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Research paper

Duplication of *Dio3* genes in teleost fish and their divergent expression in skin during flatfish metamorphosis

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ABSTRACT

Deiodinase 3 (Dio3) plays an essential role during early development in vertebrates by controlling tissue thyroid hormone (TH) availability. The Atlantic halibut (*Hippoglossus hippoglossus*) possesses duplicate *dio3* genes (*dio3a* and *dio3b*). Expression analysis indicates that *dio3b* levels change in abocular skin during metamorphosis and this suggests that this enzyme is associated with the divergent development of larval skin to the juvenile phenotype. In larvae exposed to MMI, a chemical that inhibits TH production, expression of *dio3b* in ocular skin is significantly up-regulated suggesting that THs normally modulate this genes expression during this developmental event. The molecular basis for divergent *dio3a* and *dio3b* expression and responsiveness to MMI treatment is explained by the multiple conserved TREs in the proximal promoter region of teleost *dio3b* and their absence from the promoter of *dio3a*. We propose that the divergent expression of *dio3* in ocular and abocular skin during halibut metamorphosis contributes to the asymmetric pigment development in response to THs.

1. Introduction

The thyroid hormones (THs), thyroxin (T4) and 3′,5,3-triiodothyronine (T3), are pleiotropic hormones that control development, growth and metabolic homeostasis in vertebrates (Brown and Cai, 2007; Buchholz, 2015; Darras et al., 2015; Laudet, 2011; Mullur et al., 2014; Power et al., 2001; Sinha et al., 2014; Tata, 2006). These hormones are released from the thyroid follicles in vertebrates and in responsive tissue bind to nuclear TH receptors (TRs), which function as hormone-activated transcription factors and regulate gene expression (Buchholz, 2015; Sachs et al., 2000). T4 is the predominant hormone secreted by the thyrocytes and is converted to T3, the biologically active hormone, by deiodination in the peripheral tissues. A unique family of selenoproteins in chordates, the iodothyronine deiodinases (Dio), are responsible for activation and inactivation of THs (Bianco et al., 2002).

The deiodinases are thioredoxin fold-containing selenoenzymes a group of transmembrane enzymes (Dio1-3) that tightly regulate the cellular availability of THs in peripheral tissues by intracellular activation or inactivation of T4 and T3 (Kohrle, 1999). Three deiodinase isoforms with a highly conserved function have been identified in most vertebrates. Dio1 and Dio2 are activating enzymes that convert the prohormone, T4, into the active isoform, T3, by removing an iodine from their outer-ring structure (outer-ring

Abbreviations: 3′UTR, 3′ untranslated region; ANOVA, analysis of variance; *begain*, brain-enriched guanylate kinase-associated gene; D3KO, D3-deficient mice; Dio, iodothyronine deiodinases; Dio1, iodothyronine deiodinase type 1; Dio2, iodothyronine deiodinase type 2; Dio3, iodothyronine deiodinase type 3; *dlk1*, delta-Like 1 homolog (Drosophila); dpsf, days post start feeding; DPX, 2-chlorobenzaldehyde oxime; *dync1h1*, dynein cytoplasmic 1 heavy chain 1; EDTA, ethylenediamine tetraacetic acid; EST's, expressed sequenced tags; *hsp90aa1*, heat shock protein 90kDa 1 alpha (cytosolic) class A member 1; IFAPA, Instituto de Investigación y Formación Agraria y Pesquera; IRD, inner-ring deiodination; JTT, Jones-Taylor-Thornton model; KD, Knock-down; MH, myotome height; ML, Maximum Likelihood; MMI, methimazole; MS222, ethyl 3-aminobenzoate methanesulfonate salt; NCBI, National Center for Biotechnology Information; NJ, Neighbor joining; ORD, outer-ring deiodination; ORF, open reading frame; PCR, polymerase chain reaction; *ppp2r5c*, protein phosphatase 2 regulatory subunit B' gamma; PTU, 6-n-propyl-2-thyouracil; qPCR, Quantitative real-time PCR; RACE, rapid amplification of cDNA ends; *rtl*, retrotransposon-Like 1; SECIS, selenocystein insertion sequence; SB, Abocular skin side; SEM, standard error of the mean; SL, standard length; *slc25a47*, solute carrier family 25 member 47; ST, Ocular skin side; T_2, 3,3′-diiodothyronine; T_3, 3′5,3-triiodothyronine; rT_3, 3,3′,5′-triiodothyronine; T_4, thyroxin; TF, transcription factor; THs, thyroid hormones; TRs, nuclear TH receptors; TREs, thyroid response elements; TSGD, teleost specific genome duplication; *wars*, tryptophanyl-TRNA synthetase; *wdr20*, WD repeat domain 20; *wdr25*, WD repeat domain 25.

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deiodination, ORD), (Bianco and Larsen, 2005; Gereben et al., 2008; Schweizer and Steegborn, 2015). Dio3 is an inactivating enzyme that reduces TH levels by converting the prohormone T4 into rT3 (3,3′,5′-triiodothyronine) by inner-ring deiodination (IRD) and T3 into T2 (3,3′-diiodothyronine), (Bianco and Larsen, 2005; Bianco and Kim, 2006; Bianco et al., 2002; Gereben et al., 2008). An important difference between deiodinases in fish and terrestrial vertebrates is that in the former gene duplicates for *dio2* and *dio3* have been identified as a consequence of the teleost-specific whole genome duplication (Jaillon et al., 2004; Orozco et al., 2012; Ravi and Venkatesh, 2008; Volff, 2005). Furthermore, the deiodinases from fish have specific functional characteristics including; i) their resistance to inhibition by 6-n-propyl-2-thyouracil (PTU), and ii) the variable effect of dithiothreitol (DTT) on ORD of different teleost deiodinases (Klaren et al., 2005, 2012; Orozco et al., 2012, 2003; Sanders et al., 1997).

In mammals, modifications in Dio3 abundance are associated with thyroid pathophysiological conditions (reviewed by Darras et al., 2015; Dentice and Salvatore, 2011). In hypothyroidism *dio3* expression decreases whereas in hyperthyroidism it increases (Hernandez and Germain, 2002). High levels of *dio3* mRNA are associated with cardiac disorders (Pol et al., 2010; Wassen et al., 2002). During development, Dio3 plays a major role in controlling the tissue availability of T3 (Darras et al., 1992; Debaveye et al., 2005) and high levels of T3 in D3-deficient mice (D3KO) are linked to growth retardation, neonatal lethality and persistent congenital hypothyroidism (St Germain et al., 2009). Dio3 and Dio2 are essential for foetal and early neonatal brain development (Galton et al., 2014) and for brain cell maturation in mice (Friesema et al., 2012). Dio3 also plays an important role in the development of retinal and auditory function and skeletal myogenesis in mammals (Dentice et al., 2013). The conserved role of Dio3 in regulating TH availability through its role in T3 degradation, especially during early development, has only recently been confirmed in teleost fish. In zebrafish the *dio3* paralogs (*dio3a* and *dio3b*) encode two highly similar enzymatically active proteins with high affinity for THs (Guo et al., 2014). Knock-down (KD) studies of the duplicate *dio3* genes in zebrafish provide indirect evidence of the importance of T3 availability for successful embryonic development (Bohnsack and Kahana, 2013; Heijlen et al., 2014). The similar phenotypes of *dio3a* and *dio3b* KDs (perturbed swim bladder development and muscle development/function) indicates that the genes have overlapping functions, although KD of *dio3b* produces a more severe phenotype (Heijlen et al., 2014; Houbrechts et al., 2016). The role of Dio3 in other fish and during later stages of development has not been described.

Metamorphosis in fish is a late developmental event that is associated with changes in external phenotype as fish transition from the larval into the juvenile state. In flatfish (Pleuronectiformes), this process is particularly extreme as they undergo dramatic morphological reorganization as they change from a symmetric larva to an asymmetric juvenile (Power et al., 2008). As fish become asymmetric, the skin also acquires asymmetric characteristics and the upper side becomes pigmented (ocular side) and the bottom side (abocular or blind side) is devoid of pigmentation (Power et al., 2008). In several species of flatfish asymmetry in pigmentation is regulated by THs and in the olive flounder (*Paralichthys olivaceus*) (Yoo et al., 2000) and the spotted halibut (*Verasper variegatus*) (Tagawa and Aritaki, 2005), administration of T4 in early metamorphosis stops the formation of adult type melanophores on the blind side and disrupts the normal asymmetric pigment pattern. In the Senegalese sole (*Solea senegalensis*), the whole body expression of *dio2* and *dio3* is regulated during metamorphosis, and the concentration of T3 and T4 is correlated to *dio3* expression (Isorna et al., 2009).

In the Atlantic halibut (*Hippoglossus hippoglossus*), changes in TH levels and the expression of thyroid receptors (TRs) are correlated with the transition from prometamorphosis to the metamorphic climax in larvae (Galay-Burgos et al., 2008). During these stages, *dio2* and *dio3* expression is linked to TH levels; however unlike the Senegalese sole no significant differences occur in the expression of *dio3* in skin during the establishment of pigmentation asymmetry (Campinho et al., 2012a). One of the possible explanations for the conflicting results obtained for *dio3* expression during flatfish metamorphosis may be related to the recent identification in fish of duplicate *dio3* genes and their possible divergent function. To test this hypothesis, we analyzed *dio3* genes in the recently released transcriptome of the Atlantic halibut (Alves et al., 2016) and other flatfish, teleosts and terrestrial vertebrates to establish an evolutionary model for *dio3* genes. To elucidate how the same hormone can have divergent effects in the same tissue we took advantage of the divergent ocular and abocular skin phenotype of flatfish. The large size of the Atlantic halibut and their slow metamorphosis (occurring over approx. 58 days) permitted analysis of *dio3* gene duplicates for the first time in ocular and abocular skin of individuals at different stages of metamorphosis. *In silico* promoter analysis and manipulation of THs *in vivo* using methimazole (MMI) revealed that the *dio3* duplicates are differentially regulated by THs during metamorphosis. Overall the results suggest that *dio3b* gene expression in skin is repressed by THs during metamorphosis. Divergent expression levels and patterns of dio3b in ocular and abocular skin presumably modify TH availability, and context specific maturation program are triggered.

2. Materials and methods

2.1. Larvae and sampling for natural metamorphosis

Atlantic halibut larvae were supplied from a normal production cycle by the aquaculture company Fiskeldi Eyjafjarðar Ltd (Iceland). Samples were collected by a qualified member of staff from a standard commercial production cycle (Galay-Burgos et al., 2008) undergoing normal metamorphosis. The legislation and measures implemented by the commercial producer complied with Directive 98/58/EC (protection of animals kept for farming) and production and sampling by an experienced worker were optimised to avoid unnecessary pain, suffering or injury and to maximise larval survival.

Fish were euthanized using a lethal dose of MS222 (50 mg.l^{-1}, ethyl 3-aminobenzoate methanesulfonate salt, Sigma-Aldrich, USA) and individual larvae and juveniles were stored in RNA later (Life Technologies, Carlsbad, USA) at −20 °C until further analysis. Larval stages were classified by measuring the myotome height (MH) and standard length (SL), (Saele et al., 2004). Atlantic halibut samples from developmental stages: 5 (premetamorphosis); 6 and 7 (prometamorphosis); 8 (proclimax metamorphosis), 9 (climax metamorphosis) and post-metamorphic juveniles were used in the study. Larvae from stage 9 were further sub-divided into 9A, 9B and 9C to represent early, mid- and late climax stages as described in Alves et al. (2016). Pigmentation first becomes evident in stage 7 larvae, which acquire symmetrical low intensity light grey mottling. A shift to more intense and asymmetric pigmentation starts at the climax of metamorphosis (stage 9).

To characterise the tissue distribution of *dio3a* and *dio3b* the gills, liver, brain, eye, heart, duodenum, pyloric ceca, intestine, rectum, operculum, ocular skin, abocular skin, fin, cranial bone and white muscle were dissected from post-metamorphic juveniles (n = 4 per stage) and stored in RNAlater (Life Technologies). For expression studies during metamorphosis, whole-larvae from stages 5 to 9C and post-metamorphic juveniles (n = 5 per stage)

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