

# Heat shock gene expression and function during zebrafish embryogenesis

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## Abstract

Recent work in the zebrafish, *Danio rerio*, indicates that heat shock genes are expressed in unique spatial patterns under non-stress conditions. In particular, *hsp90α* is expressed during the normal differentiation of striated muscle fibres, and *hsp70-4* is expressed during normal lens development in the eye. Furthermore, disruption of the activity of either of these genes or their protein products gives rise to unique embryonic phenotypes that result from failures in proper somitic muscle development and lens development, respectively. Embryonic *hsp70-4* expression is also activated in a cell-specific manner following heavy metal exposure. This has allowed for the development of a *hsp70-4/eGFP* reporter gene system in stable transgenic zebrafish that serves as a reliable yet extremely quick indicator of cell-specific toxicity in the context of the multicellular, living embryo.

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## 1. Introduction

The zebrafish (*Danio rerio*) is a small tropical aquarium fish well known to home aquarium enthusiasts. Many of the features that have made it a popular addition to aquariums are also reasons it emerged as a model system for the examination of embryonic development of vertebrates. Today, it is solidly established in this capacity [1,2]. One of the appealing features of the zebrafish is the relatively low space requirements of an aquarium facility on a per animal basis. Thus, a colony of zebrafish can be maintained in a fraction of the space required for a comparable colony of mammals or larger fish models such as trout or salmon. Fertilized eggs can be obtained on demand by maintaining the fish on a constant photoperiod schedule. Together, these features allow users to obtain a large number of embryos for experimental manipulation without any seasonal limitations. The embryos themselves develop rapidly (somitogenesis is completed within the first day following fertilization) and a comprehensive staging series has been published [3]. Manipulations of environmental conditions are easily carried out since the embryos develop ex utero, a feature which greatly facilitates the study of environmental influences on development. The relatively large size of the embryos al-

lows for the introduction of molecules such as reporter genes and morpholino-modified antisense RNA by microinjection, and their translucency allows for the monitoring of internal morphological changes and transgene expression in both living and fixed whole embryos. This feature also makes the embryos amenable to the technique of whole mount in situ hybridization analysis to rapidly examine the three dimensional patterns of tissue-specific gene expression. Finally, the zebrafish has been the focus of successful broad scale and smaller, targeted mutagenesis screens [4,5], as well as insertional mutagenesis screens [6] in an effort to identify genes that regulate embryonic development. Combined with the recent effort to complete sequencing of the zebrafish genome, these studies are providing valuable information regarding the molecular cascades that regulate development of fish and other vertebrates.

Many of the features described above also make the zebrafish an attractive model for examining the expression and function of heat shock genes during embryonic development. Information about heat shock protein (hsp) expression and function in this system has increased substantially in the past decade, and much of this can now begin to be integrated into the mechanistic framework of developmental pathways. In this review, we summarize recent data suggesting that zebrafish hsps play critical roles during normal developmental processes, and on the use of a living transgenic indicator strain of zebrafish in the analysis of cell-specific toxicity of heavy metals during embryogenesis.

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## 2. The heat shock response of zebrafish embryos

Zebrafish embryos develop normally within a temperature range of approximately 23–33 °C and are usually maintained at 28–29 °C under laboratory conditions. There is a proportional increase in the rate of development with higher temperatures over this range [7,8]. Exposure to a temperature of 34–35 °C for 2–3 h results in higher death rates and increases the number of developmental anomalies [8–10]. Embryos are most susceptible to heat shock at early cleavage stages and acquire increased resistance as they progress through the blastula and gastrula stages. As well, brief exposures of 2–20 min to higher temperatures of 37–41 °C gives rise to a number of developmental abnormalities [7,11].

In order to initially characterize the zebrafish heat shock response at the molecular level, Northern blot analysis was utilized to examine the expression of heat shock genes *hsp10* (*cpn10*), *hsp60* (*cpn60*), *hsp47*, *hsp70*, *hsp90 $\alpha$* , and *hsp90 $\beta$*  at different stages of development under both control and heat shock conditions [12–17]. Post-blastula and later stage embryos first exhibited inducible heat shock protein mRNA accumulation following a one hour heat shock at 34 °C with maximum induction occurring at 37 °C. At 40 °C, embryos at all stages of development from gastrula through to post-hatching died rapidly and exhibit weak induction of hsp mRNA. This suggests that heat protective mechanisms cease to be effective in zebrafish embryos at temperatures above 37 °C. Interestingly, these expression patterns correlate with the aforementioned embryological data with respect to stress temperature, the associated level of heat shock gene expression and the appearance of developmental anomalies. For the most part, zebrafish heat shock genes were expressed in cells throughout the embryo following exposure to a severe (37 °C) heat shock [14–18], and this response appears to be mediated through the activity of heat shock factor 1 (HSF1) similar to other eukaryotes [19]. However, exposure to a number of different chemical toxins such as ethanol, arsenic, and cadmium activated several zebrafish heat shock genes in only a small number of cells within the embryo [14,20,21]. This suggests that differences in the mechanisms through which some cells sense different forms of environmental stress and subsequently activate heat shock gene expression. Consequently, we have utilized hsp expression as an indicator of cell-specific toxicity of such compounds (see later in this review).

## 3. Heat shock genes are expressed in unique spatial and temporal domains during normal zebrafish development under non-stress conditions

Our laboratory has been particularly interested in the expression and possible function of heat shock proteins as molecular chaperones during normal developmental processes. To this end, we have made extensive use of in situ hybridization analysis to examine tissue-specific patterns

of heat shock gene expression in zebrafish embryos under non-stress conditions. This approach serves as a first step in attempting to determine what role(s) different hsps may be playing during embryogenesis. Surprisingly, we have found that a number of zebrafish heat genes are expressed in very specific spatial and temporal patterns in early embryos. Importantly, subsequent studies of two of these hsps (*hsp70-4* and *hsp90 $\alpha$* ) have revealed that these expression patterns are indicative of tissues that require these hsps for their normal development.

## 4. *hsp90 $\alpha$* is required for normal somitic muscle development in zebrafish embryos

Members of the eukaryotic *hsp90* family regulate post-translational events within cells by interacting with and modulating the activity of important cellular signaling molecules such as steroid receptors and transcription factors (recently reviewed in [22]). The importance of *hsp90* is underscored by recent studies suggesting that it serves as an evolutionary capacitor of morphological changes during embryogenesis [23–25]. Vertebrates express two *hsp90* genes, *hsp90 $\alpha$*  and *hsp90 $\beta$* , and studies in zebrafish and other systems indicate that these genes are differentially regulated [12,18]. For example, whole mount in situ hybridization analysis with gene-specific probes revealed that constitutive *hsp90 $\alpha$*  mRNA was restricted primarily to a small subset of cells within the pre-somitic paraxial mesoderm, somites and pectoral fin buds of developing zebrafish embryos (Fig. 1; [18]). Importantly, these *hsp90 $\alpha$* -expressing cells also expressed the *myoD* gene, and expression of the *hsp90 $\alpha$*  gene was down-regulated along with *myoD* in mature muscles of the trunk at a time when levels of mRNA encoding the muscle structural protein  $\alpha$ -tropomyosin remain high [18,26]. *myoD* was originally identified by its ability to convert cultured CH3 10T1/2 fibroblasts to a myoblast phenotype [27]. It belongs to a family of conserved proteins known as myogenic regulatory factors (MRFs) that are expressed in skeletal myoblasts and myotubes. MRFs are known to play a pivotal roles at different points in the myogenic program whereby proliferating myoblasts exit the cell cycle, fuse to form multinucleate muscle fibres, and begin to express components of the muscle contractile apparatus [28,29]. The expression pattern of zebrafish *myoD* suggests that it plays a similar role during early myogenesis [30]. Data obtained in several mutant strains exhibiting abnormalities in somite formation confirmed that the *hsp90 $\alpha$*  gene is specifically expressed within cells of the paraxial mesoderm lineage which go on to form striated trunk muscle [26]. Specifically, expression of the *hsp90 $\alpha$*  gene in *flh* embryos is activated in axial mesoderm, which would normally form notochord, but becomes respecified to a striated muscle fate. This respecified axial mesoderm also expresses other muscle-specific markers [31,32]. As well, expression of the *hsp90 $\alpha$*  gene is not detectable in mis-migrated paraxial mesoderm cells of

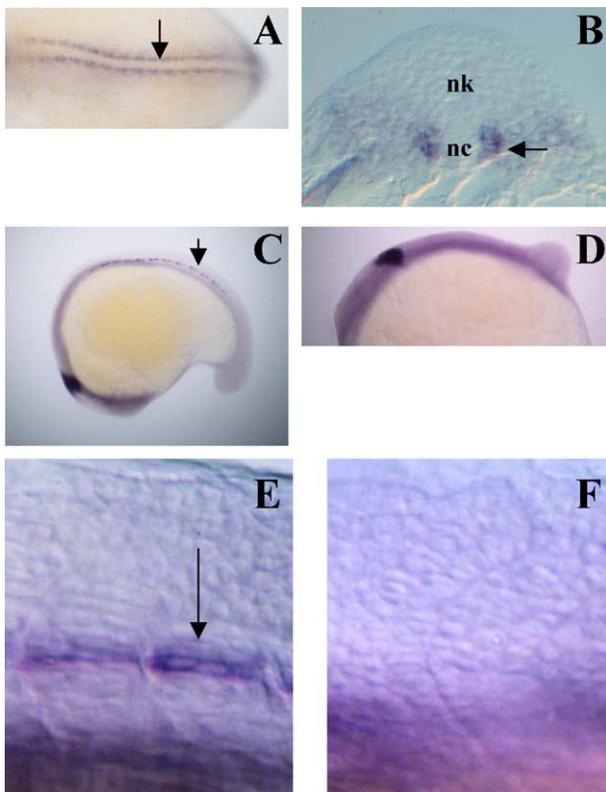


Fig. 1. Expression of *hsp90α* in developing muscle cells (A and B), and the inhibition of muscle pioneer formation in geldanamycin-treated embryos (C–F). The *hsp90α* gene is strongly expressed in the progenitors of muscle pioneers known as adaxial cells (arrow in panels A and B). Following geldanamycin treatment, zebrafish embryos display a characteristic shortened trunk/tail somitic phenotype (compare panels C and D). This phenotype appears to arise from a failure of the development of muscle pioneers, which can be distinguished from other muscle cell types by their strong expression of the *eng-2* gene (arrow in panels C and E, but absent in panels D and F). However, activation of the *eng-2* gene at the midbrain-hindbrain boundary is normal in GA-treated embryos, indicating that GA does not have a general inhibitory effect on *eng-2* expression. (A) 16-somite embryo, dorsal view. (B) Cross-section of pre-somitic mesoderm in 16-somite embryo. (C and E) Low and high magnification lateral views of untreated embryo. (D and F) Low and high magnification views of GA-treated embryo. (A and B) In situ hybridization detection of *hsp90α* mRNA. (C–F) In situ hybridization detection of *eng-2* mRNA. nc, notochord. nk, neural keel. (Adapted and reprinted from [35], with permission.)

*spt* embryos [33] that would normally form muscle. These mis-migrated paraxial mesoderm cells do not express the *myoD* gene and do not form striated muscle.

In contrast to *hsp90α* mRNA, *hsp90β* mRNA is detectable within the CNS and other tissues at both control and heat shock temperatures, and is not specifically restricted to *myoD*-expressing cells [18]. This further supports the suggestion that these two genes are subject to different mechanisms of regulation within developing embryos and probably play different functional roles.

The expression pattern *hsp90α* suggests that it may be playing a specific role in the normal process of muscle de-

velopment. Since zebrafish embryos are very amenable to treatment using pharmacological agents, we took advantage of the well-characterized and highly specific *hsp90*-binding activity of the benzoquinoid ansamycin geldanamycin to examine the embryonic impact of inhibiting *hsp90* [34,35]. *Hsp90* was the only protein with which geldanamycin interacts in zebrafish protein extracts, and treatment of early embryos with geldanamycin prior to and during somitogenesis resulted in a reproducible short trunk and tail phenotype in over 80% of the embryos (Fig. 1; [35]). Importantly, the defect was associated with a failure to form an early type of somitic muscle cell known as a muscle pioneer (Fig. 1; [35]). Muscle pioneers are the earliest muscle cells to differentiate in zebrafish embryos, and can be distinguished from other somitic muscle cells by their strong and specific expression of the engrailed gene (*eng-2*; [36]). Muscle pioneers and their progenitors, known as adaxial cells, are also the earliest cells of the somitic muscle lineage to specifically activate expression of the *hsp90α* gene [18,26]. The geldanamycin-induced phenotype was also very similar to that observed in several zebrafish mutants that have defects in somite formation due to a failure in notochord development [35]. The somitic defects in these mutants are thought to arise by a failure in development of muscle pioneers, as these cells rely on signals from the notochord for their specification. However, unlike the mutants, geldanamycin-treated embryos exhibited relatively normal development of notochord [35]. Thus, the somitic phenotype of geldanamycin-treated embryos is most likely caused by improper development of the *hsp90α*-expressing muscle pioneers.

## 5. The zebrafish *hsp70-4* gene is expressed specifically during lens formation, and is required for normal differentiation of lens fibres

The *hsp70* family has been the most widely studied of the heat shock proteins. Members of this family are present in all intracellular compartments and are known to play a pivotal role in protein folding and translocation within these locations [37]. In vertebrates, the *hsp70* gene family consists of both stress inducible (*hsp70*) and constitutively expressed (heat shock cognate or *hsc70*) members, a categorization based predominantly on information obtained in cell culture studies. However, such a clear designation does not necessarily hold true when examining these genes *in vivo* in the zebrafish embryo.

A PCR-based approach was used to clone members of the zebrafish *hsp70* gene family [14]. One of the clones obtained, designated *hsp70-4*, exhibited strong heat-inducible expression in a wide range of cell types during several stages of embryonic development. Surprisingly, this gene was also strongly and very specifically expressed during normal lens development under non-stress conditions (Fig. 2; [38]), and this was the only tissue in which constitutive *hsp70-4* ex-

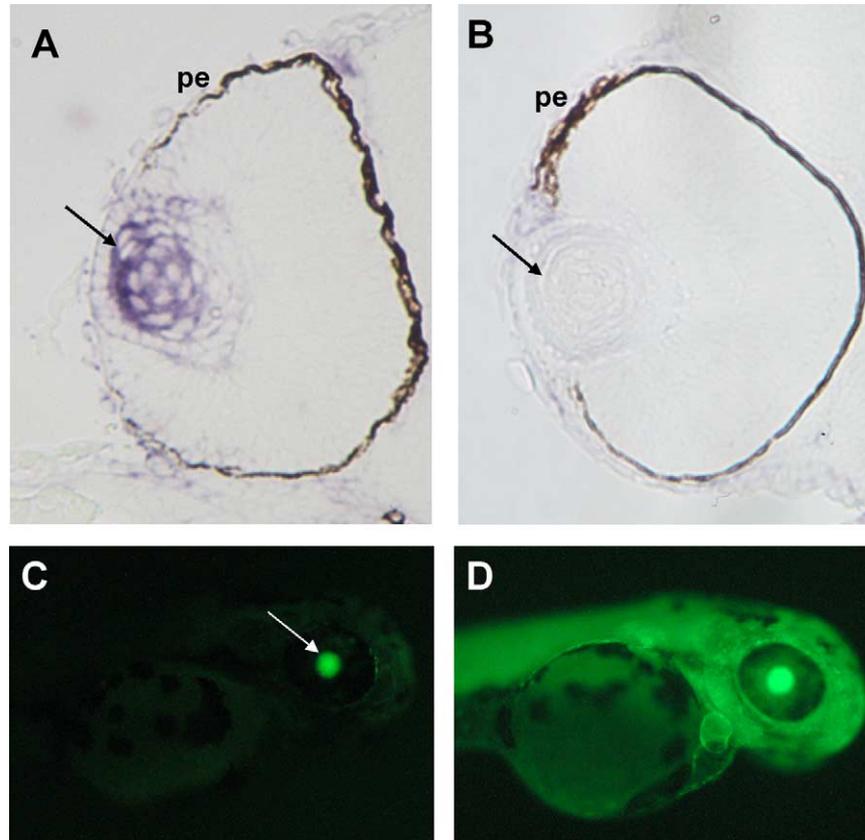


Fig. 2. Specific localization of *hsp70-4* expression to the developing lens. *hsp70-4* mRNA is strongly expressed in the developing lens during fibre differentiation at 38 hpf (arrow in A) but is no longer detectable in the more mature lens during the third day of development (arrow in B). Similarly, under non-stress conditions eGFP fluorescence is only visible within the lens of transgenic larvae carrying a stably integrated *hsp70-4/eGFP* transgene (C), but is detectable in all cells following heat shock at 37 °C (D). (A and B) Cross-sections of eye. (C and D) Lateral view of live embryos viewed under a fluorescence microscope using the appropriate GFP filter. pe, pigmented epithelium. (Reprinted from [38], with permission.)

pression was observed. Lens-specific expression of *hsp70-4* coincided closely with the initiation of lens fibre differentiation (28 hpf), and expression was downregulated substantially following secondary lens fibre differentiation in 2–3-day-old embryos. In addition, GFP fluorescence in a strain of transgenic zebrafish carrying a 1.5 kb region of the *hsp70-4* promoter driving expression of an *eGFP* reporter gene was visible only in the lens under non-stress conditions. As expected, fluorescence was visible throughout the embryo following heat shock. Thus, a 1.5 kb region of the *hsp70-4* promoter is both necessary and sufficient to direct lens-specific expression of the *hsp70-4* gene, and to activate the gene in all cells during heat shock. As discussed later, this promoter also contains the regulatory regions required for heavy metal-induced *hsp70-4* gene expression [21].

The localization of *hsp70-4* expression to the developing lens of zebrafish embryos suggests a specific role for the gene during the formation of lens fibres, or for some other function carried out specifically by these cells during this time period. The relative simplicity of the lens, as well as its accessibility in zebrafish embryos provide a unique opportunity to examine the role of a heat shock protein in a

defined cell type in a vertebrate embryo. To this end, experiments in which the translation of *hsp70-4* mRNA was inhibited by microinjection of morpholino-modified antisense oligonucleotides (MOs) have been carried out. MOs are synthetic DNA analogs used as an *in vivo* gene targeting tool to eliminate the expression of a particular gene. MOs function by inhibiting translational initiation [39]. In this approach, an antisense sequence is targeted against the 5' end of the mRNA of interest. Complimentary base pairing will result in a double stranded RNA:MO hybrid that will sterically inhibit scanning of the RNA by the 40S ribosomal subunit, effectively blocking translation, and eliminating the production of protein. This method overcomes many of the shortcomings of previously developed antisense technology, and represents the first viable sequence-specific gene inactivation method in zebrafish [40,41]. Its efficacy has now been widely demonstrated for a number of different zebrafish genes [41,42].

MO-mediated inhibition of *hsp70-4* gene expression gave rise to a reproducible small eye phenotype in zebrafish embryos microinjected with the antisense *hsp70* MO (Evans and Krone, unpublished data). The lens and retina

of 52-h-old microinjected embryos exhibited an immature phenotype when compared to a normal 52-h-old eye. This phenotype is not apparent at 24 h post-fertilization, but is readily visible by 48 h. Thus, it coincides closely with the period of *hsp70-4* gene expression, and suggests that the gene plays a critical role in normal eye development. Further experiments have confirmed this to be the case, and shown that the effect is lens autonomous as would be expected for a gene expressed exclusively within the lens. (Evans and Krone, unpublished data). The zebrafish *arl* mutant has a similar lens autonomous phenotype [43], and it will be interesting to determine whether *hsp70-4* and *arl* function in the same molecular pathway.

In contrast to the *hsp70-4* gene described above, several *hsp70* gene family members most similar to the mammalian heat shock cognate (*hsc70*) family have also been isolated. Zebrafish *hsc70* is expressed strongly during normal development and exhibits only a slight increase in expression following heat shock [44]. However, *hsc70* transcripts are enriched in the developing CNS and a fraction of the somites, and the gene is strongly induced during fin regeneration. To date, a specific requirement for *hsc70* during zebrafish embryogenesis has not been demonstrated.

## 6. The zebrafish *hsp47* gene is co-expressed with type II collagen (*col2a1*)

The gene encoding the collagen-specific chaperone *hsp47* (see review by Nagata in this issue) is expressed in only a few tissues of early embryos, and the pattern of expression correlates closely in both a temporal and spatial manner with that of the gene encoding type II collagen (*col2a1*; [45,46]). In particular, the expression of these two genes is co-ordinate in both a spatial and temporal manner in the developing notochord of early stage embryos. In later stage embryos, precartilagenous cells of the otic capsule and fins also co-express the two genes. However, not all cells expressing the type II collagen gene also express *hsp47*. For example, the floor plate and hypochord express *col2a1* but not *hsp47*. Conversely, the lens expresses *hsp47* but not *col2a1*. These data support a model in which the regulation of these genes is subject to multiple and non-overlapping mechanisms of regulation. More recent evidence indicates that *hsp47* expression is regulated in part by bone morphogenetic protein (*bmp-2*) signaling pathways (Sperber and Krone, unpublished data), a result which is supported by cell culture studies in the mouse [47]. The potential role of *hsp47* in zebrafish embryos is being investigated using a morpholino knockdown approach similar to that described above for the *hsp70-4* gene. Given the data obtained for *hsp47* knockouts in mouse, it would not be surprising if *hsp47* also plays an important role in collagen processing events within the cells of zebrafish embryos.

## 7. Cell-specific induction of an *hsp70/eGFP* transgene in a live zebrafish model suitable for rapid developmental toxicity profiling

Heat shock genes have long been of interest as potential markers of stress exposure in toxicological studies, including vertebrate developmental and reproductive toxicology [48–50]. A significant amount of work has focussed on using RNA or protein blot analyses in culture systems, whereas current knowledge of the potential impact of toxicants at the level of individual cells within the intact embryo is more limited. Furthermore, many of the assays used for in vivo studies, such as in situ hybridization or immunohistochemistry, are time-consuming and laborious. This makes them difficult to apply to larger scale screening projects, and when carrying out mechanistic studies that examine multiple stages of embryonic and larval development. However, a recently developed assay system based on the line of transgenic fish carrying a stably integrated *hsp70-4* promoter linked to an enhanced green fluorescent protein (eGFP) reporter gene promises to greatly enhance the use of heat shock gene expression in toxicological studies [21]. In contrast to in situ hybridization and immunohistochemical assays, the assay of eGFP activity requires only a few minutes under a fluorescent microscope, and offers greater sensitivity in detecting individual cell populations that express the transgene. Using the *hsp70-4/eGFP* stable line of transgenic zebrafish, Blechinger et al. [21] showed that eGFP activity was an accurate and reproducible indicator of *hsp70-4* promoter activation, and that the transgene faithfully mimicked cell-specific activation of the endogenous *hsp70-4* gene in larvae exposed to cadmium (Fig. 3; [21]). In particular, the transgene was activated in epidermal and gut epithelial cells and olfactory sensory neurons at low concentrations of cadmium, and in liver and pronephros (primitive kidney) at high concentrations. Importantly, the transgene responded in a dose-dependent manner at concentrations similar to those observed for morphological indicators of early life stage toxicity, and was sensitive enough to detect cadmium at doses below EC<sub>50</sub> and LC<sub>50</sub> values as determined by classical morphological indicators.

A major (and justifiable) criticism of reporter gene assays in toxicological studies is whether a relationship exists between the appearance of reporter gene product and a real effect on the organism at the cellular, individual, or population level. Without such a link, it is difficult to justify the use of a transgenic system as a biomarker for use in ecological risk assessment. In subsequent studies, we have found that the olfactory receptor neurons that strongly express eGFP at and below EC<sub>50</sub> range concentrations (as assessed by classical morphological indicators), also activate cell death pathways (Blechinger and Krone, unpublished data). Interestingly, a recent report has shown that olfaction-mediated predator avoidance behaviours are disrupted in cadmium-exposed rainbow trout [51], and that this correlates closely with cadmium uptake in the olfac-

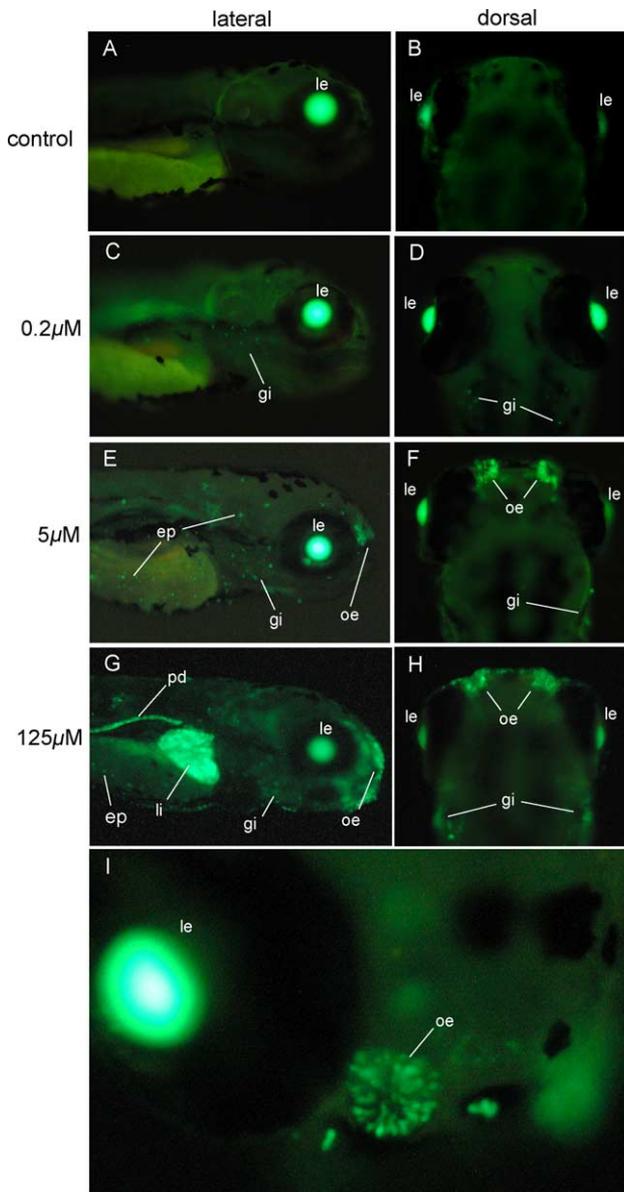


Fig. 3. Exposure of *hsp70-4/eGFP* transgenic zebrafish larvae to cadmium results in a dose-dependent increase in reporter gene expression (eGFP fluorescence). Fluorescence in the lens of all larvae represents residual eGFP from constitutive lens expression earlier during development. At 0.2  $\mu\text{M}$  cadmium (C and D), *hsp70-4/eGFP* expression is evident in isolated cells of the developing gill region similar to that observed for endogenous *hsp70*. At 5  $\mu\text{M}$  cadmium, the expression has expanded to include cells of the developing skin (E) as well as the olfactory organs, visible as localized regions of bilateral fluorescence in the anterior region of the head (F, magnified in panel I). The highest dose of 125  $\mu\text{M}$  displays prominent eGFP fluorescence in both in the liver and pronephros (G) besides the gill, olfactory region, and skin (H). Controls did not show any cadmium-induced *hsp70*-GFP expression (A and B), while background autofluorescence in the yolk region is evident at all doses (A, C, E, and G). All images are of living larvae. le, lens; gi, gills; ep, epithelial cells of developing skin; oe, olfactory epithelium; li, liver; pd, pronephric ducts. (Reprinted from [21], with permission.)

tory system. Thus, reproductive and other behavioural effects that rely on proper olfaction can be disrupted following cadmium exposure, and current work is examining whether *hsp70-4/eGFP* transgene expression can be used as an early indicator of conditions that will give rise to such an effect. This work relies on one of the major strengths of the using GFP transgenes in toxicological studies, that is, the assay is carried out in living embryos without the addition of any assay reagents. As a result, direct correlations between conditions that enhance *hsp70-4* promoter activation and subsequent morphological and behavioral effects can be made hours, days, or even weeks later in the same individual animals.

Given the range of chemical stressors that are known to be capable of inducing *hsp70* gene expression in other systems, it is likely that similar relationships will be found between *hsp70/eGFP* expression, endogenous *hsp70-4* gene activation, and subsequent effects such as cell death. Indeed, we have recently found that both arsenic and mercury can also induce transgene activation in a cell-specific manner (Salisbury, Blechinger, and Krone, unpublished data). Thus, this system should serve as a good indicator strain for assessing the overall toxicity of mixtures of compounds that impinge on the stress response mechanism in sensitive cell populations. The stable nature of this transgenic line will also allow for rapid and reproducible toxicological profiling of embryos and larvae throughout development.

## 8. Conclusions

Zebrafish heat shock genes are differentially regulated in a complex tissue-specific manner during normal development. The studies reviewed here, along with those currently being obtained in other animal systems (see other reviews in this issue), strengthen the argument that some heat shock proteins fulfill specific roles during embryonic development as opposed to serving simply as general housekeeping genes. The identification of possible chaperone targets of *hsp70* and *hsp90* must be carried out in order to elucidate what, if any, post-translational role these proteins may play in the mechanisms that regulate development of early zebrafish embryos. Comparison of phenotypes induced by *hsp* inhibition to those observed in the many mutagenesis screens should assist in integrating specific *hsp*s into the molecular pathways that direct zebrafish embryogenesis.

Zebrafish embryos respond in a complex manner to environmental stress. Recent data suggest the presence of different stress-inducible regulatory mechanisms for heat shock genes, and that such mechanisms appear to operate in a cell and stress-specific manner. Furthermore, these data illustrate the complexity of the stress response in a multicellular organism and the importance of determining cellular specificity of *hsp* induction when carrying out comparative analyses of different stresses and different genes. It is possible that these differences are due to differential activity

of HSF1, the transcription factor which is known to play a major role in the stress-induced activation of heat shock genes, or to the presence of different zebrafish HSF isoforms in different cell types [52,53]. Alternatively, differential access of HSF to heat shock gene promoters due to changes in chromatin structure could explain the results obtained. In any event, cell-specific expression of heat shock gene activation holds considerable promise for the study of mechanisms of toxicity in the context of the multicellular, developing embryo.

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