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Expression pattern of the expanded noggin gene family in the planarian *Schmidtea mediterranea*

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ABSTRACT

Noggin genes are mainly known as inhibitors of the Bone Morphogenetic Protein (BMP) signalling pathway. Noggin genes play an important role in various developmental processes such as axis formation and neural differentiation. In vertebrates, inhibition of the BMP pathway is usually carried out together with other inhibitory molecules: chordin and follistatin. Recently, it has been shown in planarians that the BMP pathway has a conserved function in the maintenance and re-establishment of the dorsoventral axis during homeostasis and regeneration. In an attempt to further characterize the BMP pathway in this model we have undertaken an *in silico* search of noggin genes in the genome of *Schmidtea mediterranea*. In contrast to other systems in which between one and four noggin genes have been reported, ten genes containing a noggin domain are present in *S. mediterranea*. These genes have been classified into two groups: noggin genes (two genes) and noggin-like genes (eight genes). Noggin-like genes are characterized by the presence of an insertion of 50–60 amino acids in the middle of the noggin domain. Here, we report the characterization of this expanded family of noggin genes in planarians as well as their expression patterns in both intact and regenerating animals. *In situ* hybridizations show that planarian noggin genes are expressed in a variety of cell types located in different regions of the planarian body.

1. Results and discussion

Noggin was first identified as a secreted molecule that induces dorsal development in *Xenopus* embryos (Smith and Harland, 1992). Later, noggin genes were shown to directly inhibit the BMP pathway by sequestering the BMP ligand in an inactive complex so that it does not recognize its receptor (Zimmerman et al., 1996; Groppe et al., 2002). Although this family was initially characterized in vertebrates, in which from one to four genes have been identified (Smith and Harland, 1992; Valenzuela et al., 1995; Fürthauer et al., 1999; Fletcher et al., 2004; Eroshkin et al., 2006) several noggin genes have recently been reported in various invertebrate species including sponges, cnidarians, planarians, ascidians, sea urchin and lancelets (Ogawa et al., 2002; Müller et al., 2003; Satou and Satoh, 2003; Lapraz et al., 2006; Matus et al., 2006; Molina et al., 2007; Yu et al., 2007). In addition to noggin, other molecules that function as extracellular antagonists of the BMP pathway are chordin and follistatin (Piccolo et al., 1996; Fainsod et al., 1997). Interestingly, the overexpression of some of these inhibitors individually results in strong defects in formation of the dorsoventral axis (Smith and Harland, 1992; Sasai et al., 1994; Fainsod et al., 1997; Bauer et al., 1998). However, in order to block the formation of dorsal structures the co-inhibition of these molecules is required (Khokha et al., 2005; Dal-Pra et al., 2006), suggesting that some antagonists of the BMP pathway function redundantly. In addition to its function in the establishment of dorsal structures, noggin plays an important role in processes such as the formation and patterning of neural tissues (reviewed in Gaulden and Reiter, 2008), the differentiation of somitic mesoderm (Tonegawa and Takahashi, 1998), and skeletogenesis (reviewed in Rosen, 2006).

Recently, the BMP pathway has been implicated in the re-specification and maintenance of the dorsoventral axis in planarians (Molina et al., 2007; Orii and Watanabe, 2007; Reddien et al., 2007). Planarians are known for their high regenerative capabilities (Newmark and Sánchez Alvarado, 2002; Agata, 2003; Reddien and Sánchez Alvarado, 2004; Saló, 2006; Cebrià, 2007). After amputation, molecular and physiological changes that include apoptosis, morphogenesis and proliferation and differentiation of the neoblasts (the planarian totipotent stem cells) remodel old tissues and re-build the missing structures (González-Estevez et al., 2007; Pellettieri and Sánchez Alvarado, 2007; Eisenhoffer et al., 2008). After silencing the homologues of some of the elements of the BMP pathway such as the ligand *BMP* or the intracellular effectors *Smad1* and *Smad4*, the planarian dorsal side is ventralized and dorsal duplication of the central nervous system occurs (Molina et al., 2007; Orii and Watanabe, 2007; Reddien et al., 2007). Here

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we report the characterization of the planarian noggin gene family. Surprisingly, searches in the planarian *Schmidtea mediterranea* genome resulted in the isolation of ten genes containing a noggin domain. The analysis of the expression of the planarian noggin family indicates that these genes are expressed in a variety of cell types, such as neural, gastrodermal and mesenchymal cells; moreover, two planarian noggins show complementary expression patterns as they are expressed either dorsally or ventrally at a submuscular level throughout the body. The presence of an insertion in some of the noggin homologues together with their broad and distinct expression patterns indicates that noggin genes have expanded and diverged within the planarian lineage.

1.1. Isolation of an expanded family of noggin genes in Schmidtea mediterranea

To identify homologues of antagonist elements of the BMP pathway we performed *in silico* searches of the genome and ESTs databases of the planarian *S. mediterranea* (see Section 2). In contrast to vertebrates, which have two to four noggin genes, or cnidarians (one or two genes), we found ten putative planarian noggin genes that fall into two categories. The noggin group includes two members that contain a well-conserved noggin domain and have therefore been named *Smed-nog1* and *Smed-nog2*. In the second group there are eight genes that are more divergent and show an insertion of about 50–60 amino acids within the noggin domain, between the 5th and 6th conserved cysteine residues (underlined in Fig. 1A). A similar insertion is found in a noggin-like gene previously reported in the planarian *Dugesia japonica* (Ogawa et al., 2002). As that gene was termed *Djnlg* (*Dj noggin-like-gene*), the eight new genes identified in *S. mediterranea* were named *Smed-nlg1* to *Smed-nlg8*. Phylogenetic analysis confirms that both *Smed-noggin* and *Smed-noggin-like* genes belong to the noggin family and that they constitute two monophyletic groups (Fig. 1B), probably originating from internal duplications within the planarian lineage. This pattern of lineage-specific duplicated genes in planarians has also been reported for other gene families such as piwi/argonaute, GSK-3, and β-catenin (Reddien et al., 2005; Adell et al., 2008; Gurley et al., 2008; Iglesias et al., 2008; Palakodeti et al., 2008; Petersen and Reddien, 2008).

1.2. Expression pattern of noggin genes in intact planarians

In order to analyse the expression pattern of the planarian noggin genes we performed *in situ* hybridization on intact animals. No signal was detected for the genes *Smed-nog2*, *Smed-nlg4* or *Smed-nlg6*, even though RT-PCR experiments indicated that they are expressed (data not shown). As previously reported (Molina et al., 2007), *Smed-nog1* is expressed ventrally along the central nervous system (CNS), around the body margins, at the base of the pharynx and around the mouth (Fig. 2A). Dorsally, *Smed-nog1* is expressed in a few cells around the midline (arrowheads in Fig. 2B).

Smed-nlg1 is expressed in cells located at the periphery of the brain, close to the lateral branches that project from the cephalic ganglia to the head periphery (arrowheads in Fig. 2C). A similar pattern has been described in the species *D. japonica* (Ogawa et al., 2002), although in that species the brain lateral branches are more defined and distinctive that in *S. mediterranea* (Agata et al., 1998; Cebrià, 2007). Immunostaining with anti-myosin heavy chain and anti-synaptotagmin antibodies indicates that *Djnlg* gene is not expressed in either muscle or nerve cells (Ogawa et al., 2002). *Smed-nlg1* is also expressed at the neural ring of the distal part of the pharynx (arrows in Fig. 2C), similar to what has been observed for *Djnlg* (Ogawa et al., 2002).

Smed-nlg5 is specifically expressed throughout the gastrovascular system (Fig. 2D). The blind gut of *S. mediterranea* has three branches, one that runs anteriorly and finishes between the photoreceptors, and two posterior branches that run in parallel up to the tip of the tail. These three branches are connected at the anterior end of the pharynx and show a complex pattern of secondary ramifications (Fig. 2D).

Smed-nlg2 and *Smed-nlg3* show similar expression patterns throughout the mesenchyme (Figs. 2E–F). *Smed-nlg2* and *nlg3*-expressing cells are found surrounding the three branches of the gastrovascular system, resembling the expression patterns of several neoblast (planarian stem cells) genes (Reddien et al., 2005; Salvetti et al., 2005; Guo et al., 2006; Oviedo and Levin, 2007). Likewise, in the post-pharyngeal region of intact planarians *Smed-nlg2* and *Smed-nlg3* are expressed along three stripes of cells, two lateral around the posterior gut branches and a central stripe in between those gut branches (Fig. 2E and F). No expression is observed within the pharynx. *Smed-nlg2* is not expressed in the head region either (Fig. 2E). However, in contrast to what is most typical for a neoblast marker, a few *Smed-nlg3*-expressing cells are found in front of the photoreceptors (arrows in Fig. 2F). Upon irradiation planarian neoblasts are specifically eliminated and therefore the expression of neoblast markers is down-regulated (Eisenhoffer et al., 2008). However, the expression of *Smed-nlg2* and *Smed-nlg3* is not affected even seven days after irradiation, a time at which neoblast markers are lost (data not shown); this suggests that these genes are expressed in post-mitotic cells, or at least in irradiation-insensitive cells.

The *Smed-nlg7* gene is expressed in discrete cells on the ventral side below the body wall musculature (Fig. 2G, J and arrows in Fig. 2K). Remarkably, no *Smed-nlg7*-expressing cells are found either along the ventral midline or in the most anterior part of the head (Fig. 2G). Moreover, there are more *Smed-nlg7*-expressing cells in the pre-pharyngeal region than in the post-pharyngeal region (Fig. 2G). In addition to this expression along the ventral side, *Smed-nlg7* is expressed in discrete cells around the branches of the gastrovascular system (Fig. 2H and arrowheads in Fig. 2I). *In situ* hybridizations on histological cross-sections show that *Smed-nlg7*-positive cells are found around the dorsal side of the gut branches (Fig. 2J and arrowheads in Fig. 2K), and no signal is detected on the ventral side of the gastrovascular system.

Finally, *Smed-nlg8* is expressed in discrete cells distributed throughout the dorsal side below the body wall musculature as seen after *in situ* hybridizations on whole-mount (Fig. 2N) and histological sections (Fig. 2O). Fewer positive cells are found around the midline of the tail region (arrows in Fig. 2N). Ventrally, *Smed-nlg8*-expressing cells are detected as two stripes of cells: one around the cephalic ganglia (arrows in Fig. 2L and P) and the other between the brain and the body margin (arrowheads in Fig. 2L). *Smed-nlg8* is also expressed around the mouth opening located on the ventral surface, and through which the pharynx is normally evaginated (Fig. 2M and Q).

1.3. Expression pattern of noggin genes during regeneration

Planarians are a well-known model for studying regeneration. In order to analyse the expression of planarian noggin genes during this process we performed *in situ* hybridizations on trunk pieces regenerating a new anterior region (Fig. 3, upper panels) and on head pieces regenerating a new posterior region (Fig. 3, bottom panels). Similarly to what has been described for intact animals, no signal was detected for *Smed-nog2*, *Smed-nlg4* and *Smed-nlg6* (data not shown). After amputation, the neoblasts adjacent to the wound proliferate, giving rise to the regenerative blastema where new structures will differentiate (Sánchez Alvarado and Kang, 2005; Handberg-Thorsager et al., 2008). During the first few days of regeneration the blastema is clearly distinguishable from the pre-existing tissues because of its lack of pigmentation.