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Expression of *Ol-foxi3* and Na^+/K^+-ATPase in ionocytes during the development of euryhaline medaka (*Oryzias latipes*) embryos

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ABSTRACT

Osmoregulation is a vital function that is essential to all vertebrates. Ionocytes are epithelial cells responsible for this function and have been extensively studied in adult teleost fish gills. The euryhaline medaka (*Oryzias latipes*) has recently emerged as an investigative model because of its ability to acclimatize easily to water presenting various salinities. However, no studies to date have focused on the development of ionocytes in medaka embryos. We first analyzed the distribution of ionocytes in the skin and gills during development, using a specific marker of differentiated ionocytes (the Na^+/K^+-ATPase pump, or NKA). Strikingly, we were able to identify two ionocyte domains on the yolk surface ectoderm, that we named the Vitellin Zone (VZ) and the Lateral Zone (LZ). In zebrafish, ionocyte differentiation has been shown to be controlled by two forkhead-box genes, *foxi3a* and *foxi3b*. We cloned the medaka *foxi3* ortholog which appeared to be highly similar to *foxi3b*. Whole-mount *in situ* hybridizations performed on medaka embryos revealed that *Ol-foxi3* is expressed in differentiated ionocytes of the pharyngeal endoderm, the branchial arches and the yolk epidermis, as well as in epibranchial placode territories. We further focused on the expression patterns of the yolk epidermis and compared the expression of *Ol-foxi3* with that of the non-neural progenitor marker p63. We evidenced that *Ol-foxi3* is expressed in progenitor cells which are first of all located uniformly in the VZ and then transitorily clustered in the LZ. Taken together, these data contribute to a clearer understanding of osmoregulatory tissue ontogenesis in euryhaline fish.

1. Results and discussion

Ion transport is a vital function that plays an essential role in the osmoregulation of body fluids in all vertebrates. The epithelia responsible for this function are located in several organs, including the kidney collecting duct, the amphibian urinary bladder, the gut, the inner ear, the fish gills and the skin (Takagi, 1997, for a review see Brown and Breton (1996), Evans et al. (2005) and Hwang and Lee (2007)). All transporting epithelia contain a subset of specialized cells called ionocytes (formally called mitochondria-rich cells or chloride cells) (Keys and Willmer, 1932), that transport various ions across the epithelia down gradients created by the strongly expressed plasma membrane enzymatic complex Na^+/K^+-ATPase (NKA) pump (Blanco and Mercer, 1998; Hwang and Lee, 2007; Therien and Blostein, 2000). Medaka (*Oryzias latipes*) is a small fish model (for a review see Wittbrodt et al. (2002)) that displays particular adaptability to various water salinities (Inoue and Takei, 2002, 2003). The medaka can survive and develop normally when transferred from fresh water to sea water. For this reason, this euryhaline fish has recently emerged as an interesting model for osmoregulation studies (Kang et al., 2008; Wu et al., 2009). However, no studies to date have focused on the development of skin and gill epithelial cells during embryonic development in the medaka.

The yolk skin epidermis contains several types of cells, including ionocytes, and develops after gastrulation from the non-neural ectoderm. In the pharyngeal embryonic region, the ectoderm contributes to formation of the branchial apparatus, which develops as a result of coordinated interplay between the pharyngeal ectoderm, the pharyngeal endoderm, the mesoderm and the epibranchial placodes. More specifically, the pharyngeal ectoderm gives rise to the gill epithelium, whereas the pharyngeal endoderm forms the epithelium that lines the pharynx and contributes to the formation of associated

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organs. Both the gill epithelium and pharynx epithelia contain ionocytes. During vertebrate evolution, development of the pharyngeal apparatus has undergone a number of modifications. In terrestrial vertebrates, the parathyroid gland evolved as a result of the internalization of fish gills during the transition from an aquatic to a terrestrial environment (Graham et al., 2005). Nevertheless, it has been shown that the *foxi* class genes constitute a conserved feature of pharyngeal development and play a significant role in the establishment of branchial development (Graham, 2001; Solomon et al., 2003b). *foxi* class genes belong to a large and highly conserved gene family that encodes winged helix/forkhead-box transcription factors. They have been analyzed in detail in mouse and zebrafish. In mouse, three *foxi* genes have been identified and shown to display distinct and dynamic expression patterns in early craniofacial epithelia, including the otic vesicles (*foxi1*), cranial ectoderm (*foxi2*), early placodes of the surface ectoderm and the pharyngeal endoderm (*foxi3*) (Ohyama and Groves, 2004). Gene knock-out in the mouse revealed that Foxi1(−/−) mice display no abnormal development of the inner ear, but an absence of ionocyte differentiation (Hulander et al., 2003). In the zebrafish, two genes have been identified as being expressed in the epibranchial placodes and otic vesicles (*foxi1*), or in the chorda-mesoderm and pharyngeal arches at a later stage (*foxi2*). Zebrafish also present two members of the *foxi3* gene, *foxi3a* and *foxi3b*, that are exclusively expressed in yolk epidermis ionocytes. Recent studies have found that *foxi3* genes control ionocyte differentiation (Chang et al., 2009; Esaki et al., 2009; Hsiao et al., 2007; Janicke et al., 2007, for review see also Hwang and Perry (2010)).

In order to gain a clear understanding of skin and gill ionocyte development in the medaka, we first of all analyzed the distribution of ionocytes during the development of skin and gill epithelia, using NKA immunostaining. We then cloned the medaka ortholog of zebrafish *foxi3* key genes and examined its spatio-temporal expression during development, with a specific reference to ionocytes.

1.1. Na^+/K^+-ATPase-rich cell distribution during medaka embryonic development

In order to determine the stages of emergence of ionocytes in embryonic skin and gills, the time course expression of the NKA pump was analyzed by immunostaining on whole mount embryos. NKA immunoreactivity was first observed at st.20, in a few scattered cells on the yolk epidermis (Fig. 1A). As development proceeded, an increasing number of NKA-immunoreactive cells (NKA-ir cells) could be detected on the yolk epidermis (Fig. 1A–F). Interestingly, at st.30 (35-somite stage), a higher density of NKA-ir cells was detected in a salt-and-pepper pattern in the axial yolk epidermis on both sides of the trunk (Fig. 1C). This area was named the Lateral Zone (LZ), while the surface covering the whole yolk was named the Vitellin Zone (VZ). Subsequently, LZ and VZ could easily be distinguished from the difference in NKA-ir cell density (Fig. 1C–F). Furthermore, at st.25 (18-somite stage) and st.30 (35-somite stage), NKA-ir cells could also be detected in the pharyngeal endoderm and in the first gill slit (also called the lateral gill chamber, Fig. 1G and H). Subsequently, from st.35 onwards, NKA-ir cells were detected on the epithelium of branchial arches as they develop (Fig. 1I and J). At the larva stage, numerous NKA-ir cells were found to be located on the developing primary gill filaments (Fig. 1K).

1.2. Cloning and expression analysis of Ol-foxi3 during medaka embryonic development

We then focused our study on the *foxi3* transcription factor and its expression in the skin and gills during development. For this purpose, we cloned the medaka *foxi3* ortholog detected by analyzing the medaka genome. Only one sequence related to zebrafish *foxi3* genes could be identified and further sequence alignments enabled the construction of a phylogenetic tree (Fig. 2A). Full-length sequences of the zebrafish FOXI3a and FOXI3b displayed 56.5% amino acid identity. The Fugu FOXI3 (Solomon et al., 2003b) is 55.3% and 71.5% identical to the zebrafish FOXI3a and FOXI3b, respectively. Although the Ol-FOXI3 protein resembles the zebrafish FOXI3a (56.5% identity), it is clearly more closely related to the Danio FOXI3b and Fugu FOXI3 (70.8% and 81.3% identity at the amino acid level, respectively). A multiple alignment of FOXI3 proteins (Fig. 2B) showed the forkhead conserved domains and confirmed that it is the medaka *Ol-foxi3* ortholog.

The expression pattern of *Ol-foxi3* was analyzed by whole-mount *in situ* hybridization (WMISH) in medaka embryos and larvae. At st.18 (late neurula stage), *Ol-foxi3* mRNA expression was first detected in the anterior embryonic ectoderm flanking the midbrain–hindbrain boundary (MHB) region, which corresponds to the epibranchial (EB) placodes (Fig. 3A and I). This expression domain is maintained at st.20 (4-somite stage, Fig. 3B) but was no longer detected at st.25 (18-somite stage, Fig. 3C). From that stage, embryos displayed *Ol-foxi3* expression in the pharyngeal endoderm (Fig. 3C–F). Indeed, lateral views of embryos at different stages display a high level of expression in the pharyngeal endoderm that gradually diminishes until st.35, where it is eventually only expressed in scarce cells (Fig. 3J–N). Conversely, from st.30, a punctuated pattern of *Ol-foxi3* was detected in the developing pharyngeal ectoderm (*i.e.* branchial arches epithelium) that progressively extends from the rostral to the caudal extremities as the pharyngeal arches develop (Fig. 3K–N). Finally, from st.18, *Ol-foxi3* was also expressed in single cells scattered in the VZ domain of the yolk epidermis (Fig. 3A and H). This spotted and specific staining is congruent with an expression in ionocytes (see Fig. 1). Consistently with the ionocyte distribution described in Fig. 1, additional *foxi3*-positive cells were also observed at st.25 in the inner most part of the LZ (Fig. 3C). This staining gradually spreads as the embryo develops, whereas the *Ol-foxi3* expression level in the VZ slightly diminishes. At the larva stage, *Ol-foxi3* expression was observed in the ionocytes of both the gill epithelium and the yolk epidermis (Fig. 3O and H).

In order to confirm that *Ol-foxi3* is expressed in NKA-ir cells, we performed double fluorescent WMISH using *Ol-foxi3* and *atp1α1a.1* probes (Fig. 4). Indeed, the NKA complex contains a catalytic α subunit and a stabilizing β subunit (Jorgensen, 1974). In order to detect NKA at the gene level, the *atp1α1a.1* gene encoding the NKA α1 subunit was cloned. Further expression analysis on adult gills enabled to demonstrate that *atp1α1a.1* does indeed reflect NKA distribution (data not shown). At st.27–28 and at larva stage, double fluorescent WMISH confirmed a co-expression of *Ol-foxi3* and *atp1α1a.1* in the pharyngeal endoderm and the first gill slit (Fig. 4A–C), the skin ectoderm (Fig. 4D and F) and the gills (Fig. 4D and E). At st.20, no expression of *atp1α1a.1* was detected in *Ol-foxi3*-expressing cells in the EB domain (data not shown).

1.3. The skin epidermis displays two territories of ionocyte progenitors

One of the most striking features of *Ol-foxi3* and NKA expression patterns is the existence of two ionocyte domains on the yolk epidermis, the VZ and LZ. The expression of NKA in the VZ, which was apparent at st.20, is delayed when compared with the onset of *Ol-foxi3* expression (compare Fig. 1A and Fig. 3A). This delay was also observed in the LZ, where NKA-ir cells appeared at st.30 and *Ol-foxi3* expression was seen as early as st.25 (compare Fig. 1C and Fig. 3C). Starting from this observation, we analyzed the co-expression of *Ol-foxi3* and *atp1α1a.1* in the LZ in more detail, between st.25 and st.30 (Fig. 5). As expected, we observed that *Ol-foxi3* is