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Heat shock response and shape regulation during newt tail regeneration

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ABSTRACT

Regenerating newt tail has recently been found to react to hypergravity in a stable and reproducible way – by curving downwards. Such morphogenetic effect of non-specific physical factor applied to a complex structure of an adult animal is a rare phenomenon with unknown molecular basis. For the first steps of unraveling this basis we've chosen heat shock proteins (HSPs) as promising candidates. Morphometrical analysis of tail regeneration was performed in aquarium (control), on substrate (relative hypergravity) and in aquarium under weekly application of heat shock. HSPs were inhibited pharmacologically during regeneration in aquarium and on substrate. *Hsp70, 90* gene expression and protein localization were analyzed in the studied conditions. Weekly application of heat shock to newts regenerating tails in otherwise normal conditions led to development of curved tails (both upwards and downwards), suggesting that similar mechanisms are at play in both hypergravity-altered and heat shock-altered morphogenesis. Heat shock protein inhibitor KNK437 didn't affect tail shape during normal regeneration, but prevented the formation of tail curve in appropriate conditions. It was shown that HSP70 and HSP90 proteins are present in muscle and connective tissue of intact tails as well as regenerates, but only appear in epidermis in hypergravity-altered regenerates and heated tails. Based on our data, we hypothesize, that different external factors (e.g. hypergravity and heat shock) are received, analyzed and transmitted further to affect morphogenesis by similar mechanisms that utilize a set of HSP in epidermal cells.

1. Introduction

Classical model organisms of regeneration research (such as amphibians) have lots of benefits that are applicable in other areas. For example, they have long been used as model objects in gravitational biology. These studies started in pursue of understanding mechanisms of egg cytoplasm reorganization, but it soon became evident that amphibian models are also useful to study broader questions of axis formation and morphogenesis. It was shown that although *Xenopus leavis* development can proceed in spaceflight and simulated microgravity, some transient morphological differences from normal embryos occur under such conditions (De Mazière et al., 1996; Neff et al., 1993; Souza et al., 1995; Ubbels, 1988; Ubbels et al., 1994). Simulated hypergravity led to axial bifurcation when applied at certain early stages (Black and Gerhart, 1986; Neff et al., 1990) and to transient morphological aberrations when applied at later ones (Neff et al., 1993). *Pleurodeles waltl* entered gravitational research with French, Russian and German experiments of the 1980-s; regeneration of its organs in space was studied at IDB RAS in Moscow (Anton et al., 1996; Grigoryan et al., 2008, 2006, 1998, 1992; Mitashov et al., 1996).

Morphogenetic effect of hypergravity was first observed in Foton M3 spaceflight experiment in 2007 that included three groups of animals: aquarium control in the lab, flight group in containers with moist substrate onboard the satellite and synchronous control in the same containers in the lab. While regenerates in the further two groups appeared normal (highly symmetrical), regenerates in the latter one were curved downwards (Grigoryan et al., 2008). Conditions of the synchronous control group can easily be reproduced in the lab, so this effect became a separate line of research at IDB RAS in Moscow (Radugina and Grigoryan, 2012). Tail curve was proven to occur stably when newts were placed on moist substrate rather than in aquarium after tail amputation. Morphometrical tools for quantitative analysis of tail shape alteration were developed; histological studies were performed and showed that not only the overall tail shape, but also the regenerating spinal cord and vertebrate column were curved downwards. It was also noticed (and confirmed with 5-bromo-2′-deoxyuridine assay) that apical epithelium thickens when tails regenerate on substrate. Interestingly, hypergravity experiments with a centrifuge led

Abbreviations: HSP, heat shock protein; Δg, altered gravity regime (collective abbreviation for micro- and hypergravity); ΔK, coefficient of tail shape alteration (please see Materials & Methods for detailed explanation); dpa, days post amputation; qPCR, quantitative polymerase chain reaction; IHC, immunohistochemistry; RT, room temperature; HS, heat shock

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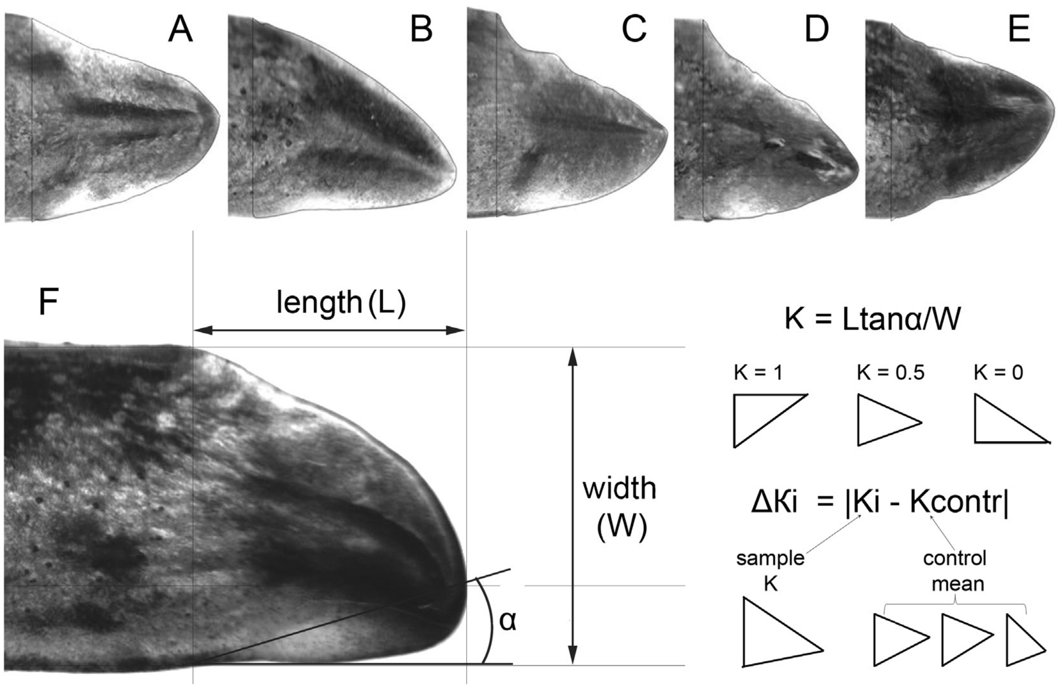

Fig. 1. Typical appearance of 49 day old regenerates. Samples from aquarium control (A), substrate group (B) and group undergoing weekly heat shock in aquarium (C, D, E). F – morphometrical analysis of tail regenerates.

to the same tail curves, supporting the hypothesis that altered tail shape on moist substrate is due to increased weight of the animals compared to partial weightlessness (hypogravity within an order of magnitude) normally experienced by them in aquarium (Grigoryan et al., 2017).

Thus, both hypergravity and microgravity, resulting in altered weight of the animal and consequential changes in its interactions with the surrounding matter, can affect animal development in various ways. It is especially interesting to find molecular mechanisms responsible for reception of altered gravity, transmission of information about it through the organism and cellular responses to it. Research in gravitational biology paid lots of attention to stress molecules, e.g. heat shock proteins, or HSPs. Data are scarce and were obtained using many different model organisms (from plants to human cells), but suggest that HSPs can be affected by micro- and hypergravity (Carlsson et al., 2003; Cubano and Lewis, 2001; Gillette-Ferguson et al., 2003; Grigoryan et al., 2008; Huin-Schohn et al., 2013; Ishihara et al., 2008; Kumei et al., 2003; Minois et al., 1999; Ohnishi et al., 1998; Rizzo et al., 2002; Shimada and Moorman, 2006; Zupanska et al., 2013). It has also been demonstrated that certain HSPs accumulate in developing tissues in the absence of stress factors (for example, the review by Heikkila (2010) summarizes such data for Amphibia) and can be crucial for proper morphogenesis in Fungi (Tiwari et al., 2015), *Hydractinia* (Duffy et al., 2012), *Xenopus laevis* (Brown et al., 2007), *Danio rerio* (Rosenfeld et al., 2013). Abundant synthesis of HSPs has been demonstrated in the regenerating limb of *N. viridescens* (Carlone and Fraser, 1989; Tam et al., 1992) and tail of *A. mexicanum* (Lévesque et al., 2005). In some cases, HSP expression has been proven necessary for blastema formation and proper regeneration (Makino et al., 2005).

Heat shock itself is a well-studied stress factor that is easy to arrange in the lab; besides, temperature is known to affect newt regeneration (Turner and Tipton, 1973 – reviewed in Connelly, 1977; Schauble, 1972; Radugina, Grigoryan, unpublished observations) and morphogenesis in different species (multiple examples are known; Cooke and Elsdale (1980) demonstrated temperature's effects on amphibian segmentation, for instance). In this regard we were interested to check if HSPs are affected by our experimental conditions (relative hypergravity on moist substrate, compared to aquarium) and to find out if heat shock can also affect tail morphogenesis during regeneration. Similar shape alterations resulting from exposure to such different unrelated factors would suggest universal underlying mechanisms that may be interesting and valuable beyond the limits of this particular model.

2. Materials and methods

2.1. Animal care and operations

10–14 months old *Pleurodeles waltl* (Michahelles, 1830) were obtained from IDB RAS breeding facility. Animal handling was performed in concordance with EU Directive 2010/63/EU for animal experiments and RAS bioethical guidelines. Newts were kept in water-filled tanks under natural day-night regime and room temperature (RT). Newts were anesthetized for 10 min with 1:1000 water solution of MS-222 immediately before amputation of distal 1/3 of the tail. All operated animals survived and developed tail regenerates. Gravity-dependent tail shape changes were induced by keeping tail-regenerating newts (substrate group) in containers on water-infused hygroscopic mat, as previously established (Radugina and Grigorian, 2012). Twice a week all newts were transferred into water-filled containers for feeding (for about an hour).

2.2. Heat shock treatments

Heat shock was used as a separate experimental procedure (applied weekly during regeneration) and as a mean of creating a positive control for PCR and IHC (applied to intact newts before tissue collection). Temperature for heat shock treatments was determined as 32 °C using standard procedure (Hutchison, 1961). Heat shock was performed as follows: 1) heating from RT to 26 °C at rate of 2 °C/10 min; 2) heating from 26 °C to 32 °C at rate of 1 °C/10 min; 3) maintenance of 32 °C for 60 min; 4) passive cooling to RT.

2.3. Morphometrical analysis of tail regenerates

Morphometrical analysis was performed on lateral-view