the remaining 44 *N. vectensis* genes were used to query the JGI *N. vectensis* EST database to confirm gene models and to identify genes with evidence of transcript expression.

For a broad phylogenetic analysis of *N. vectensis* SDRs, human SDR protein sequences were downloaded from the NCBI database (Table 1). *N. vectensis* and human SDRs were aligned using MUSCLE (Edgar, 2004) with manual correction. The alignment was trimmed to a highly conserved region (between the GxxxGxG and PGxxxT motifs), corresponding to the entire cofactor binding domain and a portion of the substrate binding domain (reviewed by Mindnich et al., 2004). Maximum likelihood

analyses were run using RAxML (version 7.0.4, Stamatakis, 2006) with a Blosum62+G matrix (model determined by AIC criteria with ProtTest v1.4, Abascal et al., 2005). Trees were visualized and illustrated with FigTree v1.1.2 (http://tree.bio.ed.ac.uk/software/figtree/).

Breitling et al. (2001) previously compared gene structures and analyzed the evolutionary relationships among *Hsd17b4* genes from three animal species (*Homo sapiens, Drosophila melanogaster, Caenorhabditis elegans*) and three fungi (*Candida albicans, Neurospora crassa, Glomus mosseae).* We extended this comparison by querying gene models from five invertebrate genome sequencing projects (ascidian: *Ciona intestinalis*, gastropod: *Lottia gigantea*, annelid: *Capitella sp. I*, anemone: *Nematostella vectensis*, placozoan: *Trichoplax adhaerens*) as well as the choanoflagellate *Monosiga brevicollis .* For these organisms we used the human *HSD17B4* as the query sequence for BLASTp searches. Top hits for each organism were used to search for more completely annotated gene models and ESTs. We aligned and completed phylogenetic analyses as described above using a WAG+G model of protein evolution. Because these homologous proteins share a relatively high degree of sequence similarity, we used larger section of the alignment (approximately 530 residues).

3.2 Intron/exon structures

We annotated the *Hsd17b4* locus for the 12 species listed in the above section. Transcripts were determined from BLAST matches and modified to include 5' and 3' untranslated regions with EST sequences where available. Intron-exon structure was determined by aligning the transcripts to the assembled genomic sequence for each species. Gene structure was illustrated with GenePalette v1.21 (Rebeiz and Posakony, 2004).

3.3 Quantitative PCR

Oligonucleotide primers were designed to amplify *Hsd17b4* from *N. vectensis* (forward 5'-TGGAGATTACAACCCCTTGC-3', reverse 5'-TGCAAGATCGGTTTGGATAAG -3'), as well as 18S ribosomal RNA (18S, forward 5'-GACTCAACACGGGGAAACTC-3', reverse 5'-GCACCACCACCCATAGAATC-3'). The amplicon for each gene spanned an intron, and primers produced predicted amplicons of 72 bp for *Hsd17b4* and 87 bp for 18S. Serially-diluted plasmids containing the amplicon of interest were used in qPCR reactions to quantify PCR efficiency and to calculate the number of molecules per reaction (as in Tarrant et al., 2006). qPCR was perfomed using iQ SYBR Green Supermix in a MyCycler Real-Time PCR detection system (Bio-Rad). For each gene, standards were run in triplicate wells and experimental samples were run in duplicate wells (technical replicates) on a single plate. The PCR mixture consisted of 11.5 μl of molecular biology grade distilled water, 12.5 μl of IQ SYBR Green Supermix, 0.5 μl of 10 μM gene-specific primers, and 0.5 μl of cDNA. PCR conditions were: 95°C for 3 min; 40 cycles of 95°C for 15 s and 64°C for 45 s. The number of molecules per μl for each gene of interest was calculated by comparing the threshold cycle (C_t) from the sample with the standard curve.

To measure developmental gene expression in *N. vectensis,* 3-5 groups of animals were collected from five broad developmental stages: embryos (0.5-2 days post fertilization, dpf), early larvae (3-4 dpf), late larvae (7-11 dpf, not metamorphosed), newly metamorphosed juveniles (8-23 dpf), and adults (>100 dpf). From each sample, total RNA was extracted using RNA Stat-60 (Tel-Test) and the Aurum total RNA mini-kit (Bio-Rad), as we have done previously (Tarrant et al., 2008). Total RNA (1 μg per 20 μl reaction) was used in cDNA synthesis reactions with the Iscript kit (Bio-Rad), according to the manufacturer's instructions.

4. Results

4.1 Diversity of N. vectensis SDRs

Phylogenetic analysis of human and *N. vectensis* SDRs indicated a number of orthologs shared between the two species (Figure 1). Overall, the human SDRs had a similar topology as reported in the recent analysis by Belyaeva and Kedishvili (2006). Our analysis strongly supported single *N. vectensis* orthologs of *HSD17B4* (Nv217427) and *HSD17B8* (Nv241360). *N. vectensis* does not appear to have any homologs to the RODH-like SDRs.

Some *N. vectensis* proteins clustered strongly with two or more proteins from human suggesting potential ancestral genes that later diversified somewhere in the lineage leading to humans. Nv97735 grouped with *HSD17B1* and prRDH. Four additional *N. vectensis* genes had strong support with this grouping, which would be consistent with an independent radiation in *N. vectensis.* Nv213829 clustered with *HSD11B2* and *HSD17B2* with intermediate support.

In other cases, multiple *N. vectensis* SDRs corresponded to a single human SDR. In four cases that we observed, two or more closely related *N. vectensis* genes were present on the same scaffold (Figure 2, Clusters A – D). The transcriptional orientation is in the same direction for all but one *N. vectensis* SDR (i.e., Nv123699, Cluster A). The largest cluster we observed was for seven *N. vectensis* SDRs spanning 40 kb on JGI scaffold 202. Nv104066 and Nv166265 are supported as duplicated homologs of *HSD17B12* and are on the same assembled scaffold (Cluster C). Similarly, Nv163220 and 163219 grouped with *HSD17B10* and are also located in the same section of the genome (Cluster D). Some *N. vectensis* genes grouped with high support to groups of human SDRs. For example, Nv81839, 99227 and 130181 clustered with human *HSD11B1* and SDR7 (also called retSDR4) but the evolutionary relationships are less certain.

4.2 Hsd17b4: Evolutionary patterns, gene structure and N. vectensis developmental expression

Phylogenetic analysis of *Hsd17b4* in diverse fungi, animals, and the choanoflagellate supported a complex evolutionary history for this gene. Our selected genes are all *HSD17B4* homologs, indicated by a strongly supported *Hsd17b4* clade compared with outgroup sequences (*HSD17B8* and *HSD17B10*, data not shown). Our analysis provided strong support for the monophyly of a fungal clade and a clade of animal and choanoflagellate genes (Figure 3). The relationships within the animal and choanoflagellate clade were variable with relatively weak support for many nodes and were not congruent with the evolutionary history of these species. *N. vectensis* grouped with moderate support with an assemblage of animals including human. To ensure that these phylogenetic relationships were not a result of our trimmed alignment, we ran additional phylogenetic analyses with different portions of the protein. We observed the same general pattern with separate clustering of the fungi and the animal plus choanoflagellate but with variable topology within the animal and choanoflagellate clade.

We annotated the gene structure for *Hsd17b4* for 12 species (Figure 4). The human gene contains 24 exons and spans more than 80 kb of genomic sequence. Previously reported intron-exon structures for two animals (*D. melanogaster* and *C. elegans*) and three fungi suggested a considerably more compact genes with fewer exons (Breitling et al., 2001). We initially annotated the *N. vectensis Hsd17b4* based on overlapping EST sequences and found that the anemone's gene structure was similar to the

invertebrates and fungi with five exons and a genomic footprint of less than 4 kb. Perhaps surprisingly, when we annotated the gene structure for other invertebrates and for the choanoflagellate *M. brevicollis*, we found that these taxa all have large exon numbers, similar to human, but with more compressed genomic size due to smaller introns.

Hsd17b4 expression increased during *N. vectensis* development (Figure 5) with the highest expression in adults. 18S ribosomal RNA expression did not significantly vary among developmental stages.

5. Discussion

In previous studies, incubations of tritiated substrates with cnidarian homogenates have provided convincing evidence for the presence of diverse enzymes with the capability of metabolizing steroids, particularly enzymes with 17β-HSD activity (Gassman, 1992; Slattery et al., 1997; Tarrant et al., 2003). Of note, the endogenous functions of and substrates for these enzymes are unknown and the corresponding genes have not yet been identified.

With its sequenced genome and ease of culture, the anemone *N. vectensis* provides an extremely valuable model for investigating the evolution of steroid metabolism and for developing testable hypotheses regarding gene evolution and function. Our analysis has revealed that *N. vectensis* contains a rich complement of SDRs, including homologs of genes that metabolize steroids and retinoids in other animals. Sequence similarity does not always correspond to functional similarity. For example, 17β-HSD activity has been shown to evolve several times within the SDR superfamily as well as in the AKR superfamily (Baker, 2001; Penning, 1997). With this caveat in mind, it is illustrative to examine the evolutionary relationships between vertebrate and cnidarian SDRs.

Within the *N. vectensis* genome, we identified single orthologs of *HSD17B4* and *HSD17B8*. *Hsd17B4* genes have previously been identified in fungi, choanoflagellates, protostomes and deuterostomes (Breitling et al., 2001). *Hsd17B8* homologs are present in both protostomes and deuterostomes, and the sequences are surprisingly well-conserved between humans and *C. elegans* (Baker, 2001). Both 17β-HSD4 and 8 are predominantly oxidative enzymes that can convert estradiol to estrone in the presence of NAD^+ (de Launoit and Adamski, 1999; Luu-The, 2001). Structural, functional, and evolutionary analyses of the *Hsd17b4* clade have indicated that the primary substrates for 17β-HSD 4 enzymes are long- and branched-chain fatty acids, and 17β-HSD 4s most likely play only a minor physiological role in steroid metabolism (reviewed by Breitling et al., 2001). 17β-HSD 8 has been hypothesized to play a similar role in fatty acid metabolism, although additional functional studies are needed (Baker, 2001; Moeller and Adamski, 2006). Many cnidarian taxa are rich in dietary lipids, including sterols, wax esters and free fatty acids (Fukuda and Naganuma, 2001; Hamoutene et al., 2008). Shallow reef-building corals often have extremely high lipid concentrations, although a substantial proportion of these lipids are derived from their dinoflagellate symbionts (Grottoli et al., 2004; Harland et al., 1993; Stimson, 1987). It is likely that fatty acids and/or other dietary lipids are substrates in *N. vectensis* for *Hsd17b4* and possibly *Hsd17b8.* In this context, it is interesting that *Hsd17b4* was most highly expressed in adults, indicating a possible ontogenetic difference in lipid metabolism. In culture, we have observed that medium or large polyps (>5 mm length) feed more easily on brine shrimp and minced mussels and thus grow more rapidly once they cross a size threshold. Thus, high adult expression of *Hsd17b4* would be consistent with greater metabolism of stored lipids to fuel growth and/or reproduction.

We identified *N. vectensis* genes related to other multifunctional *HSD17B* genes known to metabolize both steroidal and non-steroidal substrates (*HSD17B10* and *HSD17B12*). Three *N. vectensis* genes clustered with *HSD17B10*, including two genes on the same scaffold (Cluster D); this pattern is consistent with diversification in the *N. vectensis* lineage. 17β-HSD 10 can oxidize steroids (at several positions, including the 17β-OH dehydrogenation of estradiol and 3α-OH dehydrogenation of androgens), bile acids and fatty acids (Shafquat et al., 2003; Yang et al., 2005). In mammals, 17β-HSD 10 also binds β-amyloid proteins and may play a role in neurodegenerative diseases, such as Alzheimer's disease. The neural role of β-amyloid proteins is phylogenetically conserved (Cao et al., 2008; Niwa et al., 2008), although it is not known whether the interactions between 17β-HSD 10 and β-amyloid proteins are similarly conserved. Lethal phenotypes associated with *scully* (*D. melanogaster* gene homologous to *HSD17B10*) mutants indicate an essential role of 17β-HSD 10 in β-oxidation of lipids in gonadal and neural tissues (Torroja et al., 1998). Given the generally high lipid content of cnidarian tissue and their relatively sophisticated nervous system (reviewed by Grimmelikhuijzen and Westfall, 1995; Kass-Simon and Pierobon, 2007; Tarrant, 2005), it would be of great interest to determine the roles of 17β-HSD 10 enzymes in cnidarian lipid metabolism and neural function. Two *N. vectensis* genes clustered with *HSD17B12* and formed a sister group to *HSD17B3*. 17β-HSD 12 and 3 are both reductive enzymes previously recognized to share a common ancestor (Mindnich et al., 2004). 17β-HSD 12 is a reductive enzyme that catalyzes fatty acid elongation and can transform estrone to estradiol in cell-based assays (Luu-The et al., 2006; Moon and Horton, 2003). 17β-HSD 3 catalyzes the reduction of androstenedione to testosterone (Geissler et al., 1994). It is likely that the related *N. vectensis* enzymes will facilitate fatty acid synthesis, but their catalytic activity with fatty acids, steroids and other substrates remains untested.

We did not detect clear orthologs of either *HSD17B1* or *HSD17B2*, important regulators of sex steroid activity in vertebrate tissues. *HSD17B1* is primarily expressed in female reproductive tissues and catalyzes the reductive activation of estrogens (reviewed by Mindnich et al., 2004; Moeller and Adamski, 2006). As previously reported (Baker, 2001 and references therein) and as shown by our analysis, *HSD17B1* is closely related to prRDH (photoreceptor-specific retinol dehydrogenase, also called RDH8), a reductive enzyme mediating the recycling of retinoids within the outer rods (Maeda et al., 2005). The steroidal activity of *HSD17B1* may be derived from a retinoid-metabolizing ancestor, although additional phylogenetic analysis, structural modeling, and functional studies are needed to test this hypothesis (Baker, 2001; Mindnich and Adamski, 2007). Thus, the related *N. vectensis* enzymes are likely to reduce retinoid substrates, but may also have affinity for steroids. The relationship between enzymes metabolizing retinoids and estrogens is of considerable interest because reproduction in many animals, including *N. vectensis* and other cnidarians may be stimulated or synchronized through light cues (Fritzenwanker and Technau, 2002; Hand and Uhlinger, 1992; Jokiel et al., 1985). *HSD17B2* is broadly expressed in mammalian tissues and primarily catalyzes the oxidative inactivation of androgens (reviewed by Mindnich et al., 2004; Moeller and Adamski, 2006). *HSD17B2* is closely related to *HSD11B2*, and Baker (2001) hypothesized that these genes evolved from an ancestral retinoid dehydrogenase. In *N. vectensis,* we identified one gene that clustered with *HSD17B2* and *HSD11B2* with moderate support; further analysis of this gene may provide insight into the common ancestor of *HSD17B2* and *HSD11B2*.

N. vectensis also lacks homologs for several other steroid-metabolizing enzymes. More detailed phylogenetic comparisons with multiple taxa will need to be conducted in each case to determine whether the absence is most likely to represent gene loss in the lineage leading to *N. vectensis* or

diversification in the lineage leading to humans. For example, we did not detect any clear homologs to *HSD17B7*, consistent with an observation by Baker (2001) that this gene appeared to be restricted to deuterostomes. *HSD17B7* clustered with good support with a group including several *N. vectensis* genes and human retinol dehydrogenases. These phylogenetic relationships were complex, consistent with divergence and diversification in the lineages leading to humans and/or *N. vectensis*. N*. vectensis* also does not appear to have any homologs to the RODH-like SDRs, which was expected because these genes appear to be a vertebrate-specific radiation (Belyaeva and Kedishvili, 2006)*.* Some *N. vectensis* genes (e.g., Cluster A and related genes) are highly divergent compared with the SDR family members included in this analysis. This may represent diversification within the cnidarian lineage.

In addition to functional assays, more information can still be gained through detailed bioinformatic and phylogenetic analysis of the SDR superfamily; we have built upon work by Breitling et al. (2001) and examined *Hsd17b4* evolution and gene structure in greater detail. Our phylogenetic analysis supported a monophyletic group of animal and choanoflagellate *Hsd17b4*s, but surprisingly indicated a sister relationship of the fly and choanoflagellate *Hsd17b4* genes and an outgroup position of the ascidian *Hsd17b4.* This result indicates that *Hsd17b4* has undergone a complex pattern of divergence and diversification in several lineages. Several of the nodes within the tree were relatively poorly supported, and functional studies are needed to validate and interpret the physiological significance of these groupings. More detailed analyses of individual motifs within the gene are likely to yield additional insight into these patterns (Breitling et al., 2001; Kallberg et al., 2002; Persson et al., 2003). Previous analysis had suggested that the large number of exons in the human *HSD17B4* was derived from simpler ancestral genes, as indicated by the relatively small number of exons in *D. melanogaster* and *C. elegans.* While *N. vectensis HSD17b4* has a small number of exons, other protostome (annelid and snail), placozoan and choanoflagellate genes possess a large number of exons, similar to *HSD17B4.* These results suggest that at the base of the animal tree, *Hsd17b4* was already a complex gene with a large number of exons and that this number has been reduced independently somewhere in the lineage leading to *N. vectensis* and in the lineage leading to the two ecdysozoans *D. melanogaster* and *C. elegans* (Breitling et al., 2001)*.*

In a broader survey of *N. vectensis* intron number and placement, *N. vectensis* has been shown to share a majority of intron positions with *H. sapiens* (69%), which is 3-4 times more conserved positions than other model animals (*C. elegans, D. melanogaster*). Therefore, Sullivan et al. (2006) argued that the ancestor to cnidarians and bilaterians had a relatively intron-rich genome and that animal genome structure has been conserved over long periods of evolution. Our finding of a low number of introns in *N. vectensis Hsd17b4* is surprising and not consistent with the overall observed conservation of gene structure. The annotation of *M. brevicollis* and *T. adhaerens Hsd17b4* showing a large number of introns, consistent with the gene structure in human, snail, and annelid, is strong support of the idea that the low intron number in *N. vectensis* is a secondary reduction from a more complex gene.

Cnidarians are a morphologically, physiologically and ecologically diverse group that will almost certainly vary considerably in their complement of steroid-metabolizing enzymes. It is unclear which, if any, of the *N. vectensis* SDRs we have identified is a homolog of the gene responsible for the 17β-HSD activity measured in coral homogenates. Indeed, we have not yet characterized the ability of *N. vectensis* homogenates to metabolize steroid substrates. With an unusual affinity for NADP$^+$ in an oxidative enzyme, the coral enzyme may be highly divergent. A detailed analysis of *N. vectensis* SDR motifs and predicted structures may provide additional insight into their likely cofactor preferences (Persson et al., 2003).

Functional studies are clearly needed to characterize enzymatic activities of *N. vectensis* SDRs and other invertebrate enzymes. These studies are facilitated by the recent availability of the *N. vectensis* genome but complicated by the limitations of available expression systems. Immortalized cnidarian cell lines have not yet been developed for characterizing *N. vectensis* gene products. While it is common to characterize enzyme function in heterologous systems, such as mammalian cell lines, these approaches are prone to artifacts due to differences in conditions such as incubation temperature and the availability of cofactors and chaperone proteins. Techniques have recently been adapted to manipulate (knock down or overexpress) gene expression in *N. vectensis* (Magie et al., 2007; Rentzsch et al., 2008). Targeted manipulation of gene expression provides a means to characterize *N. vectensis* enzymes in the native environment. In the coming years, bioinformatic and functional studies will provide insight into the evolution of steroid metabolism and the physiological roles of cnidarian steroids and steroid metabolizing enzymes.

Acknowledgements

Figure Legends

Figure 1. Maximum likelihood analysis of *N. vectensis* and selected Human SDRs. Gene selection for human SDRs is after Belyaeva and Kedishvili (2006). *N. vectensis* genes with sequence confirmation (via The Joint Genome Institute's EST database) are indicated with an asterisk (*). We have designated four clusters (A – D) where *N. vectensis* genes were identified on the same genomic scaffold based on the *N. vectensis* genome assembly. Bootstrap values are percentage of 1000 replicates. Bootstrap values on terminal nodes in Cluster A were omitted due to space constraints but were generally greater than 70, indicating good support. In the rest of the tree, bootstrap values below 40 were removed.

Figure 2. Genomic location of clustered *N. vectensis* SDRs. *N. vectensis* genes were annotated on scaffolds from the *N. vectensis* genome assembly, as described in the text. Arrows indicate transcriptional direction. For all but one gene (Nv123699), transcriptional direction is the same for genes on the same scaffold. Scale bars are at right for each portion of the scaffold.

Figure 3. Maximum likelihood analysis of *Hsd17b4* orthologs from select animal and fungal species as well as the choanoflagellate *M. brevicollis*. *Hsd17b4* sequences are from public database searches (see Table 2). The animal and choanoflagellate sequences group together to the exclusion of the fungal genes. Within the animal and choanoflagellate clade, the relationships among the sequences do not match the evolutionary relationships among the taxa. Bootstrap values are percentage of 1000 replicates.

Figure 4. Gene structure of *Hsd17b4* genes from selected animal and fungal species as well as the choanoflagellate *M. brevicollis*. White boxes indicate exons with coding sequence, gray boxes are exons or portions of exons containing the untranslated regions, and lines between boxes indicate introns. The evolutionary relationships of the taxa are indicated as a cladogram at left. The *Hsd17b4* gene structure is extremely variable among species. The size of the coding region is fairly well conserved (Table 2), but exon number and intron size varies considerably. Inter-exon distances in the Human *HSD17B4* are considerably larger than in any other taxa studied. *N. vectensis* has a greatly reduced number of exons which is supported as a secondary loss due to the large exon number in *T. adhaerens* and *M. brevicollis*. Similarly *D. melanogaster* and *C. elegans* have also independently reduced exon number. *D. melanogaster* and *C. elegans* have reduced the number of introns genome-wide but this reduction may also be related to the dissociation of this locus in these two species (Breitling et al., 2001).

Figure 5. Developmental expression of *N. vectensis Hsd17b4* and 18S ribosomal RNA as measured by qPCR (see text for additional details). Expression in molecules per μl cDNA indicated on the y-axis. Bars indicate the mean ± standard error of 3-5 biological replicates from the following developmental stages: embryos (0.5-2 days post fertilization, dpf), early larvae (3-4 dpf), late larvae (7-11 dpf, not metamorphosed), newly metamorphosed juveniles (8-23 dpf), and adults (>100 dpf). Expression patterns were analyzed using a one-way ANOVA followed by pairwise comparisons with Tukey's Honestly Significant Difference Test using SYSTAT 12. Letters indicate significant differences between groups at p=0.05.

Table 1. Human SDRs used in phylogenetic analysis. Sequences largely from Belyaeva and Kedishvili (2006) with some additions.

Name	Accession #
RDHL	*NP_954674*
SDRO	*NP_683695*
11-*cis*-RDH	*NP_002896*
RDH11	*NP_057110*
RDH12	*NP_689656*
RDH13	*NP_612421*
RDH14	*NP_065956*
RDH16	*NP_003699*
HSD11B1	*NP_861420*
HSD11B2	*NP_000187*
HSD17B1	*NP_000404*
HSD17B2	*NP_002144*
HSD17B3	*NP_000188*
HSD17B4	*NP_000405*
HSD17B6	*NP_003716*
HSD17B7	*NP_057455*
HSD17B8	*NP_055049*
HSD17B10	*NP_004484*
HSD17B11	*NP_057329*
HSD17B12	*NP_057226*
retSDR3	*NP_057330*
retSDR4	*NP_057113*
prRDH	*NP_056540*
RDH-E2	*NP_620419*
BDH	*NP_004042*

Table 2. Sequences used for analysis of *Hsd17b4* evolution in selected fungi, animals, and the choanoflagellete *Monosiga brevicollis*. Sequences were obtained from either NCBI GenBank or through genome browers at the Joint Genome Institute. Exon number was determined through comparison of transcripts with assembled genomes. Some of the animal (*T. adhaerens*, *Capitella sp. I*, *L. gigantea*) and *M. brevicollis Hsd17b4* sequences are gene predictions or only partially confirmed through expressed sequence tags (indicated as Y (P) in 'Confirmed' column), the annotations of these genes may be somewhat spurious due to insufficient data to confirm gene structure.

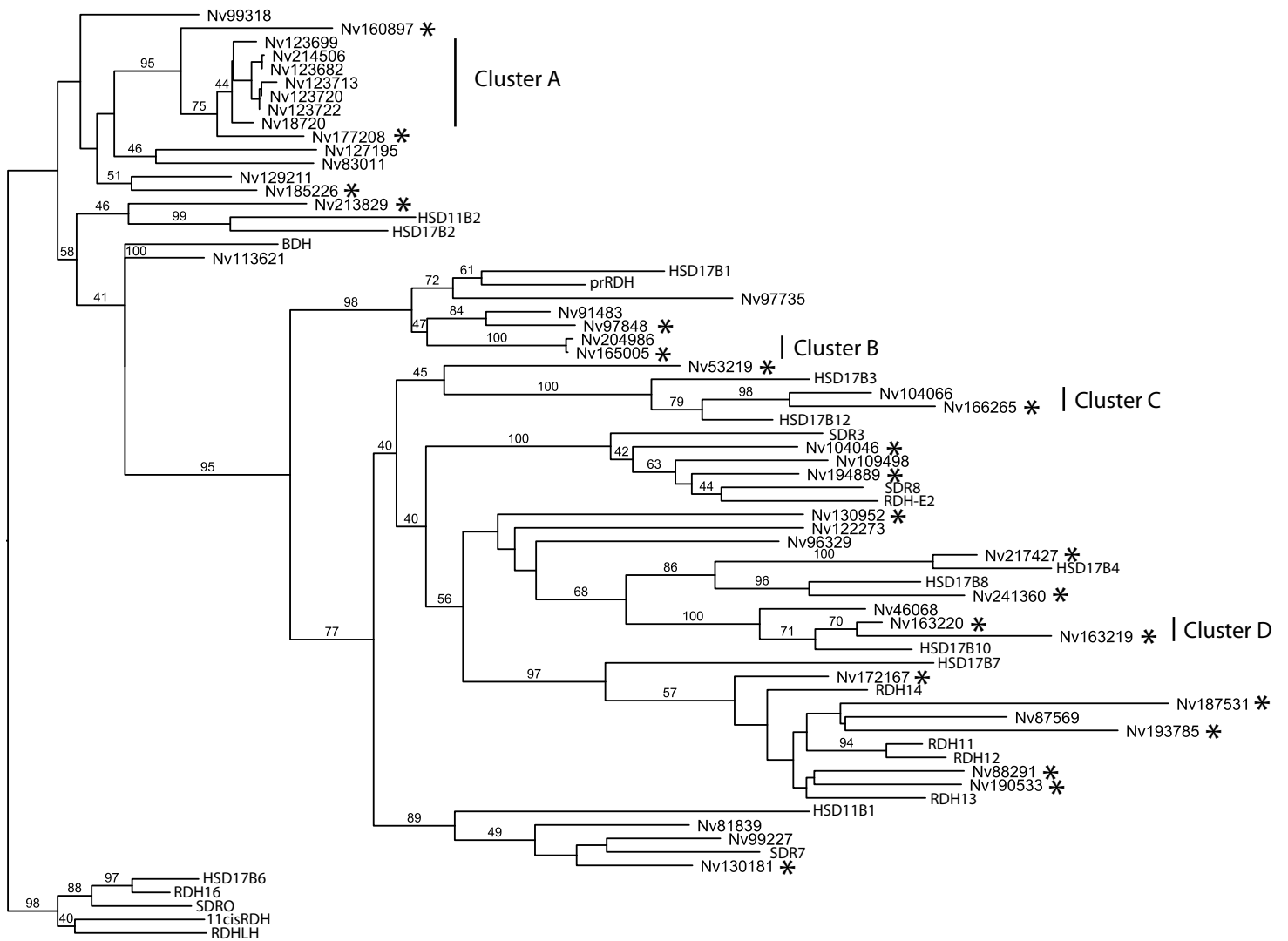| Species	Accession/JGI model	Confirmed	Exon #	Genomic/CDS Size (bp)
Candida albicans	XM_704980	Y	1	2718 / 2718
Neurospora crassa	XM_956946	Y	4	2945 / 2685
Glomus mosseae	AJ243538	Y	9	4844 / 2985
Monosiga brevicollis	fgenesh2_pg.scaffold_3000508	N	20	4462 / 2151
Trichoplax adhaerens	e_gw1.1.1286.1	N	26	7226 / 2172
Nematostella vectensis	estExt_GenewiseH_1.C_2970019	Y	5	3912 / 1869
Capitella sp. I	estExt_Genewise1.C_5110035	Y (P)	19	6719 / 2298
Lottia gigantea	fgenesh2_pg.C_sca_15000311	Y (P)	22	15141 / 2220
Drosophila melanogaster	NM_132881	Y	2	2851 / 1791
Caenorhabditis elegans	NM_076745	Y	6	1636 / 1311
Ciona intestinalis	estExt_fgenesh3_pg.C_chr_05q1023	Y	17	7613 / 2613
Homo sapiens	NM_000414	Y	24	89801 / 2208

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Candida albicans

94

Neurospora crassa

Glomus mosseae

Ciona intestinalis

Caenorhabditis elegans

20

Monosiga brevicollis

80

Drosophila melanogaster

100

Capitella sp. I

74

Lottia gigantea

61

Nematostella vectensis

33

Trichoplax adhaerens

41

32

Homo sapiens

0.1

Homo sapiens

Ciona intestinalis

Drosophila melanogaster

Caenorhabditis elegans

Capitella sp. I

Lottia gigantea

Nematostella vectensis

Trichoplax adhaerens

Monosiga brevicollis

Glomus mosseae

Candida albicans

Neurospora crassa

1000 bp
(Human: 5000bp)

Hsd17b4 bar chart. Y-axis ranges from 0.00E+00 to 2.50E+04 with gridlines at 5.00E+03, 1.00E+04, 1.50E+04, 2.00E+04, 2.50E+04. p=0.003. X-axis categories: Embryo (A), E. Larva (A), L. Larva (A), Juvenile (A,B), Adult (B).

18S bar chart. Y-axis ranges from 0 to 1.4E+10 with gridlines at 2E+09, 4E+09, 6E+09, 8E+09, 1E+10, 1.2E+10, 1.4E+10. p=0.811. X-axis categories: Embryo, E. Larva, L. Larva, Juvenile, Adult.