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Expression of zebrafish anterior gradient 2 in the semicircular canals and supporting cells of otic vesicle sensory patches is regulated by Sox10

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ABSTRACT

AGR2 is a member of the protein disulfide isomerase (PDI) family, which is implicated in cancer cell growth and metastasis, asthma, and inflammatory bowel disease. Despite the contributions of this protein to several biological processes, the regulatory mechanisms controlling expression of the *AGR2* gene in different organs remain unclear. Zebrafish *anterior gradient 2* (*agr2*) is expressed in several organs, including the otic vesicles that contain mucus-secreting cells. To elucidate the regulatory mechanisms controlling *agr2* expression in otic vesicles, we generated a *Tg(−6.0 k agr2:EGFP)* transgenic fish line that expressed EGFP in a pattern recapitulating that of *agr2*. Double immunofluorescence studies were used to demonstrate that Agr2 and GFP colocalize in the semicircular canals and supporting cells of all sensory patches in the otic vesicles of *Tg(−6.0 k agr2:EGFP)* embryos. Transient/stable transgenic analyses coupled with 5′-end deletion revealed that a 100 bp sequence within the −2.6 to −2.5 kbp region upstream of *agr2* directs EGFP expression specifically in the otic vesicles. Two HMG-binding motifs were detected in this region. Mutation of these motifs prevented EGFP expression. Furthermore, EGFP expression in the otic vesicles was prevented by knockdown of the *sox10* gene. This corresponded with decreased *agr2* expression in the otic vesicles of *sox10* morphants during different developmental stages. Electrophoretic mobility shift assays were used to show that Sox10 binds to HMG-binding motifs located within the −2.6 to −2.5 kbp region upstream of *agr2*. These results demonstrate that *agr2* expression in the otic vesicles of zebrafish embryos is regulated by Sox10.

1. Introduction

The *anterior gradient 2* gene was initially identified as encoding a protein (XAG) secreted from the *Xenopus* cement gland [1]. XAG-2 was subsequently shown to be involved in the specification of dorsoanterior ectodermal fate in *Xenopus* [2]. Unlike their orthologous protein in *Xenopus*, both human AGR2 and mouse Agr2 are primarily expressed in various endoderm-derived organs [3,4]. In addition, high expression of AGR2 has been identified in several human cancer cells, and AGR2 is considered to be a pro-oncogenic factor [5]. The role of AGR2 as a survival factor is attributed to its ability to prevent p53 transactivation in cells upon ultraviolet damage [6]. AGR2 is essential for maintaining breast cancer cell growth and survival, through its regulation of the expression of cyclin D1, estrogen receptor-α, and survivin [7]. Furthermore, AGR2 can promote adenocarcinoma cell growth through induction of amphiregulin, an EGFR ligand, in a process mediated by activation of the Hippo signaling pathway co-activator, *YAP1* [8]. The identification of metastasis-associated GPI-anchored C4.4a protein and extracellular alpha-dystroglycan as binding partners of human AGR2 have further hinted at a role in tumor metastasis [9].

AGR2 is a member of the protein disulfide isomerase (PDI) family, which contains a thioredoxin-like fold (amino acid residues CPHS) [10]. The cysteine residue within the thioredoxin-like fold forms a mixed disulfide bond with Mucin-2, indicative of an essential role in Mucin processing [11]. Mice lacking *Agr2* exhibited decreased goblet

Abbreviations: agr2, anterior gradient 2; cls, colorless; DIC, Differential interference contrast; EGFR, epidermal growth factor receptor; ER, endoplasmic reticulum; ERK, Extracellular Signal-regulated Kinase; Fgf, fibroblast growth factor; EMSA, electrophoretic mobility shift assays; HMG, high mobility group; hpf, hour post-fertilization; PCR, polymerase chain reaction; PDI, protein disulfide isomerase; RT-PCR, reverse transcription polymerase chain reaction

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cell Mucin-2, abnormal distribution of Paneth cells, elevated ER stress, and severe terminal ileitis and colitis [12]. Therefore, Agr2 is considered to be a candidate protein involved in inflammatory bowel disease [13]. In contrast to both *Xenopus XAG-2* and mammalian *Agr2*, zebrafish *agr2* is expressed in ectoderm-derived organs, such as the olfactory bulb, otic vesicles, and epidermis, as well as endoderm-derived organs, such as the esophagus, intestine, and swim bladder [14]. Knockdown of zebrafish Agr2 disrupts terminal differentiation of goblet cells in the intestine [15]; however, neither knockdown nor overexpression of *agr2* expression resulted in ER stress in zebrafish embryos. Despite the current knowledge of the roles of Agr2 in several biological processes, little is known regarding the regulation of *Agr2* expression in different organs.

Zebrafish (*Danio rerio*) has become an important model for biomedical research, due to its ease of genetic manipulation and transgenic analysis, and anatomical and physiological similarities between its organs and those of mammals [16]. Zebrafish ear placodal ectoderm is derived from groups of cells located on both sides of the ventral midline at the 50% epiboly stage [17]. Otic placodes form beside the hindbrain of embryos at the 10th somite stage. Analyses of *ace/fgf8* mutants and *fgf3* morphants showed that Fgf3 and Fgf8 have redundant roles in otic placode induction [18]. Downstream factors of Fgf signaling, such as *erm*, *pea3*, and *spry4* (the latter being an antagonist of Fgf signaling) are expressed in the otic placode, where they mediate otic vesicle induction [19,20]. Transcription factors, including *dlx3/7*, *pax2.1/2.2/8*, *eya1*, and *six4.1* are also expressed in the otic placode, to regulate early otic placode development [17]. At the 18th somite stage, otic placodes starts to form a vesicle with a lumen via cavitation [21]. A number of genes encoding HMG-box transcription factors, including *sox9a/sox9b*, *sox10*, and *sox11a/sox11b*, are expressed in otic vesicles [22,23]. Double mutant embryos of *sox9a* and *sox9b* fail to develop otic vesicles [23], while *sox10/cls* mutants form small otic vesicles [24]. These results implicate *sox* genes in the development of otic vesicles. Five sensory patches, including two maculae and three cristae, develop in the embryonic ear. The development of rostral utricular macula and caudal saccular macula, as well as the respective overlaying otolith, can be readily observed at 30 hours post-fertilization (hpf) [21]. By 48 hpf, formation of three cristae is marked by expression of *bmp2b* and *bmp4*. By 60 hpf, a semicircular canal system composed of anterior, lateral, and posterior canals develops [17]. Afferent otic neurons derived from the ventral epithelium of the otic vesicle form the statoacoustic ganglion (SAG) between 22 and 30 hpf [21]. Two distinct neuronal populations within the SAG innervate the utricular macula and saccular macula, respectively, at 42–48 hpf, and appropriate anterior–posterior segregation of utricular versus saccular otic neurons is controlled by Hh signaling [25]. A functional inner ear develops by 96 hpf.

Sensory patches contain two major cell types: hair cells and supporting cells. Specification of hair cells and supporting cells in sensory patches is controlled by Delta–Notch signaling. Mutations that disrupt Delta–Notch signaling enhance hair cell production at the expense of supporting cells [26]. Two proneural genes, *atoh1a* and *atoh1b*, are required for the differentiation of hair cells, and their activity is further regulated by Fgf and Notch [27]. Expression of *atoh1b* is necessary for the formation of tether cells (early-forming hair cells), while *atoh1a* expression is essential for the development of later-forming hair cells. Compared to hair cells, little is known about the development and function of supporting cells. One reason for this is the lack of molecular markers for supporting cells, though expression of *sox2* has been shown to be maintained in the supporting cells, and this is required for hair cell survival and regeneration [28]. Nevertheless, studies using mutants of *mnl* or *mib* have revealed that supporting cells regulate the formation of otolith, and are required for the maintenance of sensory patch epithelial integrity and the survival of hair cells [29,30].

Here, we describe the cloning of a 6 kbp genomic fragment upstream of exon 1 of zebrafish *agr2*; this fragment was used to generate a *Tg(−6.0 k agr2:EGFP)* transgenic fish line. The expression pattern of EGFP in this line recapitulated that of *agr2* in several embryonic organs. Double immunofluorescence assays were used to demonstrate that Agr2 and EGFP colocalize in the semicircular canals and supporting cells of all sensory patches in the otic vesicles of *Tg(−6.0 k agr2:EGFP)* embryos. Transient/stable transgenic analyses coupled with 5′-end deletion revealed that a 100 bp sequence within the −2.6 to −2.5 kbp region upstream of *agr2* directs EGFP expression specifically in the otic vesicles. Two HMG-binding motifs were detected in this region. Subsequent analyses using constructs with mutated HMG-binding motifs, *sox10* morpholinos, electrophoretic mobility shift assays, and double fluorescence in situ hybridization of *sox10* and *agr2* were used to demonstrate that *agr2* expression in the otic vesicles of zebrafish embryos is regulated by Sox10.

2. Materials and methods

2.1. Zebrafish strains and maintenance

Zebrafish, including ASAB wild type and *Tg(−6.0 k agr2:EGFP)* transgenic fish, were maintained as previously described [31]. Embryos were maintained at 28.5 °C, and morphological criteria were defined as described [32].

2.2. Plasmid construction

Polymerase chain reaction (PCR) was used to amplify different lengths of the region upstream of *agr2*, using a bacterial artificial chromosome (BAC RPC-71) as template. The following primer pairs were used to amplify the *agr2* upstream regions indicated in parentheses: (i) 5′-CTCGAG CTCGAGGGCGCCCATATGGATGTCCAGTTGCTGTTTAATA TTTTG-3′ and 5′-GTCGACGTCGAC CTTTTGCGTCTCTCTCTTCCACCT-3′ (6 kbp: −6038 to +1); (ii) 5′-CTCGAGCTCGAGTAGTCCCTTTATTAAT CCGGGGT and 5′-GTCGACGTCGACCTTTT GCGTCTCTCTCTTCCACCT-3′ (3.3 kbp: −3284 to +1); (iii) 5′- CTCGAGCTCGAGACAAAGACA CCAT TGATCCTCGGC-3′ and 5′- GTCGACGTCGACCTTTTG CGTCTCTCTCTTCC ACCT -3′ (2.6 kbp: −2623 to +1); (iv) 5′- CTCGAGCTCGAG ACTGTA TATGCTCTGTATGCTTAC -3′ and 5′-GTCGACGTCGACCTTTTGCG TCTCTC TCTTCCACCT-3′ (2.4 kbp: −2471 to +1); and (v) 5′- CTCGAGCTCG AGATAAAGAAGT TTAAATTTTTTTTAAACC -3′ and 5′- GTCGACGTCG ACCTTTTG CGTCTCTCTCTTCCACCT -3′ (2 kbp: −2039 to +1). All forward primers contain a *Xho* I site, while reverse primers contain a *Sal* I site. PCR products were cloned into the pT2KXIG△in Tol2 transposon vector containing an EGFP reporter [33,34].

A plasmid containing the *agr2* basal promoter region (−273 to +1) was generated by PCR using plasmid DNA from the −6.0 k-EGFP construct as template and the following primer pair: 5′- CTCGAG CTCGAG GGCCGGCC CCTAGG GGCGCGCCACATCACAGA TCAGAGCTCAGCC -3′ and 5′- GTCGAC GTCGAC GCGATCGC TCGCGA ACGCGTCTTTT GCGTCT CTCTCTTCCACCT -3′. The PCR product was cloned into pT2KXIG△in digested with *Xho* I and *Sal* I. The 200ecto-EGFP and 100ecto-EGFP constructs were generated by PCR, using plasmid DNA from the −6.0 k-EGFP construct as template, and the following primer pairs: 5′- CCTAGGCCTAGGACAA AGACACCATTGATCCTCGGC -3′ and 5′- GGCG CGCCGGCGCGCCGTAAGCATACAGAGCATATACAGTT -3′ (200ecto); and 5′- CCTAGGCCTAGG ACAAA GACACCATTGATCCTCGGC -3′ and 5′- GGCGCGCC GGCGCGCCGTGACTGAAATAATGTTG TAATTCAGG -3′ (100ecto). PCR products were cloned into the *agr2* basal promoter vector digested with *Avr* II and *Asc* I. To generate constructs containing either one or two mutated HMG-binding motifs within the −2.6 to −2.5 k *agr2* 5′-upstream region, PCR was performed using plasmid DNA from the −6.0 k-EGFP construct as template, and one of the following primer pairs: 5′- CCTAGGCCTAGG*TTTT*AGACACCATTGATCCT CGGCTG -3′ and 5′- GGCGCGCCGGCGCGCCGTGACTGAAATAATGTTGTA ATTCAGG -3′ (100ectoM1); 5′- CCTAGGCCTAGG ACAAAGACACC*AAAA*ATCCTCGG CTG -3′ and 5′- GGCGCGCC GGCGCGCCGTGACTGAAATAATGTTGTA ATTCAGG -3′ (100ectoM2); or 5′- CCTAGG CCTAGG *TTTT*AGACACC

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