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Oestrogen receptor distribution related to functional thymus anatomy of the European sea bass, *Dicentrarchus labrax*

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ABSTRACT

In jawed vertebrates, the crosstalk between immune and endocrine system as well as many fundamental mechanisms of T cell development are evolutionary conserved. Oestrogens affect mammalian thymic function and plasticity, but the mechanisms of action and the oestrogen receptors involved remain unclear. To corroborate the oestrogenic regulation of thymic function in teleosts and to identify the implicated oestrogen receptor subtypes, we examined the distribution of nuclear and membrane oestrogen receptors within the thymus of the European Sea bass, *Dicentrarchus labrax*, in relation to its morpho-functional organisation. Immunohistological analysis specified thymus histology and organisation in teleosts and described, for the first time, Hassall's corpuscle like structures in the medulla of sea bass. All oestrogen receptors were expressed at the transcript and protein level, both in T cells and in stromal cells belonging to specific functional areas. These observations suggest complex regulatory actions of oestrogen on thymic function, notably through the stromal microenvironment, comprising both, genomic and non-genomic pathways that are likely to affect T cell maturation and trafficking processes. Comparison with birds, rodents and humans supports the thymic localization of oestrogen receptors and suggests that oestrogens modulate T cell maturation in all gnathostomes.

1. Introduction

The immune system (IS) forms an integrated network destined to detect and eliminate invading pathogens or transformed cells. The immune response may be mounted by two major entities, the innate and adaptive immunity, the latter being an innovation of the vertebrate lineage (Cooper and Alder, 2006). Innate and adaptive immunity operate synergistically *via* innate immune cells (*e.g.* phagocytes), which initiate a protective response and activate adaptive immune cells (*i.e.* B or T lymphocytes) for an efficient and specific immune reaction (Boehm and Swann, 2014; Esteban et al., 2015). Pathogen recognition by innate immune cells is based on germline gene-coded receptors that confer limited and non-specific capacity. Contrariwise, lymphocytes clonally express a monoallelic somatically diversified antigen receptor conferring high antigen specificity (Boehm and Swann, 2014). Basal jawed vertebrates, such as teleosts, also display other elements of higher vertebrate immunity, including (1) lymphoid organs, such as thymus, spleen and mucosa-associated lymphoid tissues (Boehm et al., 2012), and (2) fundamental steps of thymus development and thymopoiesis (Boehm et al., 2012; Bajoghli et al., 2015). As for the latter, the thymus provides the appropriate microenvironment for T cell development, comprising proliferation, maturation and the generation of their antigen receptor repertoire (Boehm et al., 2012; Nakanishi et al., 2015). In fish like in mammals, mature and self-

Abbreviations: Ab, antibody (mAb, monoclonal Ab, pAb, polyclonal Ab); CD, cluster of differentiation; Ct, connective tissue; Ck, cytokeratin; E2, 17-β-oestradiol; Esr, nuclear oestrogen receptor; Gper, G protein-coupled oestrogen receptor; HCs, Hassall's Corpuscles; HES, Hematoxylin-Eosin-Saffron; IHC, immunohistochemistry; IS, immune system; MCs, mast cells; MyCs, myoid cells; OZ, outer zone; PAS, Periodic Acid-Schiff; PBS, Phosphate-Buffered Saline; Pcna, Proliferating Cell Nuclear Antigen; PVS, perivascular space (iPVS, inner PVS, oPVS, outer PVS); SDF-1, stromal cell-derived factor; TECs, thymic epithelial cells (cTECs, cortical TECs, mTECs, medullar TECs, LTECs, limiting TECs).

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tolerant T cells leave the thymus towards the secondary lymphoid organs in order to coordinate the adaptive immune reaction (Langenau and Zon, 2005; Nakanishi et al., 2015).

In mammals, the IS is modulated by the reproductive system *via* sexual hormones, notably oestrogens, as reflected by sexual dimorphisms in the IS performance and female autoimmune disease prevalence, but also by the high oestrogen levels during pregnancy (Hince et al., 2008; Klein and Flanagan, 2016). The thymus and T cell development are particularly targeted by oestrogenic regulation, as evidenced by numerous studies over three decades of research (Bernardi et al., 2015; Glucksmann and Cherry, 1968; Screpanti et al., 1991). Indeed, thymus structure and volume vary throughout lifetime in relation to endogenous oestrogen levels, reproductive status and age (Hince et al., 2008), not only in mammals but also in reptiles and birds (Lutton and Callard, 2006). In fish, the thymus shows a considerable seasonal plasticity of its volume (Tatner, 1996). To what extent these variations of the thymus are linked to the reproductive cycle and, consequently, to changes in hormone titres is, however, less clear (Tatner, 1996). However, oestradiol exposure has recently been shown to modify thymic volume and regionalization in juvenile European sea bass, *Dicentrarchus labrax* (Seemann et al., 2015).

In mammals, the structural changes provoked by naturally elevated oestrogen levels or by experimental oestrogen exposure have been ascribed to numerous processes in thymus, including: (1) the induction of thymocyte apoptosis (Do et al., 2002; Okasha et al., 2001; Wang et al., 2008); (2) an inhibition of thymocyte proliferation (Gould et al., 2000; Zoller and Kersh, 2006; Zoller et al., 2007); (3) decreased infiltration of T cell progenitors into the thymus (Zoller and Kersh, 2006) and (4) extensive T cell leakage through the blood vessels into the periphery (Chapman et al., 2015; Martín et al., 1995). Notwithstanding uncertainties and conflicting results obtained in mammals, it is generally accepted that oestrogens (1) modulate T cell maturation by increasing the proportion of single positive CD4+/CD8+ T cells (Bernardi et al., 2015; Erlandsson et al., 2001; Screpanti et al., 1991) and (2) block T cell maturation, as suggested by the increased proportion of immature double negative CD4-/CD8-phenotypes (Bernardi et al., 2015; Rijhsinghani et al., 1996; Wang et al., 2008). The cellular players and the respective oestrogen receptors were, however, only partially identified (Erlandsson et al., 2001; Staples et al., 1999; Wang et al., 2008). Although a growing body of knowledge on oestrogenic regulation of the teleost IS supports the idea that the immunomodulatory role of E2 probably exists across all vertebrates (reviewed in Burgos-Aceves et al., 2016; Segner et al., 2017; Szwejser et al., 2017b), the interplay between stromal and T cells as well as the extent of conservation along vertebrate evolution remains to be detailed (Segner et al., 2017).

Oestrogens mediate their effects on target cells and tissues through oestrogen receptors. These include both genomic pathways (classically associated to nuclear oestrogen receptors, Esrs) and non-genomic pathways (associated to membrane localised Esrs or to recently characterized G-protein-coupled oestrogen receptors, *i.e.* Gpers). In numerous teleost species, the Esrs are represented by three isoforms: Esr1 (also known as Erα), Esr2a (Erβ1) and Esr2b (Erβ1) (Burgos-Aceves et al., 2016). More recently, two GPER isoforms, Gpera and Gperb, have been described in some teleost species (Lafont et al., 2016; Pinto et al., 2016), indicating that both pathways can interact in teleosts (Nelson and Habibi, 2013; Pinto et al., 2014). The presence of oestrogen receptors in the thymus has been demonstrated in mammals (Nancy and Berrih-Aknin, 2005; Seiki and Sakabe, 1997; Wang et al., 2008), chicks (Katayama et al., 2014; Yonezawa et al., 2008) and common carp (Szwejser et al., 2017a). It may, therefore, be hypothesized that the different receptors may have a similar distribution in the fish thymus. The morpho-functional organisation of the thymus is well documented for the European sea bass, describing the thymic microenvironment and the expression of genes related to the T cell development (Picchietti et al., 2008, 2009, 2015), with the different steps of T cell maturation taking place in specific regions of the thymus, comparable to the processes described for mammals (Bajoghli et al., 2015).

With respect to the complex and not fully understood oestrogenic regulation of thymopoiesis, in this study we aimed at investigating how and at which point oestrogens influence T cell maturation and selection in the European sea bass (Bajoghli et al., 2015; Langenau and Zon, 2005). The expression of the three nuclear Esr isoforms and the Gper genes in thymic cells was confirmed by RT-PCR. Their localization within the thymic substructures of *D. labrax* was established in conjunction with a detailed histological analysis, using oestrogen receptor specific antisera previously validated for other teleost species (Cabas et al., 2013; Pinto et al., 2009; Szwejser et al., 2017a). The presence of both membrane and nuclear ER-isoforms in most cell types of the thymic microenvironment and their strong occurrence in certain thymic zones, such as the medulla and connective tissue, point to a functionally conserved regulation of thymopoiesis by oestrogens across all vertebrate taxa.

2. Material and methods

2.1. Animals and sampling

Fingerlings of *D. labrax* were obtained from the hatchery "L'écloserie marine de Gravelines" (Gravelines, France) and raised in the facilities of "Aquacaux" (Octeville, France) in 1800 l tanks with continuous flow of marine seawater at environmental temperatures. The animals were fed daily *ad libidum* with "Turbot label rouge" fish feed (Le Gouessant, Lamballe, France). All fish were handled in accordance with the European Union regulations concerning the protection of experimental animals (Dir 2010/63/EU).

Two different fish batches were employed for this study: three-year-old female fish with a total length of 30.6 cm ± 1.42 standard deviation (s.d.) and a weight of 391 g ± 58.02 s.d. for histology and IHC and one-and-a-half-year-old male and female fish with a total length of 21.8 cm ± 2.9 s.d. and a weight of 97.8 g ± 10 s.d. for molecular biology and cytometric analysis. Specimens were sacrificed at the end of November/beginning of December 2014 for the IHC and histological analysis and in June 2015 for molecular biology and immunofluorescence.

2.2. Leukocyte preparation

All solutions for leukocyte preparation were adjusted to 360 mOsm/kg. Dissected thymuses were cut into pieces, immersed in cold Leibovitz 15 (L-15) containing 1 mM Na_2EDTA, and forced through a 100 μm cell strainer. The cell suspension was centrifuged at 1200 *g* for 5 min at 4 °C prior to erythrocyte lysis in ammonium chloride-Tris solution for 30 min at room temperature under stirring followed by another round of centrifugation at 1200 *g* for 8 min after which the supernatant was discarded. Pellets were resuspended in L-15 and filtered through a 40 μm mesh before loading on a Ficoll gradient (Pancoll, PAN BIOTECH) at a density of 1.077 g/ml and centrifugation at 400*g* for 5 min at 4 °C. The leukocyte layer, occurring at the interface of medium and Ficoll, was collected, washed and centrifuged twice with L-15 medium at 1200 *g* for 5 min and 4 °C. The cell concentration was adjusted to 10^6 cells/ml. Before flow cytometric measurements, cell viability was estimated with trypan blue exclusion and 50 μg/ml propidium iodide (Sigma) over 10 min at room temperature.