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A conserved role of αA-crystallin in the development of the zebrafish embryonic lens

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ABSTRACT

αA- and αB-crystallins are small heat shock proteins that bind thermodynamically destabilized proteins thereby inhibiting their aggregation. Highly expressed in the mammalian lens, the α-crystallins have been postulated to play a critical role in the maintenance of lens optical properties by sequestering age-damaged proteins prone to aggregation as well as through a multitude of roles in lens epithelial cells. Here, we have examined the role of α-crystallins in the development of the vertebrate zebrafish lens. For this purpose, we have carried out morpholino-mediated knockdown of αA-, αBa- and αBb-crystallin and characterized the gross morphology of the lens. We observed lens abnormalities, including increased reflectance intensity, as a consequence of the interference with expression of these proteins. These abnormalities were less frequent in transgenic zebrafish embryos expressing rat αA-crystallin suggesting a specific role of α-crystallins in embryonic lens development. To extend and confirm these findings, we generated an αA-crystallin knockout zebrafish line. A more consistent and severe lens phenotype was evident in maternal/zygotic αA-crystallin mutants compared to those observed by morpholino knockdown. The penetrance of the lens phenotype was reduced by transgenic expression of rat αA-crystallin and its severity was attenuated by maternal αA-crystallin expression. These findings demonstrate that the role of α-crystallins in lens development is conserved from mammals to zebrafish and set the stage for using the embryonic lens as a model system to test mechanistic aspects of α-crystallin chaperone activity and to develop strategies to fine-tune protein–protein interactions in aging and cataracts.

1. Introduction

The development of the ocular lens follows a regulated program that culminates in the terminal differentiation of epithelial cells to fiber cells. During this process, the developing cells shed their organelles and express a group of water soluble proteins, the crystallins, that undergo little if any turnover (Bassnett, 1997; Beebe et al., 2001). The type of crystallins and their relative abundance is species-specific but invariably mammalian lenses have α-, β- and γ-crystallins as the main constituents at unusually high concentrations (Bloemendal et al., 2004). The optical properties of the lens, refractivity and transparency, arise from the glass-like, short-range order packing of the crystallins (Delaye and Tardieu, 1983; Tardieu, 1988). Protein–protein interactions between crystallins are tuned to be consistent with a uniform protein distribution on dimensions comparable with visible light wavelength while preventing long range order or crystallization (Benedek, 1971).

The role of α-crystallin in lens transparency has garnered intense interest owing to its function as a small heat shock protein with chaperone activity towards destabilized and unfolded proteins (Clark and Muchowski, 2000; Claxton et al., 2008; Horwitz, 1992, 2003; Koteiche and McHaourab, 2003, 2006; Kumar et al., 2009; Mchaourab et al., 2002; McHaourab et al., 2007, 2009; Sathish et al., 2003; Sathish et al., 2004; Sharma et al., 1997; Sharma and Santhoshkumar, 2009). In the context of long-lived protein solutions, it stands to reason that such an activity may be critical for proteostasis. Constantly assaulted by environmental factors, lens proteins undergo extensive age-related modifications (Hanson et al., 1998, 2000; Lampi et al., 1998, 2002, 2006; Truscott, 2005) that compromise their thermodynamic stability

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[1] This paper is dedicated to the memory of our wonderful colleague and friend, Dr. Hanane Koteiche.

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and promote the formation of large aggregates. Large protein aggregates can disturb the short range order of lens proteins and lead to light scattering thereby compromising the optical properties of the lens (Benedek, 1997). We have proposed and experimentally tested a thermodynamic model of α-crystallin chaperone activity using destabilized T4 lysozyme as a model client protein (Claxton et al., 2008; Koteiche and McHaourab, 2003; Mchaourab et al., 2002). Our model implies that once modified lens proteins cross an energetic threshold of destabilization, they become substrates for α-crystallin which sequesters them away. The progressive exhaustion of α-crystallin binding capacity by the binding of age-damaged, destabilized lens proteins eventually leads to the formation of aggregated proteins and the subsequent scattering of light.

There is little doubt that *in vitro* α-crystallin recognizes and binds destabilized proteins with high capacity. α-crystallin binding affinity and stoichiometry are regulated by changes in its oligomeric structure induced by phosphorylation and by higher temperature (Claxton et al., 2008; Koteiche and McHaourab, 2003; McHaourab et al., 2009; Reddy et al., 2000; Sathish et al., 2003). What lacks direct evidence is the *in vivo* role of α-crystallin chaperone activity in the maintenance of lens transparency and in delaying its aging. In contrast to the spectrum of model protein substrates that have been investigated, neither αA- nor αB-crystallin display high affinity to thermodynamically destabilized, aggregation-prone, γD-crystallin mutants (Liu et al., 2005; Mishra et al., 2012) shown to induce cataract in mouse models (Liu et al., 2005; Wang et al., 2007). Interpretation of the relevance of these findings is confounded by the unusually high protein concentration of lens fiber cells which shapes protein stability and interactions *in vivo* as opposed to the dilute solutions where these *in vitro* studies are typically performed. Molecular crowding leads to excluded volume effects manifested by many-fold difference in the magnitude of equilibrium affinities, rate constants of interactions and free energies of unfolding (van den Berg et al., 1999; van den Berg et al., 2000). Crowding in lens fiber cells, which has been invariably neglected in α-crystallin mechanistic models, is peculiar involving the three molecules whose interactions are to be studied. Therefore, the implications of low affinity between α-crystallin and its putative physiological targets in dilute solution are unclear. To address this unresolved aspect of α-crystallin mechanism, there is a need to develop cell- and organism-based approaches to test the chaperone hypothesis of α-crystallin role in the lens.

Zebrafish has emerged as a powerful model system for eye development and disease (Fadool et al., 1997; Fadool and Dowling, 2008). Zebrafish embryos are extracorporeal and transparent during the first few days of development. Larval embryos have relatively large eyes which become functional 3 days post fertilization (dpf) enabling the examination of lens gross morphology by bright field microscopy. Studies by the Link (Soules and Link, 2005), Clark (Greiling and Clark, 2009; Greiling et al., 2009) and Posner (Runkle et al., 2002; Dahlman et al., 2005; Posner et al., 2008) laboratories lay the morphological and proteomic foundations for zebrafish lens. The structure of the adult zebrafish lens resembles the mature human lens and the development and morphology are similar to those of mammals with few differences. Importantly from the perspective of protein–protein interactions, the zebrafish lens expresses a complement of α-, β- and γ-crystallin orthologs, as well as additional γ-crystallins which are specific to aquatic species. Therefore, it is postulated that similar molecular interactions account for lens transparency.

Similar to their mammalian orthologs to which they have extensive sequence similarity (Runkle et al., 2002; Dahlman et al., 2005); Zebrafish α-crystallins have chaperone activity *in vitro* (Dahlman et al., 2005). However, their role in embryonic zebrafish lens development and transparency is controversial (Goishi et al., 2006; Posner et al., 2013). Here, we report the results of an investigation into the roles of three α-crystallin genes, *cryaa*, *cryaba*, and *cryabb*, in the development of the zebrafish lens. We report that selective knockdown of each of the α-crystallin genes is associated with lens abnormalities. Lens-specific effects induced by the suppression of αA- and αBa-crystallin, but not αBb-crystallin, can be rescued by transgenic expression of rat αA-crystallin. These findings are confirmed and extended by characterization of the first knockout of αA-crystallin in zebrafish. We find that the lack of αA-crystallin expression is associated with morphological abnormalities and increased intensity of reflectance in zebrafish embryonic lens. That the lens phenotype of the knockout embryos can be partly rescued by the transgenic expression of rat αA-crystallin is consistent with a conserved role of its chaperone activity in lens development across species.

2. Materials and methods

2.1. Zebrafish maintenance and breeding

AB wild-type strain zebrafish (*Danio rerio*) were used. The embryos were obtained by natural spawning and raised at 28.5 °C on a 14/10 h light/dark cycle in 0.3× Danieau water containing 0.003% PTU (w/v) to prevent pigment formation. Embryos were staged according to their ages (hpf; hours post-fertilization or dpf; days post-fertilization). All animal procedures were approved by the Vanderbilt University Institutional Animal Care and Use Committee. The same feeding procedures were performed on all lines, including AB wild-type, *cryaa* knockout lines, and *cryaa*:*Rno.Cryaa* transgenic lines according to their appropriate ages.

2.2. Zebrafish transgenesis

To establish the transgenic zebrafish expressing rat (*Rattus norvegicus*) *Cryaa gene* (*Rno.Cryaa*) specifically in the lens, *Tg(cryaa*:*Rno.Cryaa,myl7*:*Cerulean)* was constructed by inserting *Rno.Cryaa* cDNA downstream of zebrafish *cryaa* promoter (0.7 kb; Kurita et al., 2003) in the pT2HBLR vector that was also contains m*yl7* promoter-driven Cerulean as the selection marker. Tol2 transposase RNA was synthesized *in vitro* by using mMESSAGE mMACHINE SP6 kit (Ambion). The mixture of 25 pg plasmid DNA and 20 pg transposase RNA was co-injected into one-cell stage embryos. Injected embryos were screened for Cerulean expression in the heart under Zeiss fluorescence microscope at 3 dpf, and then were raised to adulthood as F0 founder. Each F0 founder fish was out-crossed with AB fish and progenies with Cerulean expression in the heart were raised to establish stable F1 generation. F1s were further confirmed by PCR sequencing and one transgenic line was selected and maintained.

2.3. Morpholino knockdown of zebrafish α-crystallin genes

Translation-blocking morpholino antisense oligos (MOs) against zebrafish *cryaa* (5′-GTTGGATCGCAATATCCATAATGTC-3′), *cryaba* (5′-CCATTGTACCTTAGTTTGGAGCTGA-3′), *and cryabb* (5′-TCCATTT TGAGTCTGGGCCTCTTCT-3′) genes were designed and synthesized by Gene Tools (Philomath, OR). MOs were dissolved in sterile water. Different dosages (2.5 ng, 5 ng and 10 ng) of each MO were injected into the yolk of 1–2 cell stage zygotes, which were the progenies from *Tg(cryaa*:*Rno.Cryaa*,*myl7*:*Cerulean)* and AB fish. For each MO, a series of dosages were tested to rule out the toxic effects caused by over-injection of MOs.