

HSP90 expression in two migratory cell types during ascidian development: test cells deposit HSP90 on the larval tunic

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ABSTRACT Heat shock protein 90 (HSP90) is a ubiquitously expressed molecular chaperone that controls the folding, assembly and activity of proteins, many of which are involved in signal transduction. Recent work has shown that HSP90 is present extracellularly, indicating a heretofore under appreciated requirement for extracellular chaperoning, particularly among migratory cells. We applied immunological and surgical techniques to document the differential expression of HSP90 during ascidian development. Relative to other cell types during development, test cells and trunk lateral cells (TLCs), two migratory cell types in the ascidian *Boltenia villosa*, express elevated levels of HSP90. Late in embryogenesis, test cells deposit HSP90 onto the tunic, the second report of extracellular HSP90 during animal development. The pyrid ascidian *Halocynthia igaboja* and the styelids *Cnemidocarpa finmarkiensis* and *Botrylloides violaceus* all express HSP90 at elevated levels in larval mesenchyme, suggesting that this pattern of expression is widespread in the Ascidiaceae. We show that HSP90 expression in TLCs and test cells is coincident with the presence of HNK-1. Whereas in *B. violaceus*, cell populations expressing elevated levels of HSP90 are distinct from those expressing HNK-1, in *B. villosa* both these antigens are present in the TLCs. We evaluate existing hypotheses about test cell function and, in reference to the similarities between test cells and some of the descendants of TLCs, hypothesize that test cells are TLC descendants. Implications for the proposed evolutionary relationship between TLCs and neural crest are briefly discussed.

KEY WORDS: HSP90, test cells, TLC, ascidian

Among marine invertebrates, ascidians are unique in both their mode of larval locomotion and their possession of a protective extracellular matrix, the tunic. The tunic is composed of carbohydrate polymers and proteins that interact extensively (Smith and Dehnel, 1970). In adult and larval stages of ascidians, the tunic is secreted by epidermal cells (Wardrop 1970; Robinson *et al.*, 1986). In embryos and larvae the tunic appears to require the presence of test cells, a third unique characteristic of ascidian embryos.

Test cells are extraembryonic migratory cells that reside between the embryo and the follicular envelope of almost all ascidian embryos. The rarity of test cell analogues in the animal kingdom is conspicuous and the ontogenetic origin and function of test cells remains enigmatic. Test cells are thought by some authors to derive from the inner follicular envelope, which, in turn, arises from the germinal epithelium (Cloney, 1994 and references

therein). Another hypothesis is that test cells derive from migratory amoeboid or lymphocyte cells that differentiate into test cells and become preferentially associated with developing oocytes (Cloney, 1994). In either case, investigation of test cell origins has not yielded as much insight into their function as has that of their remarkable behavior.

Test cells are migratory throughout development, and in later stages, form large aggregates that collectively migrate over the surface of the embryo (Fig. 1). In larvae of some species, test cells become embedded in the larval tunic and, at least in *Ciona intestinalis*, retain their migratory behavior (Sato *et al.*, 1997); in larvae of other species test cells do not adhere to the larval tunic

Abbreviations used in this paper: HNK-1, human natural killer antigen 1; HSP90, heat shock protein 90; TLCs, trunk lateral cells.

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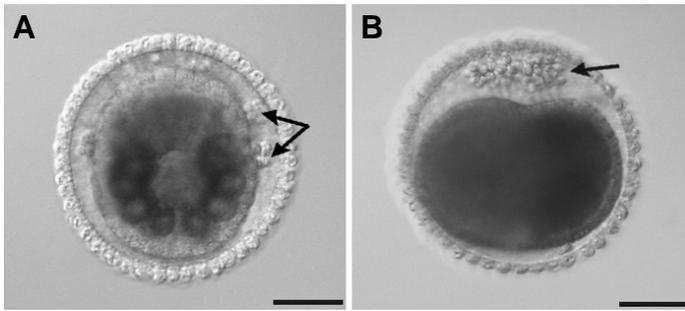


Fig. 1. Images of test cells aggregating and migrating around the outer surface of *B. villosa* embryos. (A) *Gastrula* in vegetal view. (B) *Neurula* in lateral view. Arrows in (A, B) point to test cells. Scale bars, 40 μ m.

after hatching (Cloney, 1990). Cavey (1976) demonstrated that test cells of *Distaplia occidentalis* contain and deposit granules on the larval tunic. This 'ornamentation' process was further documented for *Corella inflata* and *Styela clava* (Cloney and Cavey, 1982; Monniot *et al.*, 1992b) and subsequently in 12 additional species (Cloney, 1994).

Interestingly, a survey of 16 species representing 10 families revealed that only larvae without firmly adhering test cells bear ornaments (Cloney, 1990). In some species, silica (in the form of opal) is a component of these granular ornaments (Monniot *et al.*, 1992b). The electron density of these granules was sensitive to pronase digestion, leading to the proposition that the silica was complexed with proteins (Monniot *et al.*, 1992b). Consistent with these observations, the secretion of antigens from test cells to the tunic surface has been demonstrated using immunocytochemical approaches at both the light and the electron microscopic levels (Takamura *et al.*, 1996; Okada *et al.*, 1996). Because the nature of the antigens was not characterized in these reports, no specific proteins secreted from test cells have been identified. Test cells in *Styela plicata* contain and can be induced to release, heparin and histamine (Cavalcante *et al.*, 2000; Cavalcante *et al.*, 2002), supporting an interpretation of immune-like function. The common conclusions derived from these reports are that test cells contain and deposit molecules that modify the external surface of the larval tunic. But to what end?

Removal of test cells by dechoriation of embryos at various developmental stages indicated that test cells participate directly in morphogenesis of the dorsal, caudal and ventral fins of the larval tunic and determine its degree of hydrophobicity (Cloney and Cavey, 1982; Robinson *et al.*, 1986). Subsequently however, Cloney (1990) found that when dechoriated *Ascidia callosa* and *Corella inflata* embryos were cultured at higher densities, the

morphology of the tunic was restored; that is, the absence of test cells can be rescued. He concluded that a diffusible 'factor' from embryos is responsible for this behavior and that the function of test cells is to impart hydrophilicity to an otherwise hydrophobic tunic (Cloney, 1990). Remarkably, in *Ciona intestinalis*, removing test cells resulted in the emigration of new cells from the embryo which then re-populated the tunic in a manner reminiscent of the original test cell population (Sato and Morisawa 1999). During metamorphosis, a reduced efficiency of tail resorption (a primary metamorphic event) relative to the rotation of rudimentary juvenile tissues (a secondary metamorphic event) was also observed among larvae lacking the original test cell population (Sato *et al.*, 1999). A role for test cells in coordinating morphogenetic events during metamorphosis was thus proposed, but the possibility that the replacement cells normally participate in metamorphosis was not. Cloney (1990) discussed the finding that, despite careful removal of test cells among his dechorionated embryos, he occasionally found test cells attached to larval surfaces. He may have been observing the replacement cells reported by Sato and Morisawa (1999).

Similar to the larval tunic, the adult tunic is populated by migratory 'blood' cells, some of which are more numerous in the tunic than in the animal proper (Smith, 1970). A fraction of these cells is derived from the trunk lateral cells (TLCs) (Hirano and Nishida, 1997; Tokuoka *et al.*, 2005). At the hatching stage TLCs are positioned as two bilaterally symmetrical groups of mesenchyme in the posterior dorsal trunk region. After hatching and prior to metamorphosis TLCs migrate extensively in an anterior and ventral direction (Nishide *et al.*, 1989). As one of multiple migratory mesenchyme populations that exist in ascidian larvae, TLCs have no known function during the larval stage and thus have been presumed to be uniquely adult precursor cells. During metamorphosis, some TLCs cross the epithelium to the exterior of the animal (Nishide *et al.*, 1989) and reside in the tunic of the juvenile (Tokuoka *et al.*, 2005). Other known fates of the TLCs are listed in Table 1. In both *Halocynthia roretzi* and *Ciona savignyi* TLCs are derived from the A7.6 blastomeres, which are fate restricted at the 64-cell stage (Nishida and Satoh, 1987). Thus, although the origins of TLCs and test cells are (at least temporally) distinct, it is notable that both cell types are migratory and that each associates with the adult and larval tunics, respectively.

Here we build upon these relationships between test cells and TLCs by reporting that both cell types express elevated levels of the protein chaperone HSP90 relative to other embryonic and larval tissues in the pyurid ascidian *Boltenia villosa*. We show that test cells release HSP90 on to the larval tunic during tail elongation. We discuss the shared properties of test cells and TLCs and advance the hypothesis that test cells are descendants of TLCs. We show the ubiquity of HSP90 expression in TLCs, by documenting elevated expression in *Cnemidocarpa finmarkiensis*, *Halocynthia igaboja* and *Botrylloides violaceus*. Finally, whereas HSP90 and HNK-1 antibodies label TLCs in *B. villosa*, these markers label distinct cell populations in *B. violaceus*.

Results

Immunoreaction of test cells and trunk lateral cells to AC88

In first stage oocytes, the ooplasm is strongly immunoreactive to AC88 antiserum (Fig. 2A, arrowhead). No test or follicle cells

TABLE 1

FATES OF THE A7.6 LINEAGE IDENTIFIED BY DIFFERENT TRACING TECHNIQUES IN TWO SOLITARY ASCIDIANS

Species	Fates (function inferred from position or morphology)
<i>C. savignyi</i> ^a	-ciliary epithelium on gill slits of branchial basket -oral siphon muscle -blood cells -tunic cells and tunic granules -gut
<i>H. roretzi</i> ^b	-ciliary epithelium on gill slits of branchial basket -longitudinal mantle muscle, oral siphon muscle -blood cells

are visible at this stage. In maturing oocytes, test cells become visible in sectioned material and immunoreactivity of test cells to AC88 is detected (Fig 2A, arrows). In contrast, eggs have low levels of AC88 immunoreactivity relative to test cells (Fig. 2B). The immunoreactivity of AC88 to test cells was also documented in neurulae and midtail embryos (Fig. 2E-H). Frequently the immunoreaction is visible in a punctate pattern.

Around the time of neurulation in *B. villosa*, the test cells migrate to form clusters of cells, sometimes assembling into a single cluster (Fig. 1B), which collectively migrate(s) over the surface of the embryo. AC88 immunoreactivity in the test cells persisted beyond this stage until the mid-tail bud stage (Fig 2G). After this point we observed a striking shift in AC88 immunoreactivity from the test cells to the surface of the tunic (Fig. 2G,H). Immunoreactivity of the tunic to AC88 persisted after hatching (29-30 h post insemination at 13°C), but decreased with the age of swimming larvae (data not shown). To test whether the deposition of HSP90 from test cells was responsible for the immunoreaction of AC88 to the tunic, we dechorionated embryos at the tail bud stage (which liberated the test cells), immunostained the resulting larvae and compared these to hatched controls. No

immunoreaction to the tunic was observed among larvae from which the test cells were removed (Fig. 2 I-K).

To verify that the AC88 antibody was detecting *B. villosa* HSP90 we performed an immunoblot of total embryonic or larval proteins. A single band (sometimes resolvable into two closely migrating species) was detected by the AC88 antibody at every embryonic and larval stage tested (Fig. 3A). This band migrated further than did the band for bovine HSP90 indicating a protein of approximately 80 kD. We determined the predicted size of ascidian HSP90 by performing an *in silico* translation using ExPasy Proteomics Tools (<http://ca.expasy.org/tools/>) of *Ciona intestinalis* HSP90 identified from the JGI server (<http://genome.jgi-psf.org/Cioin2/Cioin2.home.html>). Consistent with our immunoblotting results, the predicted *Ciona* protein is 727 aa long with a molecular mass of 83.4 kD, a result also reported in Chen *et al.*, (2006). To further verify that the immunoreaction of test cells to AC88 corresponded to the presence of HSP90, twenty *B. villosa* zygotes were manually dechorionated, which liberated the test cells. Proteins from these and 20 control embryos were immunoblotted and probed with AC88. Taken together these results indicate that, relative to the zygote, test cells contain elevated

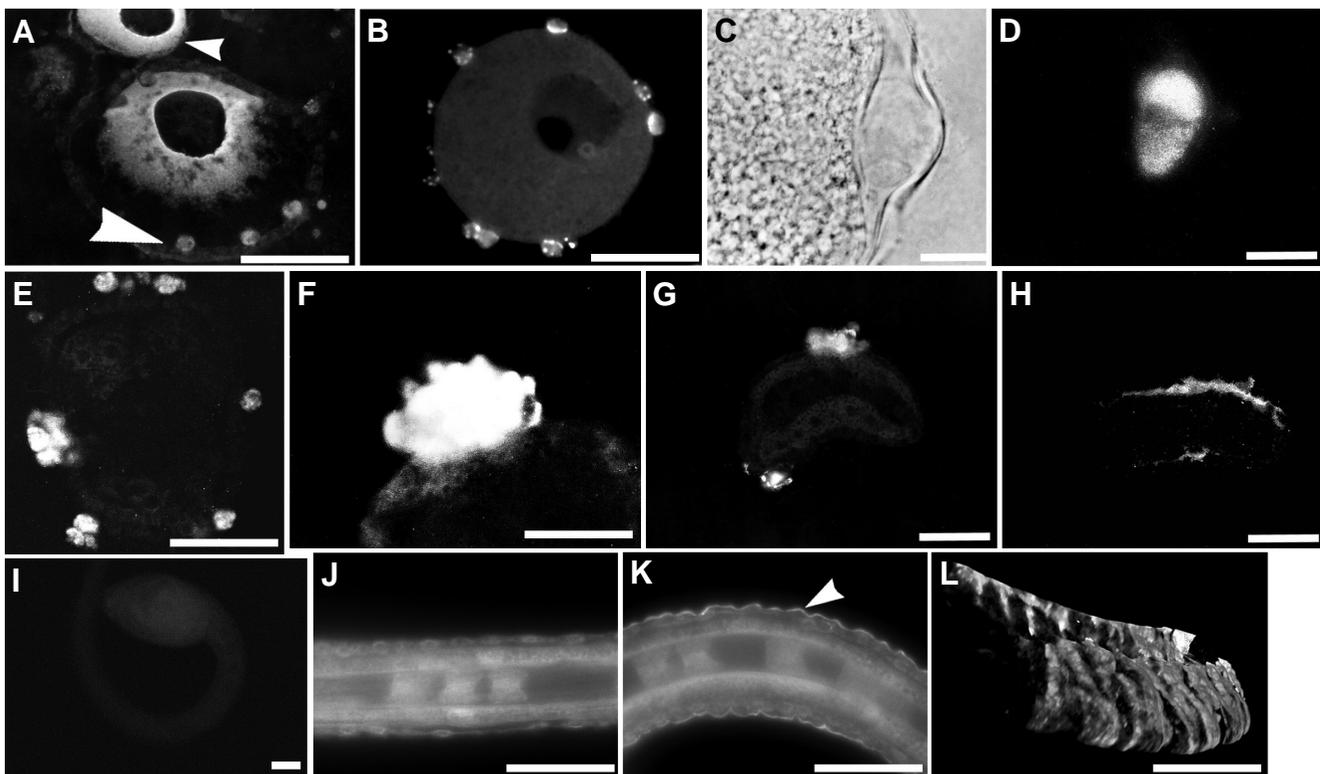


Fig. 2. Immunoreactivity of test cells and the larval tunic to AC88 during development in *B. villosa* in sectioned (A-H) and in whole mount (I-L) preparations. (A) Oocytes at two different stages of maturation dissected from *B. villosa* ovaries. The small arrowhead points to a pre-vitellogenic oocyte and the large arrowhead points to a test cell immunoreactive for AC88 in a vitellogenic oocyte. **(B)** Egg showing punctate AC88 immunoreactivity in the test cells. **(C)** Bright field view of a region of a sectioned egg containing a test cell. **(D)** The same section in (C) showing immunoreactivity exclusive to the test cell. **(E)** Section of a neurula stage embryo through the dorsal/ventral plane. Anterior is up, and note that the test cells have begun to aggregate. **(F)** Higher magnification of a cluster of test cells on the surface of a neurula stage embryo. **(G)** Sagittal section of a tailbud larva at 20 hours p.f. **(H)** Similar section of a developing tail as in (E) but at 22 hours p.f. The immunoreactivity to AC88 is no longer observed in test cells, but is now on the surface of the tail. **(I-L)** Whole mount preparations of larvae. **(I)** Negative control corresponding to panels **(J-K)** in which the primary antibody was omitted. **(J)** Larva from an embryo in which test cells were removed at the early tailbud stage. No immunoreaction to the tunic was observed. **(K)** Larva from unoperated controls. Arrowhead points to immunoreaction on the tunic. **(L)** A 3D projection of 145.27 μm of a region of tail from a hatched control larva. Anterior is to the left. Scale bars in (A,B) 70 μm ; (C,D), 10 μm ; (E-L), 50 μm .

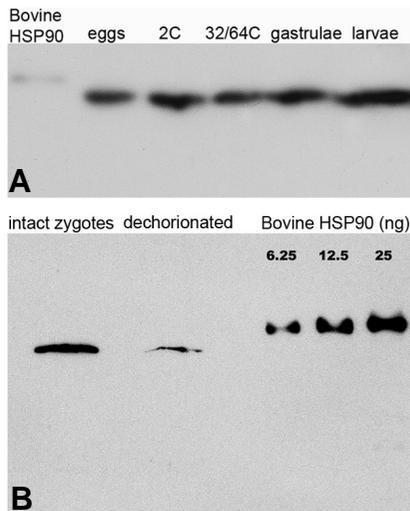


Fig. 3. Immunoblots of *B. villosa* embryonic and larval lysates probed with AC88. (A) Immunoblot of total cytosolic proteins from various developmental stages probed with AC88. A single polypeptide of ~80 kD is present in all stages. Purified bovine HSP90 was used as a positive control for the probe and as a reference for protein mobility among samples. (B) Immunoblot of total cytosolic proteins from 20 intact and dechorionated zygotes and increasing concentrations of bovine HSP90.

levels of a protein with the electrophoretic and immunoreactive properties of HSP90 (Fig. 3B). We conclude that AC88 immunoreaction is HSP90 expression and henceforth refer to it accordingly.

Elevated HSP90 expression in larval mesenchyme

The other prevalent pattern of HSP90 expression in *B. villosa* was observed in mesenchyme originating in the larval posterior trunk (Fig. 4). A punctate staining pattern became detectable in the cytoplasm of these cells within one hour of hatching (Fig. 4 A,B); larvae fixed immediately after hatching showed no elevated staining in these cells (not shown). After elevated HSP90 becomes detectable in these cells, they begin to migrate anteriorly and ventrally (Fig. 4C). In sectioned larvae immunoreactive cells are often observed between the presumptive gut and the epidermis (Fig. 4D) and in the space between the anterior larval

epidermis and the larval gut. This space becomes enlarged during larval life as the yolk in the presumptive gut is depleted. This position is maintained until metamorphosis is initiated, at which time immunostained cells can be seen exterior to the epidermis (Fig. 4E). Elevated levels of HSP90 are observed for up to 24 hours after metamorphosis (data not shown). In larvae that delay metamorphosis, immunoreactive cells are observable for longer than the elapsed time between hatching and 24 hours post-metamorphosis, indicating that the loss of HSP90 expression in these cells is associated with metamorphosis.

Larval mesenchyme corresponds to the trunk lateral cells

In the pyruid *Halocynthia roretzi*, TLCs are both migratory and occupy a position at hatching that is similar to the HSP90 positive cells that we describe here (Nishikata and Satoh, 1991). To test the possibility that TLCs in *B. villosa* express high levels of HSP90, we took advantage of the ascidian determinate cleavage pattern and ablated blastomeres in the embryo that give rise to TLCs. Cell lineage analysis indicates that the TLCs in *H. roretzi* are derived from the A7.6 blastomeres situated on the vegetal side of the embryo (Nishida and Satoh, 1987). In order to test whether the HSP90 expressing cells in *B. villosa* larvae were TLCs, we ablated the A5.2 blastomeres. These blastomeres give rise to A7.5-A7.7 (presumptive endoderm, TLCs and notochord) and the A8.15 and A8.16 blastomeres (presumptive muscle and nerve cord) (Nishida and Satoh, 1987). Because we were employing manual cytolysis methods, the A5.2 blastomeres represented the latest point in development that we could reliably identify and ablate blastomeres. Embryos lacking the A5.2 lineage continued to cleave, gastrulated, neurulated (Fig. 4F) and developed into relatively normal looking larvae with recognizable axes (Fig. 4G). Larvae lacking the A5.2 descendants were fixed and sectioned for immunocytochemistry with AC88. No immunoreactive cells were observed among larvae lacking the descendants of the A5.2

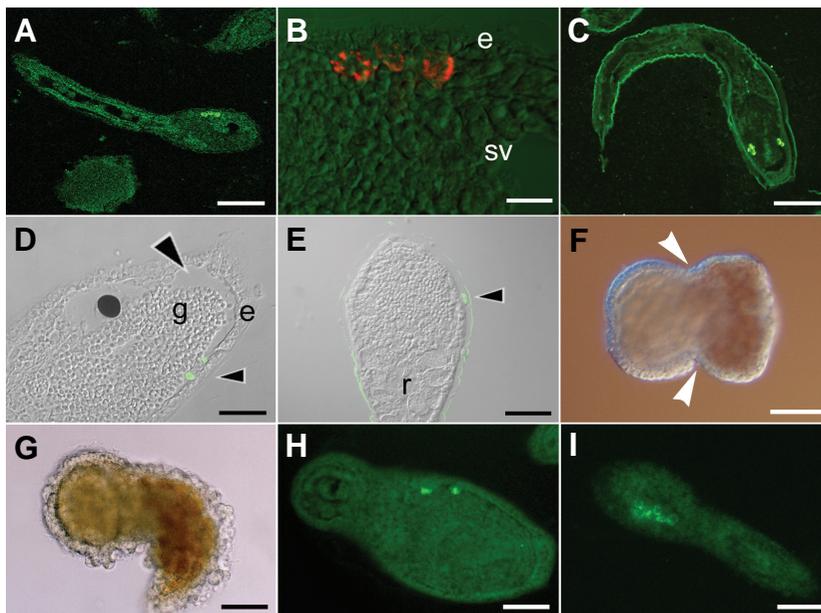


Fig. 4. Immunoreactivity of larval mesenchyme cells with AC88 antibody. (A) Sagittal section of a newly hatched larva prior to the migration of mesenchyme. (B) Confocal image of the position of pre-migratory mesenchyme relative to the dorsal epidermis (e) and the sensory vesicle (SV). Note the punctate pattern of staining. (C) A hatched larva sectioned along the dorsal-ventral axis. Two pockets of mesenchyme are positioned symmetrically around the midline as they migrate anteriorly. Although these cells are cohesive, in other preparations they migrate as independent cells. (D) Sagittal section of a hatched larva showing a single immunoreactive mesenchyme cell (small arrowhead) positioned between the presumptive juvenile gut (g) and the epidermis (e). AC88 immunoreactivity is restricted to the leading and trailing extremities of this cell. Large arrowhead points to the final position of migrating TLCs before the initiation of metamorphosis. This and the following image were created by overlaying a DIC image with a fluorescent image. (E) Section of a larva that has initiated metamorphosis. Resorbed muscle cells (r) are evident in the posterior half of the zooid. Note the position of the immunoreactive cell exterior to the epidermis. (F) A live neurula/early tailbud embryo lacking the A5.2 descendants. Arrowheads point to the position of the extirpated A5.2 lineage descendants. (G) Image of a live larva lacking the descendants of the A5.2 lineage. This and other larvae from which the A5.2 lineage was removed were sectioned and immunostained with AC88. (H) Sagittal section of the trunk region of a *H. igaboja* hatched larva immunoreactive for AC88. (I) Sagittal section of a *C. finmarkiensis* larva immunoreactive for AC88. Scale bars are 100 μ m in (A); 10 μ m in (B); 50 μ m in (C,F-I) and 25 μ m in (D,E).

lineage. Thirty-nine percent (100/279) of sections of control larvae examined contained one or more immunoreactive cells, whereas 0% (0/110) of the sections derived from larvae lacking the A5.2 lineage contained immunoreactive cells.

Elevated HSP90 expression in larval mesenchyme of other species.

To test whether the pattern of HSP90 expression in *B. villosa* was unique we performed immunostaining on three additional species. The pyurid *H. igaboja* and the styelids *C. finmarkiensis* and *B. violaceus* display a similar pattern of HSP90 expression in mesenchyme of the trunk (Fig. 4 H,I; Fig. 5 A,B). We assume that these are TLCs. The punctate character of the label supports this assumption. Interestingly, in *B. violaceus* protostigmata expressed elevated levels of HSP90 in a heterogeneous fashion (Fig. 5C). Higher magnification views show this staining to be punctate, as in HSP90 positive mesenchymal cells (Fig. 5D).

We hypothesized that the HSP90 positive cells in *B. violaceus* were the same as the HNK-1 positive cells reported in Jeffery (2006). To test this, we double stained sections of *B. violaceus* embryos and larvae with AC88 and anti-HNK-1 (5G-I). Despite their spatial coincidence, we observed no cells in which both antigens were present, indicating that, with respect to HSP90 and HNK-1, these cell populations are developmentally distinct at this stage. In marked contrast, when we conducted the same double staining experiment with sections of *B. villosa* larvae we observed that HSP90 and HNK-1 are present in the same cell, namely the TLCs (Fig 5 J-L). To our surprise, we also observed that the test cells in *B. villosa* eggs are immunoreactive to anti-HNK-1 (Fig. 5 M-O). Double staining experiments indicated that while HSP90 and HNK-1 are both present in the test cells their patterns of immunoreactivity are distinct, suggesting that their subcellular localizations do not overlap. As with AC88, the tunic of hatched larvae was immunoreactive to HNK-1 (data not shown).

Discussion

In addition to folding viral proteins involved in oncogenic transformation (Xu and Lindquist, 1993), HSP90 is upregulated several fold among a wide variety of mammalian tumors (Tsutsumi and Neckers 2007 for review). Taken together with a recently identified mitochondrial specific chaperoning complex in tumor cells (Kang *et al.*, 2007) a significant role for extracellular HSP90 in cancer cell biology is evident. As a result of this intimate relationship between HSP90 function and neoplastic transformation and cell migration, some HSP90 inhibitors representing distinct chemical classes of molecules are in various stages of clinical trials as human anti-cancer drugs (Tsutsumi and Neckers, 2007).

Recent years have seen interest in molecular chaperones in general and HSP90 in particular extend from cell biology, biochemistry and biomedical sciences into development, evolution and ecology (e.g. Sass *et al.*, 1996; D'Sousa and Brown, 1998;

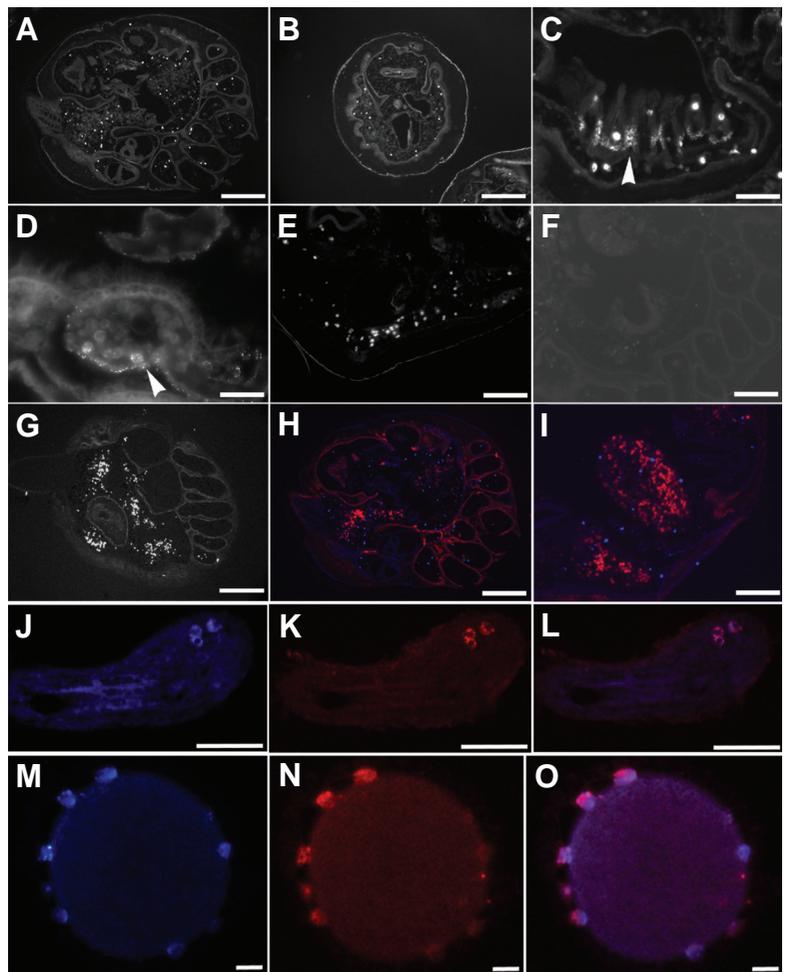


Fig. 5. Sections of *B. violaceus* (A-I) and *B. villosa* (J-L) larvae and *B. villosa* eggs (M-O) immunolabeled with AC88 and anti-HNK-1 antibodies. (A) Longitudinal section of the trunk of a larva. Immunoreactive cells are distributed throughout the trunk. (B) Transverse section of the trunk. (C) Protostigmata in which immunoreactivity is heterogeneously distributed (arrowhead). (D) Higher magnification view, showing the punctate staining present in both epithelium of the protostigmata and mesenchyme (arrowhead). The absence of staining on the opposite surface of the protostigmata corresponds to the absence of immunoreactive mesenchyme. (E) Positive control section for (F) a section treated with AC88 antibody preadsorbed with human HSP90. Both these images were collected using identical settings, but the brightness in F was increased to demonstrate the abolition of staining. (G) Longitudinal section immunostained with anti-HNK-1. (H,I) Sagittal sections double stained with HNK-1 (red) and AC88 (blue). No overlap in immunoreactivity was observed between these two populations of mesenchyme. (J-K) Sagittal sections of *B. villosa* hatched larvae stained with AC88 and HNK-1 antibodies. (L) Merged images of J and K indicating that the immunoreaction corresponds to the same cells. (M) *B. villosa* egg stained with AC88. (N) Same section as in (A) stained with HNK-1. (O) Merged image from (M,N). Scale bars are 100 μm in (A, B, G, H); 20 μm in (D, M-O); 50 μm in (C, E, F, I-L).

Rutherford and Lindquist 1998; Roberts and Feder, 1999; Bishop *et al.*, 2001; Bishop *et al.*, 2002; Gunter and Degnan, 2007). Incisive inquiry into HSP90 function in animal development has historically been incidental (e.g. Ding *et al.*, 1993; Cutforth and Rubin 1994) due in part to a lack of interest in what are perceived to be merely housekeeping genes (and therefore outside of the interest of most developmental biologists) but also to the difficulty

in studying HSP90 function using forward genetics in animal models; mutant alleles of *hsp83* were rarely viable in fruit flies. The construction of strains of *D. melanogaster* with different heteroallelic combinations of *hsp83* mutants allowed a nuanced and thorough investigation of HSP90 function in relation to morphogenesis (Rutherford and Lindquist, 1998). In this landmark paper Rutherford and Lindquist (1998) showed that, through its chaperoning function, HSP90 masked the phenotypic effects of mutations in protein coding genes. This masking effect was revealed by genetically or pharmacologically lowering HSP90 levels or from environmental stress. This and subsequent findings (Queisch *et al.*, 2002; Milton *et al.*, 2003; Milton *et al.*, 2006; Suzuki and Nijhout, 2006; Yeyati *et al.*, 2007; Connolly and Hall, 2008) have placed HSP90 at a focal point in the relationship between environmental stress and genotypic versus phenotypic change and has provided a (still somewhat vague) mechanistic basis for genetic assimilation and canalization (Waddington, 1953a,b). Despite these advances, detailed reports of developmental function for HSP90 are rare but when elucidated they are fascinating (Ding *et al.*, 1993; Cutforth and Rubin; Song *et al.*, 2007) and serve to remind us about the false dichotomy between housekeeping and regulatory protein function during development. Taken together with recent work describing differential spatial and temporal expression of HSP70, HSP90 and HSF during development of a lophotrochozoan (Gunter and Degnan, 2007) it is clear that ongoing investigations into cell type specific function of molecular chaperones during development will be fruitful if not methodologically challenging.

Although considered to be exclusively a cellular protein, a growing literature is describing an extracellular distribution (and in some cases, a function) for HSP90. Originally identified as a tumour specific antigen in mice (Ullrich *et al.*, 1986), HSP90 was identified as a secreted component of metastatic mammalian cells where it performs chaperoning roles for matrix metalloproteinase 2 (Eustace *et al.*, 2004). A novel interaction between extracellular HSP90 and HER-2, a member of the ErbB family of receptor tyrosine kinases involved in cellular differentiation, migration, and cancer progression has recently been described (Sidera *et al.*, 2008). Extracellular distributions of other components of the canonical HSP90 based chaperoning complex, including HSP70, p23 and Hop, have also been documented (Eustace and Jay 2004; Lee *et al.*, 2006). Mammalian dermal fibroblast and neural cells deposit HSP90 into the extracellular matrix where it is required for migration and cytoskeletal modifications (Sidera *et al.*, 2004; Li *et al.*, 2007). Evidently, extracellular HSP90 is not a condition unique to cancer cells and may therefore have more general function during morphogenesis than currently appreciated.

Using urochordates, we demonstrate a second example of an association between elevated HSP90 expression and extracellular HSP90 in the context of cellular migration. This indicates that the condition of extracellular HSP90 is not restricted to neoplastic cells, nervous systems, or mammals, but is an emerging shared property of some migrating cells may therefore have more general function during morphogenesis.

Test cells and the larval tunic

Test cells express elevated levels of HSP90 from late stage oogenesis until the mid-tail stage. Immunoblot analyses verified

the specificity of the antibody and indicated a single protein or two closely migrating proteins of ~80kD that cross-react with AC88. A comprehensive bioinformatic analysis of genes encoding HSP90 indicates that the *Ciona intestinalis* nuclear genome contains two, one of which is predicted to encode a protein of 83.3 kD (Chen *et al.*, 2006). By manual dechoriation we removed test cells from the surface of zygotes and showed that such embryos contain less HSP90 than control embryos. The HSP90 was restricted to test cells until the mid tail stage at which point it simultaneously became undetectable in test cells and appeared on the larval tunic indicating that *B. villosa* test cells contain and deposit HSP90 on the larval tunic. The removal of test cells from tail bud embryos resulted in the abrogation of immunoreactivity on larval tunics, verifying the observation that test cells deposit HSP90 on the larval tunic. Given that some test cells in *C. intestinalis* undergo apoptosis (Maury *et al.*, 2006), it remains possible that the observed deposition on to the tunic of HSP90 by *B. villosa* test cells is the result of apoptosis and is therefore not an active mechanism by which test cells to modify the larval tunic. In contrast to the findings of Maury *et al.* (2006) widespread apoptosis of test cells in *B. villosa* has not been observed to occur (Bates, 2004). Moreover, the cytoplasm of apoptotic cells is always surrounded by membrane, precluding the release of cellular contents into the immediate cellular, or in this case acellular, milieu.

The present results extend those of Takamura *et al.* (1996) and Okada *et al.* (1996) in which the larval tunic includes a proteinaceous component generated, in part or in whole, by the test cells. The antigens reported in Takamura *et al.* (1996) and Okada *et al.* (1996) have not been characterized and thus, HSP90 is the first identified protein that is deposited from the test cells on to the larval tunic. The mechanism by which test cells release their contents is not known, but test cells of *Styela plicata* can be induced to release heparin and histamine by the mammalian mast cell secretagogue compound 48/80 (Cavalcante *et al.*, 2002), invoking GTPase and phospholipase C- and D- activity (Chahdi *et al.*, 1998a,b, 2000; Mousli *et al.*, 1990; Senyshyn *et al.*, 1998).

Two observations indicate an evolutionary association of test cells and larval tunics: (i) Embryos of larvaceans, the sister taxon to Ascidiaceans + Thaliaceans (Wada, 1992), do not contain test cells (Cloney, 1990) and do not have a larval stage, (ii) Embryos of the direct developing ascidian *Molgula pacifica* do not contain test cells (Young *et al.*, 1988; Bates and Mallet, 1991; Cloney, 1994). For reasons that are still not clear, but are almost certainly related to the deposition of materials by test cells discussed above, this association may be required to render the tunic an adaptive structure.

Cloney's hypothesis that test cells render the tunic hydrophilic or 'wettable' is attractive; the external covering of any small marine organism must be sufficiently hydrophilic to avoid getting stuck at the air water interface. The removal of test cells from neurulae generated larvae with poorly formed fins and such larvae stuck to each other and to glass surfaces (Cavey and Cloney, 1982). However, experimentally induced hydrophobicity by removal of test cells may be a secondary effect and not reflect the primary function of the material released from test cells. The ascidian tunic, although historically considered to be composed primarily of carbohydrates, was shown to be 50% protein (Smith and Dehnel, 1970). If this can be generalized across species and

to the larval tunic, the role of a molecular chaperone in this external milieu becomes less peculiar in light of its emerging function as a chaperone that is capable of functioning extracellularly (Eustace *et al.*, 2004). Cloney (1990) observed that test cells of some species adhere to larval tunics, whereas in others, test cells are lost at hatching. Given this variation among species it should be possible to further elucidate any relationship between the deposition of HSP90 and the subsequent migration of test cells.

Trunk lateral cells

As one of multiple distinct populations of larval mesenchyme, TLCs exist at hatching in the dorsolateral region of the trunk. Based on the similar position at hatching of HSP90-positive cells in *B. villosa* to TLCs in *H. roretzi*, we hypothesized that the former were TLCs. This idea was tested and supported by removing the embryonic lineage that gives rise to the TLCs. We based this experiment on the assumption that TLC cell lineages between *B. villosa* and *H. roretzi* are conserved. Because these two species are in the family Pyuridae and because the TLCs are derived from the A7.6 blastomeres in both *H. roretzi* (Pyuridae) (Nishida and Satoh, 1987) and *C. savignyi* (Styelidae) (Tokuoka *et al.*, 2005), this assumption is reasonable. The A5.2 lineage was not fate restricted at the time of extirpation. Other fates besides TLCs include notochord, endoderm, tail muscle and nerve cord (Nishida and Satoh, 1987). Thus, it is possible that elevated HSP90 expression is localized to a non-TLC population of mesenchyme and that the abolition of immunoreactivity was due to the loss of conditional specification of non-TLC mesenchyme by cells of the A5.2 lineage. By virtue of their position in cleavage arrested embryos, HNK-1 labeled cells were identified as TLCs in *Ciona intestinalis* (Jeffery *et al.*, 2008). Together with the blastomere ablation experiments here, their position at hatching and the observation in *B. villosa* that HNK-1 and HSP90 are present in the same cell strongly supports the conclusion that the cells expressing elevated levels of HSP90 are TLCs.

In contrast to the test cells, TLCs are immunoreactive between hatching and metamorphosis. Loss of HSP90 expression in TLCs occurs 24 hours after tail resorption is initiated and appears to be responsive to the induction of metamorphosis. The punctate pattern of intracellular localization of HSP90 in test cells and TLCs is consistent with it being membrane bound in cisternae of the ER or perhaps in secretory vesicles such as exosomes.

It is notable that both cell types that express elevated levels of HSP90 are migratory, but not all migratory cells in larvae express elevated levels of HSP90. For example, there are four distinct lineages of mesenchymal cells in the ascidian tadpole, defined by embryonic origins, fates and gene expression patterns (Nishida and Satoh, 1987; Sotgia *et al.*, 1993; Hirano and Nishida, 1997; Tokuoka *et al.*, 2004). Only one of these appears to require elevated HSP90 for its function. Moreover, in *B. violaceus* we observed that HNK-1 positive mesenchyme, at least some of which are migratory presumptive pigment cells (Jeffery, 2006), did not express elevated HSP90, suggesting no particular requirement for HSP90 compared to their HSP90 positive mesenchymal counterparts. In *B. violaceus* we observed HSP90 expression in the protostigmata, perhaps the result of a contribution from HSP90 positive mesenchyme. Because TLCs contribute to the pharynx in *C. intestinalis* and *C. savignyi* (Hirano and Nishida,

1997; Tokuoka, 2005) and because HSP90 is expressed in TLCs in several taxa, it is likely that the HSP90 positive mesenchyme in *B. violaceus* is homologous to TLCs.

We documented the expression of HSP90 in larval mesenchyme from four species representing two of the three orders of ascidians, indicating that this may be a character shared by the entire class. In *B. violaceus*, we tested the possibility that the mesenchyme immunoreactive for AC88 were the same cells observed by Jeffery (2006) to express HNK-1 a marker of presumptive pigment cells. We observed no overlap in staining between these cells, suggesting no developmental relationship in this species at this stage. We consider it interesting that HNK-1 and HSP90 expression overlap in the relatively simple larva of a solitary ascidian but not in the highly adultated larvae of a compound ascidian. One possibility is that HSP90 and HNK-1 expressing cells in *B. violaceus* share embryonic origin but differentiate into two populations prior to hatching.

A relationship between test cells and some TLC descendants?

Because one of two competing hypotheses about the origin of test cells posits that they are trans-differentiated ameoboid or lymphocytic blood cells, and because hemocytes ('blood' cells) are one of the fates of TLCs, emerging similarities between test cells and some of the TLCs descendants warrant further consideration.

In this paper we show that the two cell types that express elevated HSP90 are migratory and associate with the tunic, suggesting functional similarities. This similarity was further demonstrated by immunolabeling experiments with anti-HNK-1, in which both test cells and trunk lateral cells were stained. A third connection between test cells and trunk lateral cells has been revealed by investigations into the presence of heparin and histamine in ascidians (Cavalcante *et al.*, 2000, 2002). In vertebrates, these molecules are associated with immunologic function and are secreted exclusively by a subtype of mast cell. There are three cell types in the ascidian *Styela plicata* that are known to contain both heparin and histamine: test cells, granulocytes (a type of hemocyte) and cells lining the pharynx (Cavalcante *et al.*, 2000; Cavalcante *et al.*, 2002; de Barros *et al.*, 2007). Again, given the 'blood' cell fate of some TLC descendants (although their specific fate with respect to function is not known) and the contribution of TLC descendants to the epithelium lining the stigmata (Hirano and Nishida, 1997; Tokuoka, 2005) the shared presence of heparin and histamine between these three cell types is intriguing. Fourth, as a peripheral observation, Takamura *et al.* (1996) noted that unidentified larval mesenchyme appeared to contain antigens that bound one of their 'test cell-specific' antibodies. They also noticed this immunoreaction in adult 'blood' cells. It is possible that the mesenchyme Takamura *et al.* (1996) observed to express the antigen recognized in the test cells were descendants of TLCs. A fifth, albeit weaker, similarity between test cells and trunk lateral cells is informed by the work of Sato and Morisawa (1999) in which it was shown that new cells emigrate from developing *C. intestinalis* embryos if the test cells are removed by dechoriation. We and others have observed that TLCs cross the anterior trunk epithelium shortly after hatching. Thus, TLCs constitute a cell population that can cross the larval epithelium and populate the exterior of the animal. Although, for

reasons of timing, Sato and Morisawa argued against the possibility that the replacement cells they observed were TLCs, this possibility has not been experimentally falsified.

We suggest that test cells and the fraction of trunk lateral cell descendants that become 'blood' cells are functionally and ontogenetically related. That is, we hypothesize that test cells are descendants of the TLCs. The ability to generate long term (genetic) labeling of trunk lateral cells will be required to falsify this hypothesis.

TLCs and the origin of the neural crest

On the basis of gene expression studies it has recently been reported that trunk lateral cells are neural crest-like (Jeffery *et al.*, 2008). Although we agree with this general assertion (i) earlier reports indicate that TLCs are part of a population of mesenchymal precursors of adult mesoderm. (Hirano and Nishida, 1997; Tokuoka *et al.*, 2004; Tokuoka, 2005) and (ii) no TLC descendants are known to differentiate as neurons. Based on the presence of heparin and histamine (Cavalcante *et al.*, 2000; Cavalcante *et al.*, 2002), test cells are proposed to be homologous with (mesodermally derived) mammalian mast cells. If test cells are in fact descendants of TLCs, and if ascidian test cells and mammalian mast cells are homologous, then TLCs appear to have properties and fates attributable to both vertebrate mesoderm and neural crest. Although anti-HNK-1 is a neural crest marker (Tucker *et al.*, 1984), the antigen from which it was derived was extracted from human large granular lymphocytes (Abo and Balch, 1981). Later this antigen was found to be identical to that which generated the antibody NC1 from extracts of quail ciliary ganglion (Vincent and Thiery, 1984). Here in a urochordate we observe that the HNK-1 antigen appears to be present in test cells and trunk lateral cells, two cell types that, respectively, have been homologized with vertebrate mesodermal and neural crest cells (Cavalcante 2002, Jeffery *et al.*, 2008). The implications for current models of neural crest origin and evolution are fascinating indeed.

Materials and Methods

Immunohistochemistry and immunoblotting

Specimens were fixed in methanol and ethanol (-20°C) for twenty minutes each and then embedded and sectioned as previously described (Bates, 1988) or stained processed as whole mounts. The anti-HSP90 antibody was monoclonal (clone AC88; Stressgen Biotechnologies, Victoria B.C.) and the antigen used for immunization was purified from bovine brain. This antibody recognizes HSP90 from species as diverse as water molds and vertebrates and was thus judged to be suitable as a heterologous antibody. Sections were incubated for two hours at room temperature in a dilution of 1:100-1000 AC88 from a 1 µg/mL stock whereas whole mounts were incubated in a 1:100 dilution overnight at 4°C. Antibody was diluted in blocking buffer (2.5-5% bovine serum albumin with 0.05-0.1% Triton-X-100 in PBS pH 7.4). After washing the primary antibody in blocking buffer, sections were incubated with goat anti-mouse IgG conjugated to FITC or Alexa 488 for 1 h at room temperature (Sigma or Molecular Probes). After final rinses in PBS, specimens were mounted in 70% glycerol in PBS and viewed using an Olympus fluorescence microscope, a Zeiss LSM410 confocal microscope or a Zeiss AxioPlan epifluorescence microscope. Various, omission of the primary antibody, the use of a non-relevant antibody or pre-adsorption of the primary antibody with a 5X molar excess of purified bovine HSP90 (Stressgen Biotechnologies Inc.) served as controls.

Immunoblotting was performed essentially as described in Bates and Bishop (1996). Samples were centrifuged briefly, seawater was aspirated with a fine pipette and samples were lysed directly in 1X Laemmli sample buffer. Lysates were centrifuged at 10,000 rpm for 5 min before placing the supernatant in a boiling water bath for 4 min prior to use or storage at -70°C. Purified bovine HSP90 was prepared for electrophoresis in the same manner and used as a positive control. Proteins were separated by electrophoresis on 8 or 10 % discontinuous polyacrylamide gels in SDS and blotted onto PVDF membrane (Bio-Rad). AC88 was applied at 1:1000 at 4°C overnight, while goat anti-mouse IgG conjugated to HRP was applied at 1:1000 or 1:2000 at room temperature for 2 h. ECL (Amersham Pharmacia) detection was carried out according to the manufacturer's instructions.

Microsurgical manipulations

To prepare material for immunoblotting, 20 zygotes were manually dechorionated using steel insect pins (Elephant brand #2 or #3) mounted on Pasteur pipettes, as described by Bates and Jeffery (1987) and then transferred to a separate Syracuse dish. After verifying that no test cells were transferred, the dechorionated zygotes were placed in microfuge tubes and processed as above. For blastomere ablation experiments zygotes were dechorionated after the first stage of ooplasmic segregation (20 minutes after insemination), and then cultured in Syracuse dishes coated with 0.9% agar. At the 16-cell stage A5.2 blastomeres were manually cytolysed by disrupting the plasma membranes with a sharpened steel needle. Shortly after cytolysis blastomeres turned white and became loosely associated with the embryo. These were removed by gently pipetting the embryo. Embryos lacking the A5.2 blastomeres were then cultured until the controls hatched, and then fixed and embedded as described by Bates (1988). For experiments that verified the deposition of HSP90 from test cells, tailbud stage embryos (18-20 hours post insemination at 13°C) were dechorionated and then cultured until the controls hatched. These and controls were fixed for whole mount immunocytochemistry as above except that larvae were incubated in primary antibody at 1:100 at 4°C overnight and secondary antibody at 1:200 overnight at 4°C.

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