ISJ 4: 1-9, 2007 ISSN 1824-307X

Research Report

A hedgehog-like signal is involved in slow muscle differentation in *Sepia officinalis* (Mollusca)

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Accepted January 10, 2007

Abstract

In the tentacle of *Sepia officinalis*, smooth-like, helical and cross-striated fibres deriving from different populations of myoblasts are present. Myoblasts appear at different times during the development and express two muscle-specific transcription factors: Myf5-like and MyoD-like factors. Myoblasts expressing Myf5 give rise to slow fibres, whereas fast fibres derive from $MyoD^+$ myoblasts. We found that a Hedgehog (Hh)-like signal was present in the central nerve cord of the tentacle from the early stages of development and in a specific population of myoblasts which are the precursors of slow muscle fibres. The model showed interesting similarities with vertebrates, in which Sonic hedgehog is a protein secreted by axial structures (the notochord and neurotube) and is involved in slow muscle differentiation and in survival of muscle precursors.

Key words: development; helical and cross-striated fibres; mollusc; muscle differentiation; Hh-like protein

Introduction

Myogenesis in vertebrates leads to the formation of specialised fast and slow fibres. The fibres derive from different populations of myoblasts that appear at different times during myotome formation. The first population of myoblasts gives raise to slow fibres, whereas a second population, which appears later, generates fast fibres (Kelly and Rubinstein, 1980; Devoto *et al.*, 1996).

During the last decades, many studies have been done to investigate how such diversification is achieved. A key point has been the discovery that the notochord and neurotube, which lie close to the somite, can induce myogenesis (Buffinger and Stockdale, 1994). Further studies showed that the myogenetic effect of the axial structures is mediated by a pool of secreted proteins (Münsterberg and Lassar, 1995; Münsterberg *et al*., 1995; Lassar and Münsterberg, 1996). Sonic hedgehog (Shh) is one of these proteins, and several studies have demonstrated that it is able to induce the formation of slow fibres (Blagden *et al*., 1997; Cann *et al.*, 1999; Barresi *et al*., 2000, Grimaldi *et al*., 2004c), probably by committing a specific population of myoblasts rather than by inducing the final differentiation of a specific fibre type (Blagden *et al*., 1997; Hughes *et al*., 1997). This hypothesis is supported by the finding that Sonic hedgehog (Shh), in mammalians, induces the formation of a specific subset of muscle progenitors expressing the muscle regulatory factor Myf5 and MyoD-like (Kruger *et al.,* 2001; Gustaffson *et al*., 2002). All such data indicate that Shh expression links slow fibres formation and Myf5 expression suggesting that Shh can induce slow fibres differentiation by activating Myf5 expression in a subset of myoblasts.

In the present work we use the tentacle of *Sepia officinalis* (Molluscs, Coephalopoda) as a model for studies of muscle development and differentiation. Tentacles are specialised cylindrical structures used by cephalopods to capture prey. They are essentially formed by three kinds of tightly packed muscles, smooth-like, helical and cross-striated, organised around a central nerve cord and surrounded by a monolayered epithelium (Kier, 1982, 1988; Grimaldi *et al*.., 2004a, b). We have previously demonstrated that, during the development, the first muscle fibres to differentiate are the slow smooth-like ones while later both helical obliquely striated and cross-striated fibres appear expressing fast myosin (Grimaldi *et al.*, 2004a). Slow and fast fibres derive from two

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myoblast types respectively marked by the specific muscle regulatory factors Myf5 and MyoD (Grimaldi *et al*., 2004b)

We have demonstrated that even if Molluscs are phylogenetically far from vertebrates, muscle development of the tentacle follows the developmental scheme of vertebrates. In addition mollusc model benefits also of the co-presence of slow smooth-like, cross-striated and helical fast fibres, easily distinguishable from a morphological point of view and deriving from different populations of myoblasts. This offers the possibility to easily follow the fate of a specific cell population from the very early stages of development.

Our results showed that an Hh-like protein is expressed during tentacle development and induces the formation of slow fibres.

Materials and Methods

To describe the development of *S. officinalis* tentacles three growth phases were selected. The animals belonging to these different phases were selected according to Boletzky (1987), by measuring their dorsal mantle length (DML), and to Lemaire (1970), by characterising for the developmental landmarks

Phase I: stage 26, according to Lamaire with DML of about 3 mm.

Phase II: stage 29 according to Lemaire with DML of about 6 mm.

Phase III: 1-2 week-old cuttlefish with DML of about 9-10 mm. Newborn pigmented cuttlefish were able to swim and to predate.

Phase IV: 4-week-old cuttlefish with a DML of 15 mm.

Analysis, in each phase, were conducted on the middle part of the tentacle, as already described (Grimaldi *et al*., 2004a, b).

Before all procedures and fixations, cuttlefish were anaesthetised with 1:1 solution of 7,5 % $MgCl_2.6H_20$ and seawater (Messenger *et al.*, 1985).

Light microscopy and transmission electron microscopy

For routine transmission electron microscopy (TEM), cuttlefish were sectioned and the tentacles removed and fixed for 2 h in 2 % glutaraldehyde in sea water at 4 °C. Specimens were then washed in sea water and post-fixed at 4 °C for 2 h with 1 % osmic acid in cacodylate buffer (pH 7.2). After standard dehydration in ethanol series, specimens were embedded in an Epon-Araldite 812 mixture and sectioned with a Reichert Ultracut S ultratome (Leica, Nussloch, Germany). Semithin sections were stained by conventional methods (crystal violet and basic fuchsin, Moore *et al.*, 1960) and were observed with a light microscope (Olympus, Tokyo, Japan). Thin sections were stained by uranyl acetate and lead citrate and observed with a Jeol 1010 electron microscope (Jeol, Tokyo, Japan).

Immunonocytochemistry

Cuttlefish were dissected in 4 °C sea water. Tentacles were removed and fixed in 4 % paraformaldehyde solution in 0.2 M phosphate buffer (pH 7.2) containing 1 % glutaraldehyde for 2 h at 4 °C (McLean and Nakane, 1974). Specimens of tentacles were then washed in phosphate buffer and dehydrated in a graded ethanol series. After dehydration they were processed, as described above, and sectioned with a Reichert Ultracut S ultratome. After etching with NaOH 3 % in absolute ethanol (Causton, 1984), sections (0.7 μm) were treated for 30 min with phosphate-buffered saline solution (PBS) containing 2 % bovine serum albumin (BSA) before the primary antibody incubation (37 °C for 45 min in a moist chamber). Primary antibodies (working dilution 1:20) were polyclonal antibody goat antihuman Shh (Santa Cruz Biotechnology, CA, USA) and monoclonal antibodies mouse antihuman slow myosin heavy chain (MyHC) and mouse antirabbit fast MyHC (Sigma). Washed specimens were incubated for 1h at room temperature with the appropriate secondary antibody (dilution 1:100) alkaline phosphatase, or indocarbocyanine (Cy3) conjugated (Jackson, Immuno Research Laboratories, West Grove, PA, USA). Secondary antibodies alkaline phosphatase-conjugated were visualised using 5-Bromo-4-Chloro-3-Indolyl Phosphate/Nitro Blue Tetrazolium tablets (NBT/BCIP, Sigma, St. Louis, MO).

Mounted slides were examined with an Olympus BH2 microscope or with a confocal laser microscope (λ 550 nm for Cy3, MRC 1024, Bio-Rad Laboratories, Hemel Hempstead, UK). Confocal images were superimposed using Photoshop 5.0 program (Adobe Systems Inc.).

Control sections were incubated with PBS/BSA without the primary antibody.

Biochemical procedures

Cuttlefish tentacles (from Phase III) were skinned and muscles were homogenised in liquid nitrogen with a mortar. Mouse embryos (11.5 dpc), used as positive controls, were homogenised following the same protocol. For SDS-polyacrilamide gel electrophoresis (SDS-PAGE), homogenates were suspended in extraction buffer (0.4 M NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 0.5 μg/ml pepstatin, 50 mM Tris HCl, pH 7.2 with 1 mM phenylmethylsulphonyl fluoride and 0.2 mM ATP freshly added); particulate material was removed by centrifugation at 13000 rpm for 10 min at 4 °C in a refrigerated Sorvall RCM14 microcentrifuge. Supernatants were denatured in sample buffer 2X (2 % SDS, 10 % β-mercaptoethanol, 0.002 % bromophenol blue, 20 % glycerol, 0.02 M Tris HCl pH 6.8) at 100 °C for 5 min.

SDS-PAGE

Analytical SDS-PAGE using 15 % acrylamide minigel was made according to Laemmli (1970). Molecular weights were determined by concurrently running broad range standards from Bio-Rad (Bio-Rad, Richmond, MA, USA). Electrophoresis was made at 200 V for 1 h. Gels were stained with 2.5 % Coomassie blue (Bio-Rad) in methanol:acetic acid:water 5:1:5.

Western blot

Proteins separated by SDS-PAGE were transferred onto Bio-Rad nitrocellulose filters according to Towbin *et al.* (1979). Before

immunostaining, the membrane was saturated with 5 % BSA in Tris-buffered saline (TBS, 20 mM Tris-HCl buffer, 500 mM NaCl pH 7.5) at room temperature for 2 h. Nitrocellulose sheets were incubated overnight at 4 °C with a polyclonal goat anti-Shh as primary antibody (Santa Cruz Biotechnology) at 1:250 dilution in 2 % TBS-BSA. After three washes of the membrane with TBS, antigens were revealed with a donkey anti-goat secondary antibody (1:1000) coupled with alkaline phosphatase (Jackson ImmunoResearch Laboratories). After washing with TBS, signal was developed with NBT/BCIP.

RNA synthesis and microinjections

Plasmid pSP64TZshh and pCS+nucβ-gal (kindly donated by Simon Hughes, King's College, London) were linearized with the appropriate restriction enzyme and *in vitro* synthesized in the presence of Cap Analog using the SP6 MEGAscript kit (Ambion) following the manufacturer's instructions. Experimental and control embryos at stage 23 (Lemaire, 1970), were respectively injected with *shh* and *lac-Z* mRNA as previously described (Grimaldi *et al*., 2004b) and cultured at 18 °C until Phase III.

Results

Morphological analysis

Phase I

At an early stage of development, corresponding at stage 26, the cuttlefish, inside the gel coat, had DML of approximately 3 mm. At this stage, the tentacle showed a simple organisation and was mainly formed by large cells loosely dispersed in the region between the superficial epithelium and the central nerve cord (Fig. 1A). The nerve cord occupied the longitudinal main axis of the tentacle and it was delimited by numerous grouped roundish cells (Figs 1A, B) showing a large nucleus and scant cytoplasm (Fig. 1B). Very few and small differentiated muscle fibres, localised at the periphery of the tentacle, were smooth-like (Figs 1A, C) expressing slow myosin (Grimaldi *et al*., 2004a).

Phase II

In a few days the cuttlefish (at stage 29) reached 6 mm DML and the tentacle organisation was changed (Grimaldi *et al*., 2004). Underneath the epithelium, roundish and elongated myocytes were recognizable, while groups of few differentiated

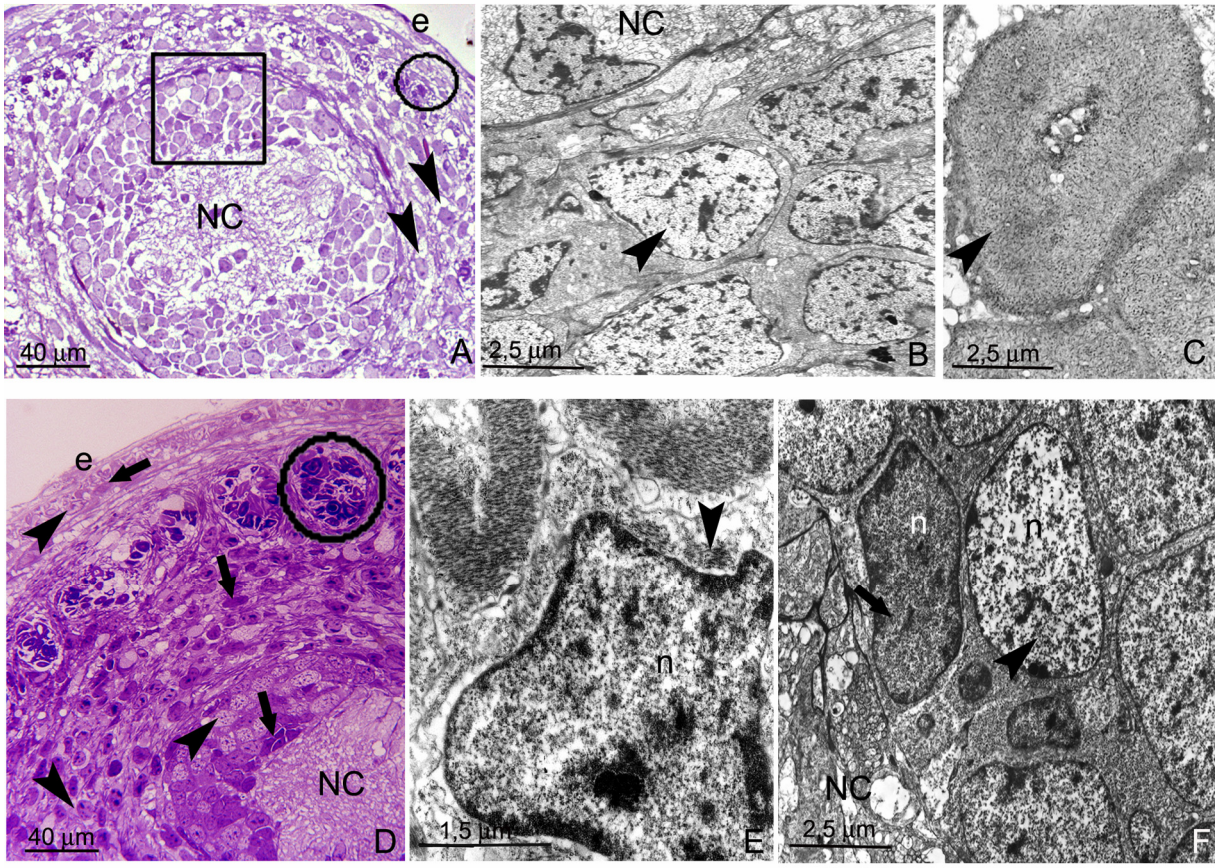


Fig. 1 A-C Phase I. Semithin (A) and thin (B, C) cross-sections of tentacle. The nerve cord (NC) occupies the central region of the tentacle and is surrounded by a thick layer of packed roundish cells (square). These cells (arrowheads) are also dispersed in the region between the epithelium (e) and the nerve cord. Small differentiated smooth fibres, are recognized underneath the epithelium (encircled area in A, arrowhead in C). **D-F** Phase II. Semithin (D) an thin (E, F) cross-sections of tentacle. Groups of smooth-like fibres (encircled area) radially disposed are clearly recognizable. The central nerve cord (NC) is surrounded by elongated (arrows in D, F) and roundish myobalsts (arrowheads in D, F) that are also visible (arrows and arrowheads) underneath the epithelium (e) and in the region between the nerve cord and the groups of helical fibres. Myocytes showing few actin and myosin filaments (arrowheads in E) are scattered. n, nucleus.

smooth-like fibres were radially disposed with respect to the central nerve cord (Figs 1D, E). At the centre of the tentacle, the nerve cord was enwrapped by two distinct layers formed by outer elongated cells irregular in shape, and inner large roundish cells (Figs 1D, F).

Phase III

In the newly hatched cuttlefish, with a 10 mm DML, the tentacle muscle organisation was complex. The sheath of cells surrounding the nerve cord was thinner and disposed into two layers. An inner layer close to the nerve cord made of cells elongated and irregularly shaped and an outer layer of roundish cells (Fig. 2A). Starting from the epithelium, external continuous muscular layer and peripheral groups, now formed by smooth-like and helical obliquely striated fibres (Figs 2B, C) were separated by cross-striated muscle fibres that also occupied the region up to the nerve cord (Figs 2A, B), forming a network of fast mononucleated fibres (Grimaldi *et al*. 2004a). Both large roundish and elongated myocytes were widely scattered (Figs 2A, B).

Phase IV

The tentacle muscular structure in a 4-week-old cuttlefish (i.e. DML of approximately 15 mm), had a fully developed muscular structure. The two populations of myoblasts previously surrounding the nerve cord had almost disappeared, and only a few of them were still visible in two restricted peripheral areas (Figs 2D, E). A few roundish and elongated myocytes were still present in the peripheral groups of muscle fibres and scattered among the cross-striated fibres (Fig. 2F).

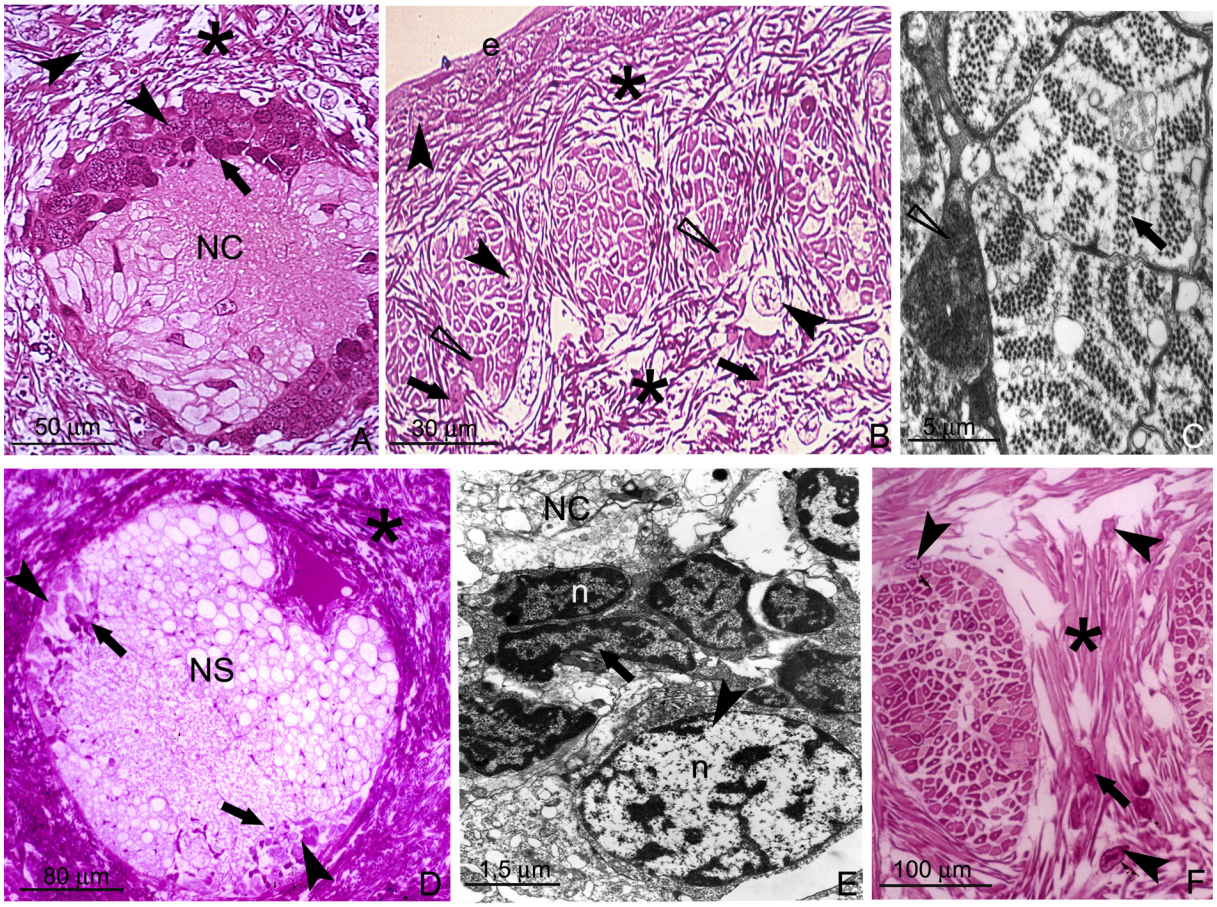


Fig. 2 A-C Phase III. Semithin (A, B) and thin (C) cross-sections of tentacle. The sheath of cells surrounding the nerve cord (NC) is thinner than in the previous developmental phase and the roundish (arrows in A and B) and elongated (arrowheads in A, B) cells are mainly scattered among the cross-striated fibres (asterisks in A and B) and among the groups of muscle (arrows in B). Helical muscle fibers with the contractile material organized in sarcomeres (arrow in C) are grouped with the smooth-like fibres (empty arrowheads in B and C) in the outer muscular layer and in the peripheral bundles. **D-F** Phase IV. Semithin (D, F) and thin (E) cross-sections of tentacle. The sheath of cells surrounding the nerve cord (NC) is extremely reduced and only a few roundish (arrowheads in D and E) and elongated (arrows in D and E) cells are still present in two peripheral areas. A few myocytes roundish (F, arrowheads) and elongated (F, arrow) are visible scattered among the cross-striated fibres (asterisk in D and F) and inside the groups of muscle.

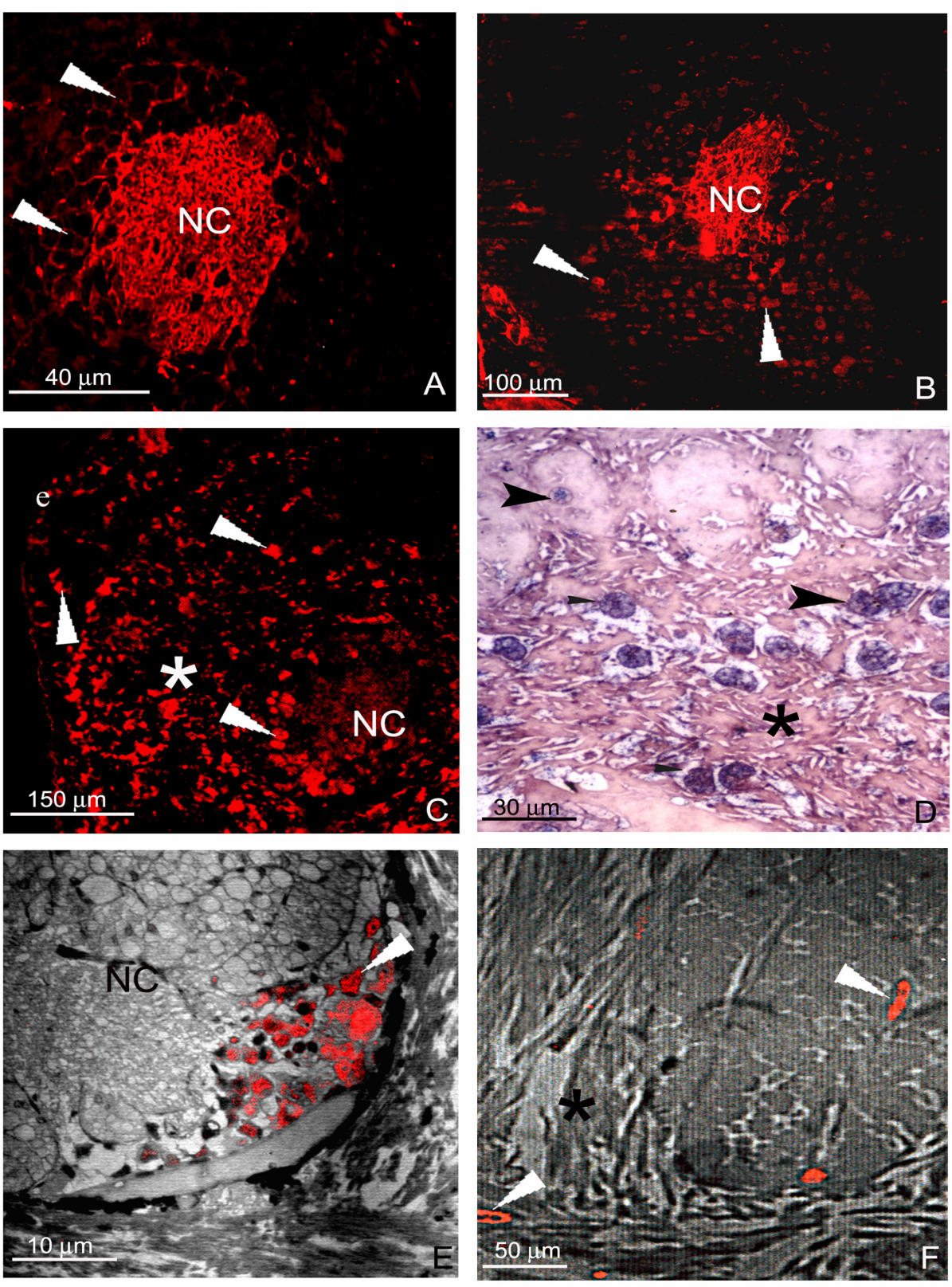


Fig. 3 A-D Phase I (A), II (B), III (C, D). Immunodetection of Hh. The expression of an Hh like signalling is revealed with both Cy3-conjugated (A-C) and alkaline phosphatase conjugated secondary antibodies (D). An high signal is detected in the central nerve axes (NC), in the roundish myoblasts/myocytes (arrowheads) close to the nerve cord, scattered among the cross striated fibres (asterisk in C and D), in the outer muscular layer underneath the epithelium (C) and in the groups (D). **E, F** Phase IV. The few roundish myoblasts (arrowheads in E and F) still present in restricted areas of the nerve cord (NC) and among the cross-striated fibres (asterisk in F) are labelled. e, epithelium

Immunonocytochemistry

In order to evaluate a possible role of an Hh-like signalling during tentacle muscle differentiation, we performed immunolocalisation experiments. Starting from early stage of development (stage 26, Phase I), Hh expression was mainly localized in the nerve cord and in the roundish cells surrounding the nerve axes (Fig. 3A). Starting from Phase II (stage 29, Fig. 3B) up to Phase III (newly hatched cuttlefish Figs 3C, D), Hh signalling greatly increased. It was detected in the nerve axes, in the roundish cells surrounding the nerve cord, in those migrating from it into the peripheral groups and in the external muscular layer underneath the epithelium. At Phase IV, Hh was expressed in the roundish myoblasts still present around the nerve cord (Fig. 3E) and in a few roundish myocytes dispersed among the cross-striated fibres (Fig. 3F).

Western blot from SDS-PAGE

Western blot analysis was performed to confirm the presence of an Hh-like protein in the developing tentacle. We compared the protein extracted from a tentacle of cuttlefish at Phase III (when the Hh-like signalling was highly expressed) with that from mouse embryo (11.5 dpc), used as positive control. Fig. 4 (lanes A, B) showed the electrophoretic patterns of tentacle and mouse embryo, respectively. Western blot analysis of both extracts separated by monodimensional SDS-PAGE showed that the anti-Shh antibody detected two bands of about 19 kDa and 44 kDa respectively (Fig. 4, lanes C, D).

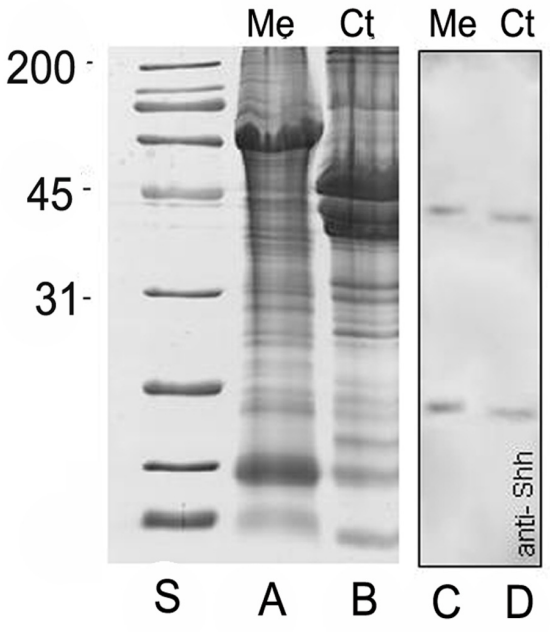


Fig. 4 Western blot from monodimensional SDS-PAGE. In the tentacle extract (Ct) of Phase III cuttlefish, the antibody anti-Shh detects two bands of about 19 kDa and 44 kDa (Lane D). Lane C: antibody specificity was compared to the positive control, mouse embryo (11,5 dpc) extracts (Me). Lane A 11,5 dpc mouse embryo extract, Lane B tentacle extract, S Standard

***hh* mRNA injection**

To investigate the effect of *hh* mRNA injection on the different population of muscle fibres, we performed a morphological analysis on cuttlefish tentacles from Phase III, when all types of muscle fibres were present. The injection of *hh* mRNA caused a massive increase in number of smooth-like slow fibres both in the outer continuous layer underneath the epithelium and in the inner groups (Figs 5A-C). This expansion occurs at expense of differentiated fast helical and cross-striated fibres, as demonstrated also by immunolocalization experiments (Figs 5A, B, D). No defects were detected injecting an equivalent amount of *lacZ* mRNA (Figs 5E-H). In fact the morphological phenotype and the signals for both anti-slow and fast MyHC antibodies resemble those already described in uninjected control animals (Grimaldi *et al.*, 2004a).

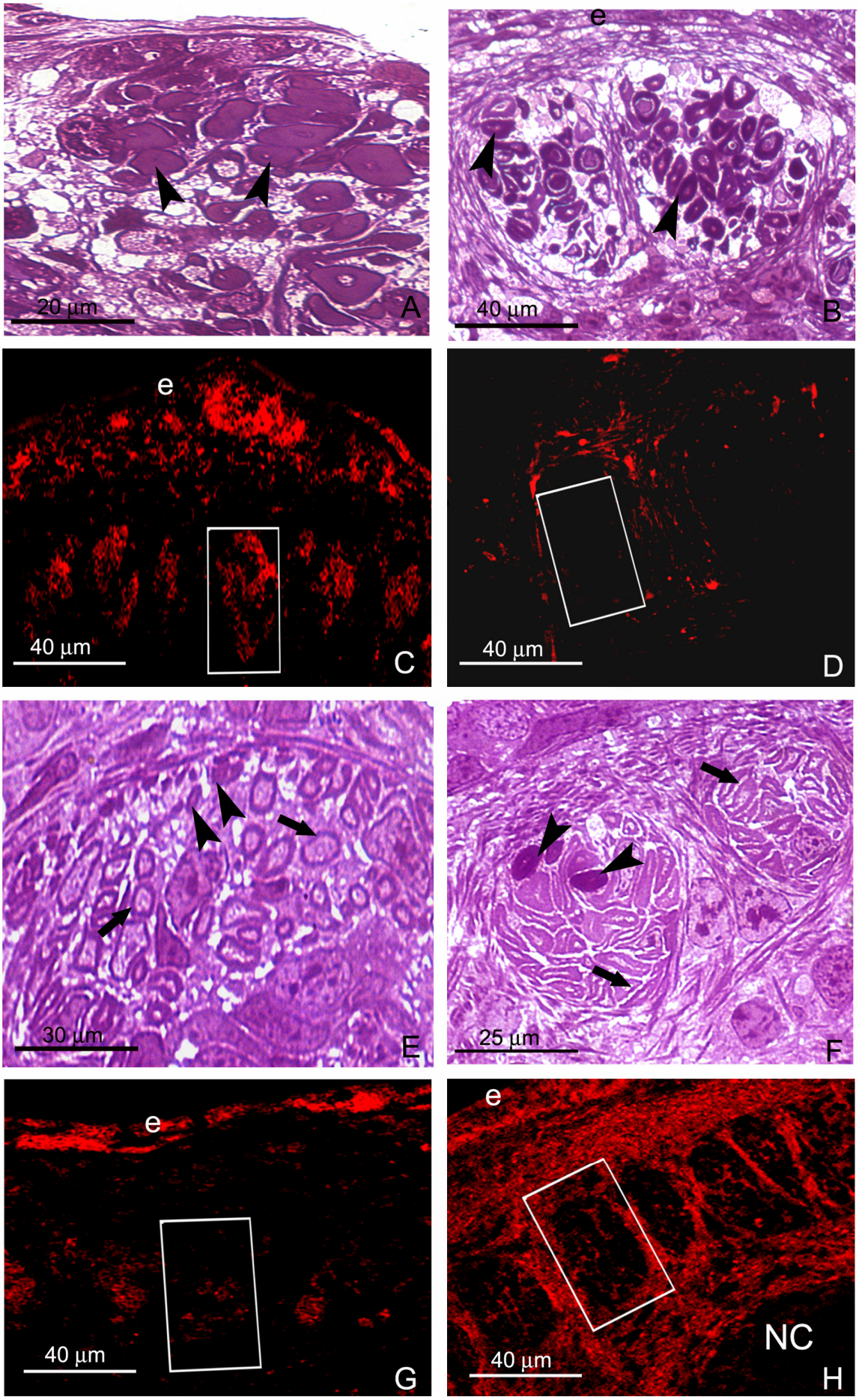
Discussion

Our results show the involvement of an Hh-like signalling in the formation and differentiation of cuttlefish muscle tentacle. Starting from the early stages (Phase I) of tentacle development the Hh-like expression is mainly localised in the nerve cord and also in the specific population of large and roundish myoblasts forming a thick layer around the central axial structure. These Hh^+ myoblasts move centrifugally from this region maintaining during migration the Hh expression that allows us to easily follow their fate during the different phases of development. Starting from Phase II these Hh^+ cells are still visible spread in the muscle layer under the epithelium and in the peripheral muscle groups especially made of smooth-like fibres. These Hh^+ migrating cells, showing actin and myosin filaments in their cytoplasm, correspond to those previously described by Grimaldi and coworkers (2004a, b) that differentiate in smooth-like fibres, express the muscle regulatory factor Myf5 and belong to slow-type.

Thus an Hh-like signal in the tentacle, confirmed by immunolocalization, seems to be involved in slow muscle differentiation as in vertebrates (Blagden *et al*., 1997; Cann *et al*., 1999; Grimaldi *et al*., 2004c).

Like vertebrates, in cuttlefish, an Hh-like expression is specific for slow fibre formation and it is not involved in fast fibre specification. Moreover *hh* mRNA over expression induces ectopic smooth-like slow fibres differentiation at expense of fast helical and cross-striated fibres. Such data are in agreement with those of Blagden *et al*. (1997) demonstrating, in zebrafish, the involvement of Shh, a member of hedgehog signal, notochord-derived, in inducing slow muscle formation at the expense of fast fibres.

The presence of Hh expression, in non axial structures, has been found also in vertebrate (chick) growing limb. In this district Shh^+ mesenchymal cells are localised in the region involved in the anteroposterior axis specification (Saunders and Gasseling, 1968) and the produced Shh factor is essential for setting up the pattern of slow fibres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◄**Fig. 5 A, B** Phase III. Semithin cross-sectioned tentacle of cuttlefish injected at stage 23 with mRNA *hh*-homolog. The outer layer underneath the epithelium (A) and the peripheral groups (B) were formed only by smooth-like fibres (arrowheads). **C, D** Phase III. Immunofluorescence staining against slow (C) and fast (D) MyHC in cross-sectioned tentacle of cuttlefish injected at stage 23 with mRNA *hh*-homolog. A massive number of slow fibres are detected in both the outer continuous layer underneath the epithelium and in the inner groups (square), while a few fibres are stained by the antibody anti fast MyHC. **E, F** Phase III. Semithin cross-sectioned tentacle of cuttlefish injected at stage 23 with mRNA *lac-Z*. Both smooth-like (arrowheads) and helical (arrows) fibres are recognizable in the outer layer underneath the epithelium and in the peripheral groups (F). **G, H** Phase III. Immunofluorescence staining against slow (G) and fast (H) MyHC in cross-sectioned tentacle of cuttlefish injected at stage 23 with mRNA *lac-Z*. At difference from the mRNA *hh* injected animals, both slow and fast fibres are detected in the outer muscular layer and in the groups (square). Moreover a large number of fast cross-striated fibres (asterisk in H) are visible. The signal for both antibodies resemble that already described (Grimaldi *et al.*, 2004a) in uninjected control animals. e, epithelium; NC, nerve cord.

during development of limb muscles (Riddle *et al*, 1993; Duprez *et al*.., 1999) and acts as a survival and proliferation factor for myogenic precursor cells during mouse hypaxial muscle (Kruger *et al.*, 2001) and in chick limb muscle development (Duprez *et al.*, 1998). The interesting feature is that in our model, unlike vertebrates, the myoblasts Hh-like positive auto-regulate their differentiation in slow fibres.

The fact that an antibody directed against vertebrate Hh protein reacted also with cuttlefish might confirm a high structural conservation of this protein in cephalopods as well. This hypothesis is also supported by the recent discovery of an Hh homolog (accession number AZ99217) in *Octopus bimaculoides* (Cephalopoda, Mollusca) whose sequence shows a high degree of conservation with the vertebrate hortolog (44 % identity and 63 % similarity). The conservation has been also confirmed by Western blot analysis on tentacle total protein extracts using the same antibody, that showed the presence of a protein with similar molecular weight compared to Shh detected in mouse embryo extract (11 dpc) used as positive control. In literature, vertebrate *shh* gene encodes a precursor transmembrane protein of about 44 kDa that undergoes an autocatalytic processing that releases two peptides: a 19 kDa amino-terminal and a 27 kDa carboxy terminal (Hall *et al*., 1997). The anti-Shh antibody (against a peptide mapping at the amino terminal of Shh protein) labels both in tentacle and mouse embryos two bands: a 19 kDa band that corresponds to the amino terminal portion of Shh and a 44 kDa band that may be the uncleaved precursor. Furthermore, by expressing *shh* in cuttlefish tentacle we also demonstrate a strong functional conservation of this gene between vertebrates and cuttlefish, as already shown by Krauss *et al*. (1993) for fish and *Drosophila.*

Summarising, the presence of a Hh-like signal in cuttlefish tentacle could suggest an evolutionary conservation of a system that might have, like in vertebrates, multiple functions. It could play an important role in myogenic process that lead, through Myf5 activation (Borello *et al*., 2006; Borycki *et al*., 1999) to the slow fibre formation; It could also act as an organiser of the spatial organization of tentacle muscles. Moreover Hh homolog could play a critical role in the survival and proliferation of myogenic precursor cells and the maintenance, at the end of development, of Hh-like expression only in very few myoblasts could a response to the constant physiological growth of molluscs.

Acknowledgements

We are grateful to Elena Bossi for injecting embryos and to Kathleen Histon for editing the manuscript. This work was supported by University of Insubria FAR 2004 and 2005 to AG.

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