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Morpholino oligonucleotide knockdown of the extracellular calcium-sensing receptor impairs early skeletal development in zebrafish

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ABSTRACT

The complex vertebrate skeleton depends on regulated cell activities to lay down protein matrix and mineral components of bone. As a distinctive vertebrate characteristic, bone is a storage site for physiologically-important calcium ion. The extracellular calcium-sensing receptor (CaSR) is linked to homeostatic regulation of calcium through its expression in endocrine glands that secrete calcium homeostatic hormones, in Ca^{2+}- and ion-transporting epithelia, and in skeleton. Since CaSR is restricted in its presence to the chordate–vertebrate evolutionary lineage, we propose there to be important functional ties between CaSRs and vertebrate skeleton in the context of that group's characteristic form of calcium-mineralized skeleton. Since little is known about CaSR in the skeletal biology of non-mammalian vertebrates, reverse transcription-polymerase chain reaction (RT-PCR), in situ hybridization and immunohistochemistry were applied to adult and embryonic zebrafish to reveal CaSR transcript and protein expression in several tissues, including, among these, chondrocytes and developing bone and notochord as components in skeletal development. Morpholino oligonucleotide (MO) knockdown technique was used to probe CaSR role(s) in the zebrafish model system. By RT-PCR assessment, injection of a splice-inhibiting CaSR MO reduced normally-spliced *Casr* gene transcript expression measured at 2 days postfertilization (dpf). Corresponding to the knockdown of normally-spliced mRNA by the CaSR MO, we observed a morphant phenotype characterized by stunted growth and disorganization of the notochord and axial skeleton by 1 dpf. We conclude that, like its critically important role in normal bone development in mammals, CaSR is essential in skeletogenesis in fishes.

1. Introduction

Ionic calcium (Ca^{2+}) is an important component in many physiological systems and is crucial for organismal function and survival. Ca^{2+}-dependent cellular processes include excitation–contraction coupling in muscle, neurotransmitter release at chemical synapses, and stimulus–secretion coupling in endocrine cells. Consequently, the concentrations of Ca^{2+} that are necessary to support properly these important functions are homeostatically regulated in both intracellular and extracellular fluid compartments. The extracellular calcium-sensing receptor (CaSR) was identified in 1993 as the physiological sensor of extracellular Ca^{2+} concentration in bovine parathyroid gland cells (Brown et al., 1993), providing a first look at this receptor-based (rather than ion channel-based) mechanism of ion detection in the extracellular fluid of animals. Subsequent studies have dramatically expanded our understanding about the structure and functions of these receptor proteins, as well as their phylogenetic restriction to the vertebrate–chordate lineage (cf. Loretz, 2008; Herberger and Loretz, 2013a).

The CaSR, as a critical regulator in vertebrate Ca^{2+} homeostasis, is expressed in many tissue types. In addition to receptor expression in endocrine cells that secrete calcium-homeostatic hormones, CaSR is expressed in kidney and intestine where it may directly influence Ca^{2+} absorption and/or secretion, and in bone where it may affect Ca^{2+} storage and mobilization (Brown et al., 1993; Brown and MacLeod, 2001; Chang and Shoback, 2004; Loretz, 2008; Loretz et al., 2009; Brown, 2010; Loretz et al., 2012). In aquatic vertebrates, the calcium homeostatic process involves some ion-transporting tissues not represented in other vertebrate classes. CaSR is expressed in ionocytes (the so-called mitochondria-rich "chloride cells") of the gill that are active in ion uptake and extrusion (Loretz et al., 2004; Hwang, 2009; Choi et al., 2011). Functional coupling in ionocytes of CaSR to transepithelial ion transport can be envisioned as a particularly relevant role for the receptor. For euryhaline fishes that naturally encounter changes in environmental salinity (and consequently in plasma Na^+, Cl^- and Ca^{2+} concentrations), CaSR expression in olfactory systems in fishes may add a useful sensory capability for detection and

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response to changes in environmental Ca^{2+} concentration (Hubbard et al., 2000, 2002; Loretz et al., 2009).

With the evolutionary emergence of the vertebrate-type mineralized skeleton, bone became the major calcium store in this taxonomic group. Our core hypothesis is that CaSRs, which are restricted in distribution and expression to vertebrate animals (and perhaps to some evolutionarily-related non-vertebrate chordates, such as tunicates and amphioxus, as a CaSR-like protein of unconfirmed function), evolved contemporaneously with mineralized bone in the vertebrate lineage. Supporting this notion of CaSR linkage to vertebrate skeletal evolution, CaSR plays a vital role in mammalian skeletal development. A tissue-specific knock-out in mouse bone resulted in severe skeletal developmental defects and stunted growth accompanied by a decrease in bone mineralization and density (Chang et al., 2008). Although CaSR is expressed in skeletal tissues of fishes (Loretz et al., 2012), there is currently no information on the role of CaSR in skeletal (cartilage and bone) development in non-mammalian vertebrates. Therefore, we investigated the location and functional role of CaSR gene (*Casr*) expression in skeletal tissues in the zebrafish model system to better understand the developmental and evolutionary biology of this receptor, and to provide crucial insight into the possibly primitive and important role(s) of CaSR in skeletal development. The zebrafish is a powerful model system for developmental and other studies owing to its fast growth and development time, large number of offspring produced from each mating, accessibility by way of oviparity of fertilized eggs and embryos for observation and experimental manipulation, and availability of genome database resources (Spoorendonk et al., 2010). In this report, we present observations on *Casr* gene and CaSR protein expression in the zebrafish skeletal system, and experimental evidence from morpholino oligonucleotide (MO) knockdown for the critical role that CaSR plays in skeletal development during early embryogenesis. Findings are interpreted in the context of mineralized bone skeleton as a key feature in early vertebrates.

Some of these results have been presented in abstract form (Herberger and Loretz, 2013b).

2. Materials and methods

2.1. Zebrafish maintenance and care

Zebrafish (*Danio rerio*) adults and embryos (wild type TU strain) were purchased from the Zebrafish International Resource Center (ZIRC; Eugene, OR, U.S.A.). Adult zebrafish were maintained and handled using standard protocols (Westerfield, 2007; Harper and Lawrence, 2011). In brief, groups of 15 to 20 adult fish were kept in 20-to-40-L tanks in a secure environmental chamber at 28 °C with a 14 h/10 h light/dark cycle. Adult fish were fed a complete granular diet (Adult Zebrafish Complete Diet, Zeigler Bros., Inc., Gardners, PA, USA) 2–3 times per day. Fertilized eggs from paired or group matings were collected shortly after spawning (during the first 30–60 min of the light phase), and embryos were maintained in suitable growth media in petri dishes until they reached the proper stages for experimental manipulation and/or observation, with ages reported as either hours (hpf) or days post-fertilization (dpf), with confirmation of developmental stage according to Kimmel et al. (1995). In the case of the MO microinjection experiments (below), embryos were held briefly (<1 h) at refrigerated temperatures (4 °C) to retard cell division pending microinjection treatment; they were promptly then returned to 28 °C. After hatching from the chorion, and beginning at about 5 dpf, larvae were fed microparticle diet (Larval Food Supplement; Zeigler Bros., Inc.). Zebrafish were euthanized by using ethyl-3-aminobenzoate methane sulfonic acid salt (tricane methanesulfonate, MS-222; Sigma-Aldrich Co., St. Louis, MO, USA; 0.03% wt/vol, with $NaHCO_3$ buffering). All standard animal maintenance procedures and experimental protocols were reviewed and approved by the University at Buffalo Institutional Animal Care and Use Committee (IACUC approval # BIO29119N).

2.2. Whole-animal, embryo and tissue specimen processing for microscopic analysis

Generally, we followed here our published techniques (Loretz et al., 2009, 2012) for fixation and paraffin embedding of both whole embryos and isolated tissues. Briefly, euthanized specimens were fixed in 4% paraformaldehyde in 0.01 M phosphate-buffered saline (PBS; 137 mmol/L NaCl, 2.7 mmol/L KCl, 8.1 mmol/L Na_2HPO_4, 1.8 mmol/L KH_2PO_4, pH 7.4) at 4 °C for 2 overnights (o/n). Embryos and fry were subsequently processed whole, but adult specimens were cut into smaller sections before further processing. After repeated washing in 70% ethanol (EtOH) to remove fixative, specimens were dehydrated in graded ethanols and benzene and were embedded in paraffin (Paraplast, McCormick Scientific, St. Louis, MO, USA) following infiltration in melted paraffin under vacuum for several hours. Specimen-containing paraffin blocks were stored in the cold until subsequent processing separately for either in situ hybridization or immunohistochemistry.

Histological sectioning of 4-dpf to 1-month-old zebrafish produced higher quality paraffin sections, relative to sections from zebrafish of ages 3 months through adulthood. The latter have larger, dense and hard mineralized bone that typically resulted in sections that were substantially less suitable for processing due to tearing. In our CaSR immunohistochemical trials on tilapia (Loretz et al., 2012) and Japanese eel (*Anguilla japonica*; unpublished data) bone-containing specimens, decalcification in EDTA solutions, in attempts to improve sectioning of mineralized hard tissues, in fact abolished CaSR immunoreactivity. Therefore, immunohistochemical and in situ hybridization, analyses were restricted to early developing embryos or juvenile specimens.

2.3. RNA isolation

Total RNA was isolated from zebrafish adults and embryos using standard phenol/guanidine isothiocyanate extraction technique. In the case of adults, tissues of interest were isolated from freshly euthanized fish and homogenized in TRIzol reagent (Life Technologies, Grand Island, NY, USA). After chloroform addition and centrifugation (12 000 *g* at 4 °C for 15 min), the aqueous layer was removed. An equal volume of isopropyl alcohol was added to the aqueous solution, which was then centrifuged (12 000 *g* at 4 °C for 15 min) to precipitate the RNA. The RNA pellet was washed with 70% EtOH, centrifuged again, dried and finally resuspended in ultrapure water. In the case of embryos and larvae, whole euthanized specimens were pooled as 10–50 individuals for processing. Instead of homogenization, embryos or larvae were triturated in TRIzol by repeated passage through a sterile 26-gauge hypodermic syringe needle until thoroughly disrupted. Subsequent processing was as described above for adult specimens.

Total RNA samples were treated with DNase I (RQ1 RNase-free DNase; Promega Corp., Madison, WI, USA) to remove genomic DNA. RNA sample quantity (as ng/μL) and quality (as OD_{260}/OD_{280}) were assessed by microspectrophotometry (NanoDrop Model 2000c UV–Vis Spectrophotometer; Thermo Fisher Scientific NanoDrop, Wilmington, DE, USA) after final reconstitution of purified RNA.

2.4. Assessment of Casr gene expression

Casr gene expression during embryogenesis was evaluated by reverse transcription-polymerase chain reaction (RT-PCR) analysis of RNA extracted from embryos at ages 6 hpf and 1, 2 and 3 dpf. For the analysis, the Access RT-PCR System kit (Promega Corp., Madison, WI, USA) was used for core reagents and enzymes, together with specific oligonucleotide primers that were designed to recognize