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MINIREVIEW

Cytoskeletal proteins and morphogenesis in planarians

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Abstract

Regeneration processes employ a series of differentiative events related to various embryonic morphogenetic phenomena in which the cytoskeleton plays a fundamental role. Planarians are an excellent model to study development mechanism because they show an exceptional physiological morphogenetic plasticity that is also at the basis of their extraordinary regenerative ability. In this paper we discuss results concerning two cytoskeleton components, actin and tubulin, during morphogenetic processes of the planarian *Schmidtea polychroa*. Comparative studies on cytoskeleton function during morphogenetic processes in evolutionary-distant animal models can offer important new insights in the fields of stem cell biology and regenerative medicine.

Key words: planarian morphogenesis; actin; tubulin

Introduction

The cytoskeleton of eukaryotic cells plays a basic role in many dynamic processes that are controlled by a network of cytoskeletal fibers constituted by three types of filaments: microfilaments made up of various actin isoforms, microtubules consisting of α- and β-tubulin, and intermediate filaments. These cytoskeletal components are associated with hundreds of proteins that regulate a rich variety of structural or dynamic machineries, crucial for many essential cellular functions including cell migration, cell division, maintenance of cell shape, cell signalling, chromosome and organelle movements. The cytoskeleton also plays a pivotal role in cell-substrate and cell-cell adhesions, supplying the structural support necessary for these contacts as well as providing the focal point for signal transductions. Important advances have been made in the last years not only in characterizing the molecular and functional mechanisms of cytoskeleton but also in defining connections between cytoskeleton disorders and pathological conditions. Cytoskeletal protein abnormalities are frequently the origin of many pathological conditions including several cardiovascular, muscle, neurodegenerative and skin diseases and cancer (Ramaekers and Bosman, 2004).

Many studies in various animal developmental models have also demonstrated the importance of cytoskeletal proteins in controlling cellular behavior during the morphogenetic and developmental processes. Tissue building which occurs during both embryo development and wound-healing events, involves a series of cellular dynamics and movements that are driven by similarly orchestrated cytoskeletal machinery (Martin and Parkhurst, 2004; Redd *et al.*, 2004). Embryonic epithelial wound repair shows striking similarities in cytoskeletal remodelling with morphogenetic events of dorsal closure in *Drosophila* and ventral enclosure in *Caenorhabditis* embryos (Martin and Parkhurst, 2004; Wood *et al*., 2002). Therefore, the study of cytoskeleton changes during wound repair can offer insights about evolutionary conserved cytoskeleton functions during embryo morphogenetic processes and viceversa.

In this review we report and discuss results concerning the involvement of cytoskeletal proteins during the morphogenetic events underlying regeneration in freshwater planarians. Planarians are a fascinating model for studying the development processes because they show a great physiological morphogenetic plasticity that is also at the basis of their exceptional regenerative ability. There is a high degree of molecular and functional conservation throughout the phylogeny in the basic

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mechanisms of the developmental phenomena: the elucidation of molecular mechanisms underlying morphogenetic plasticity in animal models such as planarians will contribute to clarify key issues in the fields of wound healing and regenerative medicine.

Developmental plasticity in planarians

The biological peculiarity of the planarian model is characterized by a continuous status of morphogenesis; its tissues are physiologically subjected to a continuous cell renewal. A striking example is its ability to either grow or de-grow depending on nutritional availability: the significant modifications in body size to which planarians are subjected always maintain the correct proportions and are reversible (Baguñà and Romero, 1981). The great developmental plasticity is also directly related to extraordinary regenerative power; it is known that planarians are able to rebuild a complete organism from a very small body region. The source of this morphological plasticity and regenerative properties is a stable population of totipotent stem-cells, called "neoblasts", that, in the adult, are the only proliferating cells (Baguñà, 1976a, b). These stem cells are able to generate all different cell types present in the adult and are responsible for physiological homeostasis and tissue renewal.

Regeneration in planarians

Regeneration is widely distributed among metazoans: a large number of species have the capacity to replace injured or lost body parts and, in few cases, to rebuild the entire organism from a tiny portion of the body. Models of regeneration are provided by cnidarian, platyhelminthes and echinodermata, that can regenerate a complete organism by bi-directional regeneration, and by urodele amphibians that can rebuild a complete appendage. Morgan (1901) described two major types of regeneration: 1) morphallaxis, that involves remodelling and re-patterning of pre-existing tissues into newly organized structures as occurs in hydra, the classical example of this form of regeneration and 2) epimorphosis, that requires active cell proliferation at the wound site and the formation of a new growth zone from which the missing structure will be regenerated and correctly patterned. This mode of regeneration can involve the dedifferentiation of cells that then proliferate and redifferentiate to form new structures, such as occurs in amphibians, and/or the activation of pre-existing stem cell populations to form a new stump, the blastema, that then differentiates into missing body parts. Planarians are capable of blastema-based epimorphic regeneration. They are triploblastic acoelomates belonging to the Platyhelminthes clade and are included as members of the Lophotrochozoa (Adoutte *et al*., 2000). Although the epimorphosis is the prevalent regenerative process in planarians, morphallactic events also contribute to the restoration throughout the re-establishment of the exact body proportion and symmetry. The two phenomena can contribute differently to regeneration process depending on the starting-conditions. As mentioned before, epimorphic

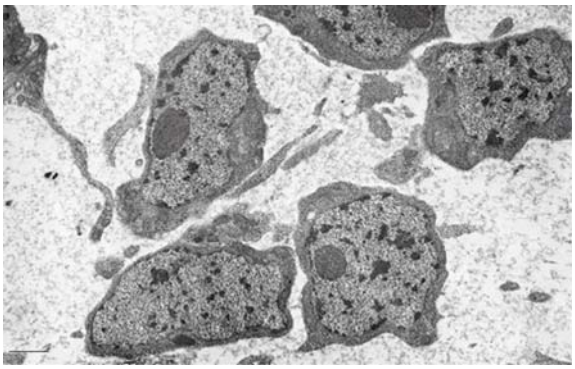

Fig. 1 Electron micrograph of a cluster of neoblasts. Bar = 2 μm.

regeneration in planarians is based mainly on a population of undifferentiated cells, the neoblasts. They are small (5-8 μm in diameter) with large nuclei and very little cytoplasm and are distributed throughout the parenchyma or mesenchyme along the whole body (Fig. 1) (Reddien and Sànchez Alvarado, 2004). When a planarian is injured a strong muscular contraction occurs immediately to minimize the wound area that is then rapidly covered by a thin layer of epithelium (Pascolini *et al.*, 1984; 1988a, b; Baguñà *et al*., 1994; Sànchez Alvarado and Newmark, 1998). Sequentially, the neoblasts proliferate close to the site of injury and migrate distally accumulating under the wound epithelium. They form a regenerative blastema, the unpigmentated structure in which missing tissues regenerate in a week (Dubois, 1949; Baguñà, 1976b; Saló and Baguñà, 1984). Once inside the blastema, the neoblasts no longer divide (Saló and Baguñà, 1984). There are two principal hypotheses concerning the source of blastema-forming cells: one is based on a transdetermination event that is supported by evidence that somatic cells can originate from undifferentiated cells committed to becoming germ cells (Gremigni and Miceli, 1980); the second is based on the totipotency of pre-existing stem cells that proliferate in response to injury. The evidence that blastema is constituted by pre-existing stem cells came from experiments of X-ray irradiation after which planarians completely lost the ability to regenerate and died within several weeks. The irradiated animals only regained their regenerative ability when injected with an enriched fraction of neoblasts (Brøndsted, 1969; Baguñà *et al.*, 1989). Moreover, BrdU labelling experiments of neoblasts confirmed that planarian stem cells contribute to the regeneration process through an active migration towards the wound region (Newmark and Sànchez Alvarado, 2000).

New molecular and genetic approaches have allowed to identify molecular markers of neoblasts and developmental genes important for both stem-cell biology and regeneration. Recently, proteins belonging to the Argonaute/PIWI family, *smedwi-1* and *smedwi-2,* with a regulative role in the production of neoblast progeny have been isolated in *Schmidtea mediterranea* (Reddien *et al.*, 2005). *DjPum*, a member of evolutionary conserved PUF

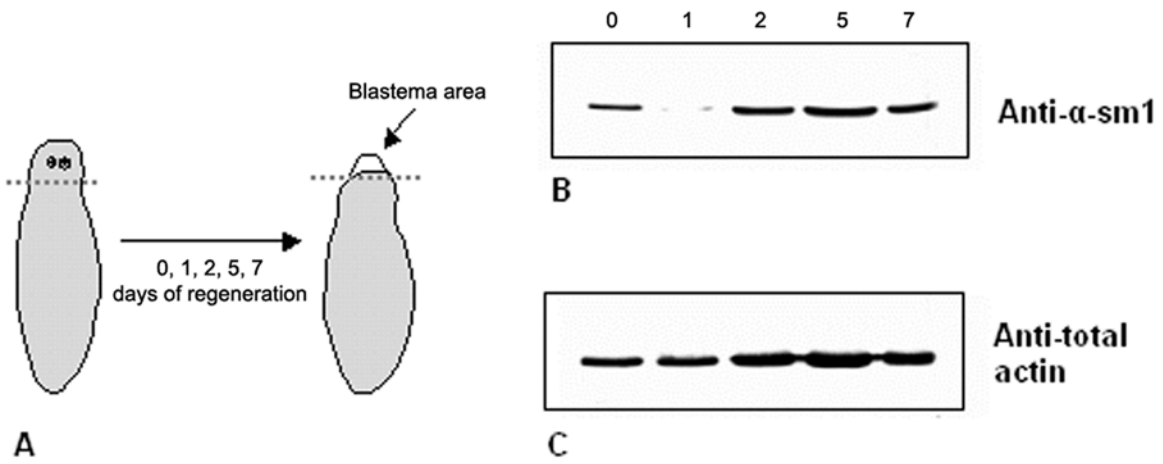

Fig. 2 A) Schematic representation of the head amputation in planarian. The blastema area at various times of regeneration is used for immunoblotting. B, C) Immunoblotting of planarian blastema area at 0, 1, 2, 5, 7 days of regeneration decorated with anti-αSM-1 (B) and anti-total actin (C) mAbs.

family of RNA-binding proteins, homologue of *Drosophila PUF gene* Pumilio, also plays an essential function in *Dugesia japonica* neoblast maintenance by supporting their mitotic proliferation (Salvetti *et al.*, 2005). The analysis of several neoblast markers, such as PCNA protein and DjPiwi-1 in *Dugesia japonica*, have suggested that a neoblast population could be heterogeneous (Ito *et al*., 2001; Rossi *et al*., 2006).

The molecular processes underlying regeneration in planarians are largely unknown. It is likely that a combination of chemical and mechanical cues stimulate blastema growth such as the triggers originating from wound epithelium, dorsal/ventral (D/V) pattern information and epidermis-mesenchyme interactions (Chandebois, 1979; Baguñà *et al*., 1988; Kato *et al*., 1999, 2001).

Cytoskeletal proteins in planarians

There is a high degree of molecular and functional conservation of the cytoskeletal proteins during animal evolution (Doolittle, 1995). Knowledge about the role of cytoskeleton proteins has increased due to immunochemical and molecular and genetic studies on animal models at different evolutionary levels such as yeast, *Dictyostelium*, *Caenorhabditis*, *Drosophila* and mice.

This paper discusses data concerning actin and tubulin, the most conserved cytoskeletal components, during morphogenetic processes of the planarian *Schmidtea polychroa*.

Actin

Actin is the major component of the microfilament system of eukaryotic organisms and plays a central role in cell shape and motility processes. In most organisms it is encoded by a multigene family encoding different isoforms regulated in a tissue-, cell type-, and context-dependent fashion (Vandekerckhove and Weber, 1984; Rubenstein, 1990; Herman, 1993). The high evolutionary conservation reflects a stringent functional and structural constraint to interact with itself, as well as with a variety of other proteins (Hennessey *et al*., 1993). Among the higher vertebrates, six isoforms are present and grouped into two classes, muscle (α-skeletal, α-cardiac, α-smooth muscle, and γ-smooth muscle) and cytoplasmic (β and γ) actins (Vandekerckhove and Weber, 1978). The distinction between muscle and non-muscle actins is a characteristic of higher animals and insects; in other invertebrate the actin isoforms are mainly similar to cytoplasmic type. It has been hypothesized that muscle actins have appeared from non-muscle actins by gene duplication and divergence twice during animal evolution (Mounier *et al.*, 1992).The major differences among actin isoforms are essentially their NH_2-terminal sequences. It has been hypothesized that this specific domain could be responsible for specialized functions due to the binding to specific actin-binding proteins. Many expressing studies have demonstrated that actin isoforms show typical modulations during cell differentiation events characteristic of both cell- and tissue development and wound repair (Sawtell and Lessard, 1989; Ruzicka and Schwartz, 1988; Darby *et al*., 1990; Gunning *et al.*, 1997). It has been demonstrated that in mammals α-smooth muscle isoactin is a marker of differentiation of both vascular smooth muscle cell during embryogenesis and regeneration, and myofibroblasts in wound healing (Darby *et al*., 1990; Serini and Gabbiani, 1999). The development of striated muscle cells also shows in myotome the transient expression of the alpha-vascular smooth actin (Woodcock-Michell *et al*., 1988; Sawtell and Lessard, 1989). α-skeletal and α-cardiac actin isoforms are involved in cardiomyogenesis (Ruzicka and Schwartz, 1988). By using immunochemical- and PCR-based approaches various actin isoforms have been characterized in the planarian *S. polychroa* (Pascolini *et al*., 1988a, b, 1992a, b; Fagotti *et al*., 1998). A specific cytoplasmic isoform has been shown to be involved in cell differentiation and

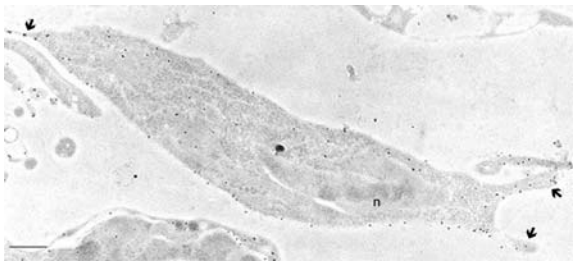

Fig. 3 Immunoelectron micrographs of migrating neoblast stained with anti-αSM-1 mAb. The labelling is mainly localized at the level of pseudopodia and filopodia (arrow). n, nucleus. Bar = 0.5 µm (Modified from Pascolini *et al*., 1992b).

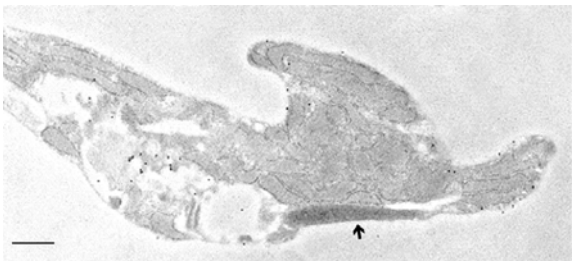

Fig. 4 Immunoelectron micrographs of differentiating myoblast stained with anti-αSM-1 mAb. The labelling is not detected in the organized myofibers (arrow). Bar = 0.5 µm (Modified from Pascolini *et al*., 1992b).

morphogenesis (Pascolini *et al*., 1992b; Di Rosa *et al*., 1994a). This isoactin was revealed by using the anti-αSM-1 mAb that selectively recognized the NH_2-terminus sequence Ac-EEED, the specific domain of the endothermic vertebrate α-smooth muscle actin (Skalli *et al*., 1986; Chaponnier *et al*., 1995). In normal planarians, this anti-αSM-1 reactive actin is localized in cytoplasmic domains of migrating undifferentiated neoblasts as well as in epithelial and nerve cells (Pascolini *et al*., 1992b; Di Rosa *et al*., 1994b). Interestingly, when a planarian is induced to regenerate, the expression of anti-αSM-1 reactive actin is up-regulated in the restricted region of blastema. There is a significant increase of this protein after two days of regeneration, it increases gradually to reach a maximum expression at about 5-6 days and then gradually decreases (Fig. 2) (Pascolini *et al*., 1992b).

Fine analysis of blastema has shown that the anti-αSM-1 reactive actin localization is associated with cytoskeletal re-arrangement that occurs during the activated neoblast migration and differentiation (Pascolini *et al*., 1992b; Di Rosa *et al*., 1994a). In migrating neoblasts, whose activation is amplified during blastema growth, the labelling was mainly localized at the level of the filamentous structures of pseudopodia and filopodia (Fig. 3). Differentiating myoblasts, that are the neoblasts committed to originate muscle fibers, also transiently express this isoactin which disappears when the cellular phenotype is differentiated and the myofibers are organized (Fig. 4). It is interesting to note that this specific expression pattern resembles those described for α-smooth muscle actin during the myogenesis process in higher vertebrates (Woodcock-Michell *et al*., 1988). The involvement of anti-αSM-1 reactive actin has also been well documented in the re-epithelialization process. The planarian epidermis consists of a single layer of columnar cells linked to one another in the apical portion by septate junctions and anchored to the basement membrane by hemidesmosomes (Fig. 5) (Hori, 1989; Di Rosa *et al*., 1994a). The basal region of epidermal cells form the characteristic processes, called 'feet', that are rich in intermediate filaments and microfilaments that are selectively labelled by an anti-αSM-1 mAb (Pascolini *et al*., 1992b; Di Rosa *et al*., 1994a).

Because of the inability of epidermal cells to divide, renewal depends on neoblasts that migrate across basement membrane from the parenchyma (Di Rosa *et al*., 1994a; Newmark and Sanchez Alvarado, 2000). This phenomenon is amplified during wound repair. In response to an injury, the wounded surface is first quickly covered by the cellular spreading of the old differentiated epidermal cells that lose their organized structure (Hori, 1991; Pascolini *et al*., 1984, 1988a, b; Baguñà *et al*., 1994; Sánchez Alvarado and Newmark, 1998). Subsequently, the wound epidermis is renewed in about 1 week by the active migration and differentiation of stem cells from the blastema (Newmark and Sanchez Alvarado, 2000). During their migration through basal lamina, the epidermal cell precursors over-express anti-αSM-1 reactive actin. They undergo a spatial and temporal cytoskeletal remodelling that involves the formation of stress-fibers in both lateral and basal cellular domains, that are specifically labelled by anti-αSM-1 (Fig. 6) (Pascolini *et al*., 1992b; Di Rosa *et al*., 1994a).

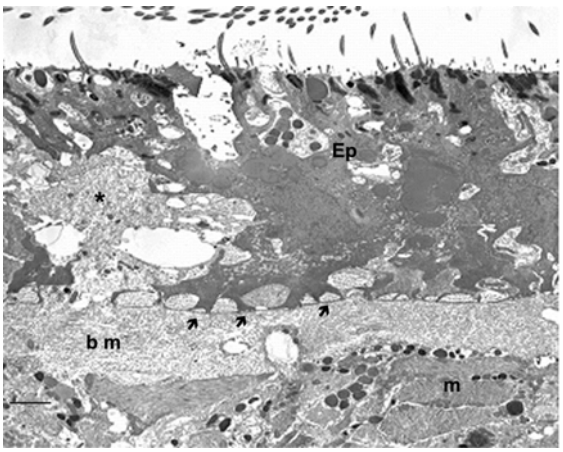

Fig. 5 Electron micrograph of intact planarian monolayered epidermis (Ep) showing basal 'feet' (arrow) anchored to the basement membrane (b m) and a differentiating epidermal cell (∗). m, myofiber. Bar = 2 µm.

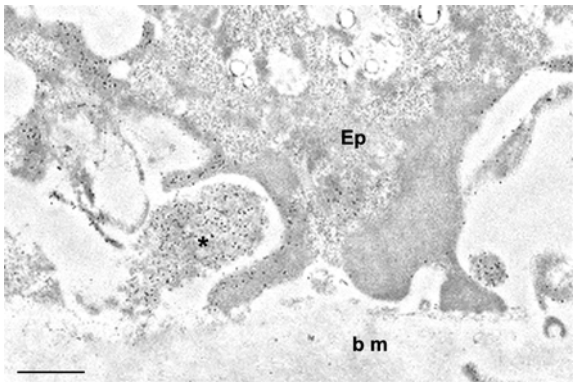

Fig. 6 Immunoelectron micrograph of regenerating planarian epidermis (Ep) decorated with anti-αSM-1 mAb: a differentiating epidermal cell (∗) appears markedly labelled. b m, basement membrane. Bar = 1 μm (Modified from Pascolini *et al*., 1992b).

These results demonstrate the modulation of anti-αSM-1 reactive actin in various planarian morphogenetic phenomena, such as blastema formation, myogenesis and re-epithelialization and strongly support the hypothesis of its specific role in cell differentiation. In particular, this specific actin is involved in neoblast activation and motility through a characteristic expression.

Tubulin

Microtubules are a ubiquitous cytoskeletal component present in all eukaryotic cells. They are formed by the self-assembly of α- and β-tubulin heterodimers and are involved in various cellular functions such as cell division, intracellular transport, maintenance of cell shape and flagellar and ciliary motility. The variety of functions can probably be attributed to the expression of different genes that are selectively expressed in specific cell types or at specific stages of development.

A tubulin cDNA (*Sp*Tub-1) has been isolated in planaria *S. polychroa*. *Sp*Tub-1 encodes for a tubulin protein which shares the highest degree of similarity with the known α-tubulin sequences (Simoncelli *et al*., 2003). The study of transcript expression shows that *Sp*Tub-1 is selectively restricted to testis tissues that consist of many elliptical follicles organized in clusters that are located on the dorsal side of the animal (Fig. 7A). Our results show that *Sp*Tub-1 mRNA was expressed in the testis at the level of spermatogenetic cells as spermatogonia, spermatocytes and spermatids (Fig. 7B). No signal was detected in the spermatozoa found inside the testis. The expression pattern suggests that *Sp*Tub-1 is a tubulin isotype specific of undifferentiated and differentiating germinal cells with a possible role in spermatogenesis.

Conclusions and perspectives

Morphogenesis includes processes in which cytoskeleton and cell migration are strongly involved. Many studies on the molecular mechanisms of physiological and pathological processes, such as embryonic morphogenesis, wound healing, immune surveillance and cancer metastasis, indicate that a coordinated organization of cytoskeletal proteins is central to cell behavior, cell migration, intracellular signalling and cell-cycle control processes.

Our studies on planarian morphogenesis have demonstrated that cytoskeletal proteins are strongly implicated in the differentiation processes, that also occur in higher animals. In particular the findings reported in planarian show that:

1) a specific actin isoform is a marker of neoblast activation and differentiation. Its modulation during morphogenetic phenomena suggests that this isoactin is necessary for regeneration. Future studies should investigate the molecular dynamic of the anti-αSM-1 reactive actin. Studies are in progress to confirm its specific involvement in cell locomotion processes. RNA interference studies should also analyze the effects of loss-of-function of this isoactin gene during both physiological and regenerative conditions;

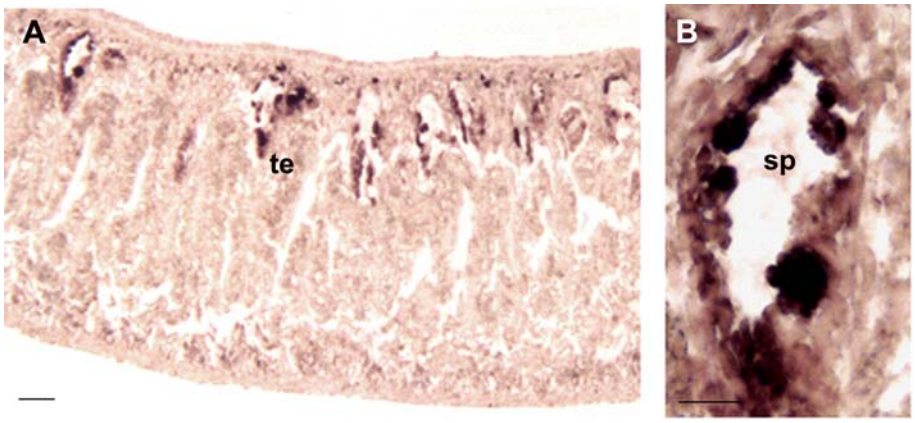

Fig. 7 A) *In situ* hybridization of *Sp*Tub-1 mRNA in planarian male gonad: the transcript is exclusively expressed in testis (te). Bar = 10 μm (Modified from Simoncelli *et al*., 2003). B) *In situ* hybridization of *Sp*Tub-1 mRNA in planarian male gonad: higher magnification showing that the signal is restricted to spermatogonia, spermatocytes and spermatidis but not in the spermatozoa (sp). Bar = 10 μm (Modified from Simoncelli *et al*., 2003).

2) a tubulin isoform is selectively expressed during spermatogenesis. Further study should focus on the mechanisms underlying germ cell fate determination. The role of this tubulin should also be studied in relation to the expression of other genes implicated in planarian gametogenesis, such as Vasa-like genes.

Greater knowledge about the cytoskeletal components and their function during morphogenetic phenomena in planarian sheds light the fundamental process of cell differentiation during animal evolution. Comparative studies of molecular and functional cytoskeletal regulation among different animal models can provide important new insights on cytoskeleton function and reveal conserved mechanisms.

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