Evo-devo: Hydra raises its Noggin

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Noggin, along with other secreted bone morphogenetic protein (BMP) inhibitors, plays a crucial role in neural induction and neural tube patterning as well as in somitogenesis, cardiac morphogenesis and formation of the skeleton in vertebrates. The BMP signalling pathway is one of the seven fundamental pathways that drive embryonic development and pattern formation in animals. Understanding its evolutionary origin and role in pattern formation is, therefore, important to evolutionary developmental biology (evo-devo). We have studied the evolutionary origin of BMP–Noggin antagonism in hydra, which is a powerful diploblastic model to study evolution of pattern-forming mechanisms because of the unusual cellular dynamics during its pattern formation and its remarkable ability to regenerate. We cloned and characterized the *noggin* gene from hydra and found it to exhibit considerable similarity with its orthologues at the amino acid level. Microinjection of hydra Noggin mRNA led to duplication of the dorsoventral axis in *Xenopus* embryos, demonstrating its functional conservation across the taxa. Our data, along with those of others, indicate that the evolutionarily conserved antagonism between BMP and its inhibitors predates bilateral divergence. This article reviews the various roles of Noggin in different organisms and some of our recent work on hydra Noggin in the context of evolution of developmental signalling pathways.

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1. Introduction

Evolutionary developmental biology (evo-devo) explores the underlying mechanisms and relationships between the processes of individual development and phenotypic changes during evolution (Müller 2007). While natural selection explains the generation of variation through macroevolution (evolution at species level), evo-devo elucidates the sources of variation at organism level that reside in conserved developmental mechanisms (De Robertis 2008). Using molecular systematics, evo-devo investigates the key transitions in animal body plan such as establishment of the dorsoventral axis, centralization of the nervous system, visual system, etc. The function and evolution of regulatory gene networks, signalling pathways and other aspects of the molecular circuitry of development are the topics of interest in contemporary evo-devo research (Müller 2007). Recent advances in experimental tools, such as recombinant DNA technology, *in situ* localization of genes/gene products and gene expression blocking methodologies, etc., have helped in gaining better insights into morphological evolution. Emergence of multicellularity from unicellularity, formation of germ layers and emergence of a central nervous system are some of the key innovations in evolution. Evo-devo allied with genetics and genomics helps to uncover the basis and origin of these innovations.

The innovations mentioned above have faced various evolutionary constraints resulting in the generation of a wide diversity of life forms. Recent studies demonstrate that such diversity and complexity are brought about through developmental mechanisms (Carroll 2008; De Robertis 2008). Organization of cells in time and space through the process of pattern formation generates variations in life

Keywords. BMP signalling; embryonic development; evo-devo; hydra; Noggin; pattern formation

Abbreviations used: BCNE, blastula Chordin- and Noggin-expressing; BMP, bone morphogenetic protein; DAN, differential screening-selected genes aberrative in neuroblastoma; DG, dentate gyrus; D-V, dorsoventral; EMT, epithelial–mesenchymal transition; evo-devo, evolutionary developmental biology; GDF, growth and differentiation factor; MIS, Muellerian inhibiting substance; RTK, receptor tyrosine kinase; SGZ, subgranular zone; SVZ, subventricular zone

We dedicate this article to the memory of Prof Veronica Rodrigues.

forms during evolution (reviewed in Salazar-Ciudad and Jernvall 2004). During early development, equipotent embryonic cells proliferate and organize into an intricate spatial arrangement on the basis of their positional values and interpretation of various signals in a coordinate system (Wolpert 1969). Seven fundamental signalling pathways, viz. Wnt, TGF-β, Hedgehog, receptor tyrosine kinase (RTK), Jak/STAT, Notch and nuclear hormone receptor signalling, are believed to drive pattern formation in animals (Gerhart 1999; Barolo and Posakony 2002).

Hydra is a classical model system in developmental biology. Several characteristics of hydra, such as its phylogenetic position as one of the most primitive organisms displaying oral-aboral polarity, nerve network, simple body plan, regeneration capacity, perpetual differentiation of cells, absence of senescence and the ease of maintenance in the laboratory and amenability to various experimental manipulations, make it a powerful model to study animal development (Steele 2002). Besides, the availability of hydra EST and genome databases (Hemmrich and Bosch 2008; Chapman *et al.* 2010) make it a model of choice to elucidate several evolutionary developmental mechanisms.

Noggin, a secreted protein, was the first molecule shown to mimic the classical Spemann Organizer by inducing neural tissue from ectoderm (Smith and Harland 1992; Smith *et al.* 1993; Zimmerman *et al.* 1996). Subsequent experiments in *Xenopus* have shown that Noggin acts as a dorsalizing signal during gastrulation by changing the fate of lateral mesodermal tissues from ventral (blood and mesenchyme) to more dorsal type (notochord, muscle, heart and pronephros) (Smith *et al.* 1993). We have isolated and characterized *noggin* from hydra. BLAST and phylogenetic analyses reveal that hydra Noggin is structurally similar to Noggins from higher vertebrates including humans. Phenotypes resulting from heterologous expression of hydra *noggin* in *Xenopus laevis* embryos confirm the conservation of bone morphogenetic protein (BMP)-antagonizing activity of hydra Noggin. Distribution of Noggin transcripts in hydra polyps and hydra pieces regenerating under different experimental conditions suggests a role of this gene during cell differentiation and patterning, probably of the nervous system. This information points to a possible origin of Noggin–BMP antagonism in hydra. Further, these observations also support recent findings of conservation of BMP signalling in cnidarians (Samuel *et al.* 2001; Reinhardt *et al.* 2004; Rentzsch *et al.* 2007).

2. Multiple roles of Noggin in development and differentiation

2.1 *BMP signalling and Noggin*

TGF-β pathway is one of the fundamental and versatile metazoan signalling pathways with a key role in development, organogenesis, stem-cell maintenance, immunity and cancer (Huminiecki *et al.* 2009). The TGF-β superfamily includes TGF-β, activin, nodal, Muellerian inhibiting substance (MIS), growth and differentiation factor (GDF) and bone morphogenetic protein (BMP) families (reviewed in Groppe *et al.* 2002). BMPs are among the major members of TGF-β family. They are the key regulators of several developmental processes including dorsoventral patterning, neurogenesis and skeletogenesis (reviewed in Graff 1997; Hartung *et al.* 2006). Various antagonizing factors such as Noggin, Chordin and Follistatin inhibit BMP signalling (Piccolo *et al.* 1996; Zimmerman *et al.* 1996; reviewed in Thomsen 1997). While BMP signalling was initially thought to have originated along with the dorsoventral (D-V) axis in bilaterians, the presence of several members of this pathway in diploblastic cnidarians has raised questions regarding the evolution of molecular mechanisms governing axis patterning (Finnerty 2003). Therefore, elucidation of this pathway in basal phyla is likely to help in understanding the basis of animal body plan.

Noggin was initially isolated in an expression screen performed to search the potential signals that induce dorsal structures in *Xenopus* embryos (Smith and Harland 1992). Noggin binds to BMP4, thus preventing it from binding to its cell surface receptors (Zimmerman *et al.* 1996; Fürthauer *et al.* 1999). In addition to Noggin, other molecules that function as extracellular antagonists of BMPs are Chordin, Follistatin and members of the differential screening-selected genes aberrative in Neuroblastoma (DAN) family (reviewed in Thomsen 1997). Overexpression of these antagonists leads to severe defects in the D-V axis (Smith and Harland 1992; Bauer *et al.* 1998). However, simultaneous inhibition of these molecules is necessary to block the formation of dorsal structures (Khokha *et al.* 2005; Dal-Pra *et al.* 2006), indicating that these antagonists function redundantly to establish the D-V axis through inhibition of BMP activity (Stottmann *et al.* 2001; Dal-Pra *et al.* 2006).

Groppe *et al.* (2002) have reported the crystal structure of human Noggin, which shows that Noggin sequesters BMP by blocking the molecular interfaces of the binding epitopes for both type I and type II receptors of BMP. The primary structure of Noggin comprises of an acidic amino-terminal and a cysteine-rich (nine cysteine residues) carboxy-terminal. Like members of BMP family, Noggin too contains a cysteine knot, suggesting the common evolutionary origin of both ligand and its antagonist (Groppe *et al.* 2002). Noggin binds BMP and induces conformational change in BMP so that the receptor-binding epitopes of BMP get masked, resulting in inhibition of further signalling (Groppe *et al.* 2002). It has been hypothesized that BMP antagonists may behave as morphogens to establish the gradients of BMP antagonism (reviewed in Paine-Saunders *et al.* 2002). Heparan sulphate proteoglycans

localize Noggin to the cell surface (Paine-Saunders *et al.* 2002). They regulate the cellular distribution of Noggin and consequently cellular responsiveness to BMPs (Paine-Saunders *et al.* 2002).

2.2 *Noggin and Spemann Organizer of Xenopus*

German embryologists Hans Spemann and Hilde Mangold demonstrated the phenomenon of 'embryonic induction' in 1924. In a classic experiment of transplantation, they showed the induction of twinned body axis when dorsal lip of the blastopore was transplanted onto the opposite (future ventral) side of the embryo. Due to the inductive properties of the dorsal lip, they termed it as an 'Organizer' (Spemann and Mangold 1924). The Organizer has since been found to possess three main properties: (1) it induces neural tissue in the overlying ectoderm, (2) it dorsalizes the mesoderm of the marginal zone, which results in the formation of somites and trunk muscles and (3) it dorsalizes the endoderm, leading to induction of a secondary gut (De Robertis and Wessely 2004). Although the concept of Organizer was experimentally demonstrated by Spemann and Mangold in 1924, it is only in recent years that the molecular characteristics of Organizer activity have been explored. Various genes, especially those encoding secreted proteins, are expressed in the Organizer (De Robertis *et al.* 2000). These secreted proteins include inhibitors of BMP pathway (Chordin, Noggin and Follistatin) or Wnt pathway (Frzb, Dickkopf and Crescent), or both (Cerberus) that bind to their respective targets in the extracellular space and prevent them from binding to their cognate receptors (reviewed in De Robertis *et al.* 2000). The Hensen's node in chick embryo, the embryonic shield of zebra fish embryo and the node of the mouse embryo exhibit Organizer properties comparable to the classical Spemann's Organizer.

Noggin was the first identified secreted factor from the Organizer (Smith and Harland 1992). In *Xenopus* embryos, expression domain of *noggin* encompasses the dorsal most cells of the marginal zone that function as the Organizer. In *Xenopus*, Noggin transcripts are contributed maternally as well as synthesized zygotically. The maternally expressed Noggin induces the overlying marginal zone to become the dorsal mesoderm, while zygotically expressed Noggin functions as a part of the Organizer (reviewed in Smith *et al.* 1993). By the late neurula stage, Noggin is expressed in axial derivatives of the Organizer, the prechordal mesoderm and notochord (Holley *et al.* 1996). Subsequent experiments have suggested that Noggin mimics the Spemann Organizer by inducing neural tissue from ectoderm (Smith *et al.* 1993). Studies by Lamb *et al.* (1993) provided evidence that Noggin induces neural tissue in the absence of dorsal mesoderm. Noggin can induce cement glands and anterior brain markers but not hindbrain or spinal cord markers (Lamb *et al.* 1993). Further, the 'neuralizing' effect of Noggin on animal caps depends on its interaction with Brachyury since Noggin acts as a dorsalizing factor in the presence of Brachyury transcripts while it induces neural tissue in their absence (Cunliffe and Smith 1994). At a concentration of 1 nM, Chordin is sufficient to induce either neural tissue or somatic muscle. In contrast, 1 nM of Noggin dorsalizes the mesoderm but a higher concentration, i.e. 10 nM, is required to induce neural tissue (De Robertis and Wessely 2004). β-Catenin induces the expression of Noggin and Chordin in a group of cells located in the dorsal animal region of the *Xenopus* blastula (Kuroda *et al.* 2004). This group of cells is termed as a Blastula Chordin- and Noggin-expressing (BCNE) region that contains both prospective neuroectoderm cells and Spemann Organizer precursors. During later development, the BCNE region forms the brain and floor plate. In addition to this, Noggin is also involved in the differentiation of the endoderm (Sasai *et al.* 1996). Thus, it is apparent that Noggin participates in the patterning of three germ layers along with other BMP antagonists in *Xenopus* (De Robertis *et al.* 2001).

2.3 *Noggin in other vertebrates and its roles other than in Organizer during embryonic development*

Since the discovery of the Organizer by Spemann and Mangold (1924), the molecular factors responsible for the neural inductive properties of Organizer have been explored (De Robertis *et al.* 2001; reviewed in Stern 2005). Several studies provided strong evidence to show the potential role of Noggin and other BMP antagonists in neural induction (Lamb *et al.* 1993; Khokha *et al.* 2005; reviewed in Stern 2005). However, the role of Noggin as a neural inducer has been challenged. It has been reported that neither Chordin nor Noggin could induce neural tissue in embryos in which FGF signalling had been blocked by the injection of a dominant negative FGF receptor (Launay *et al.* 1996). In chick embryo, the distribution of Noggin transcripts in Hensen's node, the chick Organizer, during early axial and neural stages is similar to that in *Xenopus* embryo (Connolly *et al.* 1997). However, ectopic expression of Noggin has no effect on axis formation in chick (Connolly *et al.* 1997). Further studies indicate that unlike in *Xenopus*, ectopic expression of BMP antagonists in the competent epiblast of chick does not induce the expression of any neural markers (reviewed in Stern 2005). In mouse, *noggin* is expressed in the node and its axial mesoderm derivatives, as well as the roof plate and condensing cartilage, and is required for subsequent growth and patterning of the neural tube (McMahon *et al.* 1998). Analysis of a null mutant for *noggin* in mouse revealed that Noggin is not involved in neural induction; rather, it is important for normal patterning of the vertebrate neural tube and somites (McMahon *et al.* 1998). Due to redundancy between BMP antagonists, their

incomplete knockouts and lack of sensitive assays to estimate these antagonists, it is very difficult to conclusively prove the role of BMP antagonists as neural inducers in majority of the model organisms (Khokha *et al.* 2005; reviewed in Niehrs 2005). A triple knockdown of *follistatin*, *chordin* and *noggin* leads to dramatic loss of dorsal structures and neural tissue (Khokha *et al.* 2005). Inhibition of BMP signalling by diverse neural inducers (FGF, IGF and BMP antagonists) may be a common pattern in axis specification in vertebrate development (Pera *et al.* 2003). Mouse embryonic stem cells differentiate to neural cells when treated with Noggin (Gratsch and O'Shea 2002). In Chordin$^{-/-}$ and Noggin$^{+/-}$ embryos, abnormal development of forebrain tissue has been observed (reviewed in Levine and Brivanlou 2007). These findings support the role of Noggin and BMP signalling in directing neural cell fate in mouse embryos (reviewed in Levine and Brivanlou 2007). While Noggin is a candidate molecule that confers the neural inductive properties to the Spemann Organizer, experimental manipulations in various other model organisms such as chick, zebra fish and mouse suggest a role for Noggin in later development of nervous system and elaboration of body plan.

During early development of vertebrates, somites appear in the medial portion of the paraxial mesoderm and give rise to axial structures such as vertebral cartilages and ribs as well as skeletal muscles in the trunk (Tonegawa and Takahashi 1998). In the lateral plate, Noggin regulates the levels of BMP4 to allow the differentiation of somitic mesoderm (McMahon *et al.* 1998; Tonegawa and Takahashi 1998). In mouse, Noggin is important in the pattering of neural tube and somitogenesis (McMahon *et al.* 1998). Noggin also participates in neural crest cell differentiation (Anderson *et al.* 2006). The neural inducing role of Noggin has also been explored during adult neurogenesis (Bonaguidi *et al.* 2008). The expression pattern of *noggin* in particular regions of the nervous system, such as the tufted cells of the olfactory bulb, the piriform cortex of the brain and the Purkinje cells of the cerebellum, suggests its potential role in the adult nervous system of mammals (Valenzuela *et al.* 1995). In the adult brain, neurogenesis continues throughout life in the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) as well as in the subventricular zone (SVZ) adjacent to the lateral ventricle (Bonaguidi *et al.* 2008). Noggin expressed in the SGZ of hippocampal region (Bonaguidi *et al.* 2008) and in the ependymal cells of the SVZ (Lim *et al.* 2000) of the adult brain creates a niche for neurogenesis.

The BMP/Noggin signalling pathway has also been implicated in the development of heart (Choi *et al.* 2007). Noggin is expressed in the myocardial cells of the outflow tract, atrioventricular canal and future right ventricle of the cardiac tissue (Choi *et al.* 2007). In mutant embryos, the heart is abnormal with a thicker myocardium and larger endocardial cushions (Choi *et al.* 2007), which result from increased cell proliferation. Noggin plays a critical role in the control of cell proliferation and epithelial–mesenchymal transition (EMT) during cardiac morphogenesis in the mouse (Choi *et al.* 2007). The formation of somite-derived blood vessels is controlled by BMP4 and Noggin through VEGFR-2 (*Quek-1*) in chick embryo (Nimmagadda *et al.* 2005). Several studies have revealed the potential role of Noggin during regulatory processes such as skeletogenesis (Canalis *et al.* 2003), differentiation of hair follicles (reviewed in Eroshkin *et al.* 2006), organogenesis of pituitary gland (Davis and Camper 2007), periodontium development (Kim *et al.* 2007) and morphogenesis of trachea and oesophagus (Que *et al*. 2006).

Noggin plays a key role in the formation of the skeleton by blocking the effect of BMPs in undifferentiated and differentiated cells of the osteoblastic lineage (reviewed in Canalis *et al.* 2003). It also inhibits membranous ossification and chondrogenesis (Canalis *et al.* 2003). Homozygous null mutations of *noggin* lead to severe developmental abnormalities, joint lesions, skeletal abnormalities and fetal lethality (reviewed in Canalis *et al.* 2003). In mice, overexpression of *noggin* under the control of the osteocalcin promoter leads to osteopenia and fractures (reviewed in Canalis *et al.* 2003). Although heterozygous knockout mice appear normal, heterozygous mutations in the human *noggin* locus (NOG) lead to proximal symphalangism and multiple synostosis syndrome that are characterized by apical joint fusions (Takahashi *et al.* 2001; Canalis *et al.* 2003; Groppe *et al*. 2003). Misexpression of BMPs in lymphocytes causes fibrodysplasia ossificans progressive, a rare genetic disorder of the connective tissue (reviewed in Groppe *et al.* 2003). This misexpression often occurs at the sites of inflammation and leads to conversion of muscle to bone. Although *noggin* gene does not alter in this disorder, a Noggin variant lacking the heparin-binding site is a potential therapeutic agent against BMP-mediated heterotopic ossification in mouse model of fibrodysplasia ossificans progressive (reviewed in Groppe *et al.* 2003).

2.4 *Evolutionary conservation of* noggin

Several reports from invertebrates point towards the evolutionary conservation of *noggin* from poriferans to ascidians (Ogawa *et al.* 2002; Müller *et al.* 2003; Matus *et al.* 2006b; Molina *et al.* 2007, 2009; table 1). In sponges, Noggin is associated with ‘stemness’ of cells (Müller *et al.* 2003). It is expressed in the potential embryonic cells of sponge (Müller *et al.* 2003; Schröder *et al.* 2004). Analyses of planarian (*Schmidtea mediterranea*) genome revealed the presence of 10 genes containing a *noggin* domain. These genes are expressed in a variety of

Table 1. Noggin homologues from different animals

Organism	Noggin homologue	Function	Reference
Porifera (*Suberites domuncula*)	Subdnogg-1	Maintenance of 'stemness' of cells	Müller *et al.* 2003
Cnidaria (*Nematostella vectensis*)	NvNoggin1	epithelial growth or morphogenesis	Matus *et al.* 2006b
Cnidaria (Hydra Indian spp.)	Hydra Indian spp. Noggin 1	Under investigation	Chandramore *et al.* 2010
Planaria (*Dugesia japonica, Schmidtea mediterranea*)	Djnlg, Smed nog1, Smed nlg1, Smed nlg2, Smed nlg3, Smed nlg4, Smed nlg5, Smed nlg6, Smed nlg7, Smed nlg8	pattern formation of brain, stimulation of blastema formation and dorsoventral patterning of the body during regeneration	Ogawa *et al.* 2002; Molina *et al.* 2007, 2009
Ascidia (*Ciona intestinalis*)	Partial characterization	Under investigation	Imai *et al.* 2004
Echinoderm (*Strongylocentrotus purpuratus*)	Partial characterization	Under investigation	Lapraz *et al.* 2006
Cephalochordate (*Branchiostoma floridae*)	Partial characterization	Under investigation	Yu *et al.* 2007
Fishes (*Danio rerio, Fugu rubripes*)	Noggin-1, 2, 3, 5	later development and elaboration of body plan	Fürthauer *et al.* 1999; Eroshkin *et al.* 2006
Amphibia (*Xenopus laevis, Xenopus tropicalis*)	Noggin-1, 2, 4	Dorsoventral patterning and neural induction	Smith *et al.* 1993; Lamb *et al.* 1993; Eroshkin *et al.* 2006
Birds (*Gallus gallus*)	Noggin-1, 2, 4	later development and elaboration of body plan	Connolly *et al.* 1997; Eroshkin *et al.* 2006
Mammals (mouse, human)	Noggin-1, 2	Skeletogenesis, differentiation of hair follicles, morphogenesis of trachea and oesophagus, periodontium development, organogenesis of pituitary gland, cardiac morphogenesis, Patterning of the neural tube, somitogenesis, adult neurogenesis, neural crest cell differentiation	McMahon *et al.* 1998; Canalis *et al.* 2003; Anderson *et al.* 2006; reviewed in Eroshkin *et al.* 2006; Que *et al.* 2006; Choi *et al.* 2007; Davis and Camper 2007; Kim *et al.* 2007; Bonaguidi *et al.* 2008

cell types, such as neural, gastrodermal and mesenchymal cells. *Djnlg*, a *noggin*-like gene isolated from another planarian, *Dugesia japonica*, is involved in pattern formation of the brain (Ogawa *et al.* 2002). Based on the expression pattern of *Djnlg* in the amputated region, it has been suggested that it might be involved in stimulation of blastema formation and D-V patterning of the body during regeneration (Ogawa *et al.* 2002). In addition, *noggin* is identified in sea urchin (Lapraz *et al.* 2006), amphioxus (Yu *et al.* 2007) and *Ciona intestinalis* (Imai *et al.* 2004). However, functional characterization of Noggin from these organisms has not yet been reported. A conserved role of Noggin in neural tube closure of *C. intestinalis* has been predicted in view of its expression domain in the neural folds and in the nerve cord (reviewed in Passamaneck and Di Gregorio 2005). Surprisingly, *noggin* is not found in *Drosophila melanogaster*. However, Holley *et al.* (1996) have demonstrated neurogenic activity of *Xenopus noggin* in *Drosophila*. *Xenopus* Noggin can promote various ventral ectodermal fates in a dose-dependent manner and ectopic CNS formation in *Drosophila* (Holley *et al.* 1996).

2.5 *BMP signalling in Cnidarians*

Cnidarians are considered as a basal eumetazoan lineage and an out-group of the bilaterians (Finnerty 2003). They are usually described as radially symmetrical animals with a primary oral-aboral body axis. However, recent studies suggest the existence of various symmetries such as asymmetry, biradiality, tetraradiality or even bilaterality in the cnidarians (reviewed in Finnerty 2003). Sea anemones have long been known to exhibit bilateral symmetry with a secondary axis of polarity called 'directive axis', perpendicular to the oral-aboral axis (reviewed in Finnerty 2003; Darling *et al.* 2005). Most of the genes involved in vertebrate D-V patterning are present in cnidarians (Matus

et al. 2006b). Several BMP antagonists including *noggin* are expressed asymmetrically in the directive axis of *Nematostella vectensis* (Matus *et al.* 2006a, b; Rentzsch *et al.* 2006). Two *noggin* genes, *NvNoggin1* and *NvNoggin 2*, have been identified in *N. vectensis* (Matus *et al.* 2006b). Orthologues to the two *noggin* genes (*NvNoggin1* and *NvNoggin2*) and *follistatin* (*NvFollistatin*) are expressed later during gastrulation (Matus *et al.* 2006b). *NvNoggin1* is expressed strongly in endoderm on only one side of the pharynx above the position that is destined to become the ciliated siphonoglyph and in the endodermal base and tips of the developing tentacles (Matus *et al.* 2006b). It is proposed that *NvNoggin1* may be involved in epithelial growth or morphogenesis (Matus *et al.* 2006b). *NvNoggin1*, *NvDpp* (an orthologue of *decapentaplegic/BMP2/BMP4*) and *NvChordin* are expressed on the same side of the directive axis, while *Hox* genes are expressed on the opposite side (Matus *et al.* 2006b). *NvNoggin2* is expressed weakly throughout the endoderm (Matus *et al.* 2006b). This expression pattern supports the hypothesis of evolution of mesoderm from a bifunctional mesendoderm through the regulation and modulation of TGF-β signalling (Matus *et al.* 2006b). The expression of various BMP inhibitors along with FGF has also been implicated in neural fate specification in the planula apical tuft, a sensory structure at the anterior end of the swimming stage of *Nematostella* (Matus *et al.* 2006b). Based on the asymmetrical expression of both BMP and its antagonists along the directive axis in *N. vectensis*, it has been proposed that the cnidarian directive axis might be homologous to the bilaterian D-V axis (Matus *et al.* 2006b). However, *in vivo* functional experiments are necessary to understand the functional aspects of molecules with respect to directive axis polarity (Manuel 2009).

noggin was not reported from any cnidarian other than *Nematostella* until our recent report on *noggin* from hydra (Chandramore *et al.* 2010). However, some components of TGF-β signalling pathway such as type I TGF-β family receptor, two Smad family transcription factors and a gene encoding a member of the bone morphogenetic family, *Am-BMP2/4*, have been identified in the coral *Acropora* (Samuel *et al.* 2001; Hayward *et al.* 2002). Am-BMP2/4 may be involved in axial patterning in the early coral embryo (Hayward *et al.* 2002). In addition, the presence of *BMP4* homologues in other coral species such as *Acropora muricata*, *A. digitifera*, *Favia favus* and *Platygyra sinensis* and in an actiniarian, *Actina equine*, has been reported (Hwang *et al.* 2003).

Thus, evolutionary conservation of the *noggin* gene is fairly well established. In addition, several TGF-β receptors have been isolated from the sponge *Ephydatia* (Suga *et al.* 1999), a member of the parazoan phylum Porifera, implying that TGF-β receptors originated prior to the divergence between diploblasts and Porifera at the very earliest stages of multicellular life. Genome analyses of choanoflagellate *Monosiga brevicollis* (King *et al.* 2008) and placozoan *Trichoplax adharens* (Schierwater *et al.* 2009) reveal the presence of the major components of BMP signalling. However, *noggin* is not yet reported from either of these species. Thus, the evolutionary origin of the neurogenic function of Noggin as found in *Xenopus* remains elusive because of the lack of data from the basal phyla.

2.6 *Does hydra have a Noggin?*

The evolution of anteroposterior (A-V) and D-V axes in higher organisms is one of the central issues in evo-devo. Hydra, as a representative of basal phylum, Cnidaria, provides various advantages for studying evolutionary aspects of developmental processes. Firstly, hydra is one of the earliest animals to exhibit a defined body axis in metazoan evolution. In addition, it displays a simple body plan and is thought to be the putative common radially symmetrical ancestor of higher metazoans (Meinhardt 2002). Molecules important in the generation of body axes of higher organisms are involved in the patterning of hydra as well. Noggin plays a vital role in the axial patterning of higher organisms by antagonizing BMP signalling (Zimmerman *et al.* 1996). In this regard, the possible role of molecules like Noggin, if present in hydra, is likely to shed light on the basic mechanisms of construction of metazoan body plans. Secondly, the primitive nature of the nervous system of hydra provides a framework to understand the mechanisms of origin of complex nervous system in higher animals. Hydra possesses a diffused nervous system where the sensory and ganglionic neurons and their processes are interspersed among the epithelial cells of both layers (reviewed in Watanabe *et al.* 2009). BMP and Chordin have recently been shown to play a role during axial patterning in hydra (Reinhardt *et al.* 2004; Rentzsch *et al.* 2007). Considering the 'Organizer-like' function of Noggin in higher metazoans (Smith *et al.* 1993), we reasoned that identifying a possible hydra homologue of this gene may take us a step closer to understanding the evolutionary origin of the neurogenic function of Noggin.

Another important aspect of this model is the presence of Organizer in hydra. Various transplantation and molecular studies have provided evidence for the presence of embryonic Organizer in nearly all phyla (Gerhart 2001). Existence of an Organizer-like activity in hydra was first demonstrated by Ethel Browne more than 100 years ago in 1909 (reviewed in Rentzsch *et al.* 2007). Earlier, transplantation experiments indicated that hypostome of hydra acts as an Organizer as it can induce a new axis upon transplantation (Li and Yao 1945; Yao 1945; MacWilliams 1983a, b; Broun and Bode 2002). However, hydra Organizer is different from vertebrate Organizer in several aspects

(reviewed in Broun and Bode 2002). The presence of hydra Organizer in adult organism and continuous active signalling throughout the lifespan of the animal are its distinguishing features (Broun and Bode 2002). Also, the organizing capacity appears to be physically spread through most of the body instead of being localized to a specific region (Broun and Bode 2002). It has been suggested that the hypostome region has the inductive property of an Organizer, while the body column exhibits a self-differentiating or self-organizing property (Broun and Bode 2002).

Molecular techniques have facilitated identification of several candidate genes important in the Organizer function in hydra. Similar to the vertebrate *goosecoid*, hydra *goosecoid*, *Cngsc*, is expressed in hypostome and is involved in head patterning (Broun *et al.* 1999). *HyWnt*, the hydra orthologue of *Wnt-3A* gene, is expressed in the hypostome (Hobmayer *et al.* 2000). Its spatiotemporal expression in developing buds suggests its important function in setting up the new Organizer (Hobmayer *et al.* 2000). Expression of *Hyβ-cat,* the hydra orthologues of *β-catenin* and *HyTcf* orthologues of *Tcf/Lef*, in the adult head as well as during bud formation (Hobmayer *et al.* 2000) indicates the possible involvement of Wnt pathway in hydra Organizer function similar to vertebrates (Hobmayer *et al.* 2000). Further, the role of canonical Wnt pathway in the formation and maintenance of the head Organizer in hydra is confirmed using alsterpaullone, an inhibitor of GSK-3β activity that also elevates the levels of β-catenin (Broun *et al.* 2005).

Several components of the BMP signalling have already been characterized in hydra. *HyBMP5-8b*, a *BMP5-8* orthologue, has been reported from *Hydra* (Reinhardt *et al.* 2004). A potential role of *HyBMP5-8b* has been suggested during tentacle formation and patterning of the lower body axis (Reinhardt *et al.* 2004). Chordin, one of the BMP antagonists, has been isolated from hydra. Hydra *chordin*, *HyChdl* (Chordin-like molecule), plays an important role in setting up a new head Organizer during budding and regeneration (Rentzsch *et al.* 2007). A *smad* gene, *HySmad1*, involved in nematocyte differentiation and oogenesis, has been reported from hydra, suggesting the conservation of multifunctional role of TGF-β signalling pathway between diploblastic and triploblastic metazoans (Hobmayer *et al.* 2001). Thus, occurrence of components of BMP pathway in hydra strongly supports the idea of their origin was before the divergence of cnidarians and bilaterians (Reinhardt *et al.* 2004; Rentzsch *et al.* 2007).

Origin of the Organizer, which plays an important role in axial patterning, is thought to be a major event in chordate evolution leading to a variety of body forms (Gerhart 2001). It is therefore essential to study Organizer properties and their molecular identity in basal phyla to understand the evolution of chordate body plan. Hydra provides a good model system to study 'primitive' or the earliest mechanisms of axial patterning. In order to address various aspects of evolution of axes formation and generation of body plan, we studied the hydra homologue of *noggin*.

3. Cloning and characterization of hydra *noggin*

3.1 *Isolation of* noggin *from hydra*

Whole mount *in situ* hybridization using heterologous riboprobes from *Xenopus laevis* had indicated the presence of *noggin*-like transcripts in hydra (Chatterjee *et al.* 2001). Isolation of hydra orthologue of *noggin* gene was attempted using various primer combinations such as *Xenopus noggin* and degenerate oligonucleotides designed on the basis of conserved domains of vertebrate *noggin* genes. However, this approach was not successful. With the availability of ESTs of *Hydra magnipapillata*, the database was screened for the presence of *noggin*. The coding DNA sequences of *noggin* from different organisms were used to search for homologues in the hydra EST database using TBLASTX program. *Hydra magnipapillata* EST (clone hmp_08494) showed homology to the vertebrate Noggin protein. A fragment of 514 bp was amplified from the Indian species of hydra using the primers designed on the basis of *in silico* screening of *H. magnipapillata* EST database. The PCR product was cloned in pGEMT Easy vector. BLAST analysis of the *noggin* fragment from the Indian species of hydra showed very high homology with that of *H. magnipapillata*. When translated *in silico* using Expasy-TRANSLATE program and searched for homology using BLASTP against the NCBI protein database, the fragment showed around 45%-54% homology to mouse, human and *Xenopus* Noggin (figure 1). A full-length *noggin* ORF of 693 bp was PCR-amplified with primers designed on the basis of the genomic contig sequence. The ORF fragment was cloned in pGEM-T Easy vector, sequenced and analysed using TBLASTX at the NCBI server. It showed about 48%–65% similarity with Noggin protein sequences available in the NCBI database. A full-length sequence of the Indian species of hydra *noggin* was deposited in the NCBI database (accession number: EU314928; figure 2).

The ORF of hydra *noggin* reveals all the conserved features of the Noggin family protein. It predicts a protein of 230 amino acids that contains a predicted 22-amino-acid signal peptide for secretion followed by a conserved Cysteine knots in the C-terminus. Sequence comparison of hydra Noggin with other Noggin homologues using the Clustal W program indicates that hydra Noggin has a considerable degree of conservation in the C-terminal region (Chandramore *et al.* 2010).

Figure 1. Sequence analysis of hydra Noggin. The deduced amino acid sequence of hydra Noggin was compared with representative Noggin sequences from other phyla available at the NCBI database using Clustal W. Cysteine residues, characteristic of Noggin family protein, are highlighted in blue, while other conserved residues are indicated in pink. The C-terminal part shows more conserved residues, especially all nine cysteines that are of structural relevance. Reproduced from Chandramore *et al.* 2010 with permission.

The putative amino acid sequence exhibits all the nine cysteine residues of cysteine knots in hydra Noggin. The cysteine residues Cys207-Cys215 that confer the conformational rigidity to the long finger of human Noggin (Groppe *et al.* 2002) are also conserved in hydra. The phylogenetic analysis using the MEGA 4.0 program revealed that hydra Noggin clusters with Noggins from invertebrates, such as *Dugesia*, *Suberites* and *Nematostella* (Chandramore *et al.* 2010).

3.2 *Evolutionary conservation of the function of hydra* noggin

Noggin has the ability to mask the receptor-binding epitopes of BMP and thereby inhibit further signalling (Zimmerman *et al.* 1996). Noggin causes prospective ventral mesoderm to become dorsal mesoderm (dorsalization) and prospective epidermis to become neuroectoderm (neuralization) through BMP inhibition in vertebrates (Smith *et al.* 1993). The

(A)

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ATGCTCATTTCACGTCTTACTTTTCTATTAGTGGTATACTATCTTATTGAAATTGATTGCGCTGCGTTACC
GTCTTTTAGTGATATGCTAGAGGAAACTTCACAGAAACCGGATATTAAAGCCATGATTCCTTTATTTAAAG
AAATGCAAAAACCTTCAATTGAAGAACGCAATCCTCAGAAACTTTTATATATTCTCGGAAACAGTTATGAC
TCAGAATTTTCTTCTATTGCAAAACCTCCTTCAATGAAAGCACGCGAACCCAGCACTGACGATTTAGTACC
GACATACCTTCTAGTATGAAAACACACCCAACTATGAGCGAAGAGGTCCTTAATAAAGTTTTCTGCCAAAG
TAAAAGGTAAAAAGAAGGAATTCGGGAAACGTTTAACTGAAAAAATGCAAAATTATTTATGGAACTTATCA
AAATGCCCCGTCAAGTATAAATGGATTGACTTGGGTGAAAATATTTTTCCGCAATATATCAAAAGAGGTTC
GTGTTCAAAGAAGAAAACATGCTCTTTTCCTGCTGGTATGACATGCGAAGAGTCAAAATGGAAATCTGTAA
CATATTGTTATACACATGTTTGAAGGAGTATAACTCTGTTTCTCTTGACTGTAAATGGGTCCTATGCCAGT
CAATATATTAACTGAATGTTCTTGTCAGTGTCAGAAACAAACAGAATAG
```

(B)

```
MLISRLTFLLVVYYLIEIDCAALPSFSDMLEETSQKPDIKAMIPLFKEMQKPSIEERNPQKLLYILGNSYD
SEFSSIAKPPSMKAREPSTDDLDTDIPSSMKTHPTMSEEVLNTKFSAKVKGKKKEFGKRLTEKMQNYLWNL
SKCPVKYKWIDLGENIFPQYIKRGSCSKKKTCSFPAGMTCEESKWKSVDILLYTCLKEYNSVSLDCKWRPM
PVNILTECSCQCQKQTE
```

Figure 2. Hydra *noggin* gene and protein sequences (accession number: EU314928 NCBI database). (**A**) 693 bp nucleotide sequence of hydra *noggin*. (**B**) Putative protein of hydra Noggin of size 230 amino acids.

function of a specific gene can be elucidated by studying the phenotype resulting from overexpression of that particular gene in a suitable system. However, such types of studies are difficult to carry out in adult hydra due to the lack of necessary technical information. Consequently, to assess the functional conservation of hydra Noggin, ectopic expression of hydra *noggin* mRNA in a heterologous system like *Xenopus laevis* was performed.

3.2.1 *Hydra Noggin mimics dorsalizing signal in Xenopus:* *Noggin*, when overexpressed on the ventral side of normal *Xenopus* embryos, induces partial body axes, with no head structures (Fang *et al.* 2000). About 50 pg of hydra *noggin* mRNA was injected in 30 *Xenopus* embryos at the two-cell stage. As a positive control, an equal number of embryos was injected with 50 pg of *Xenopus noggin* mRNA. Interestingly, the induction of a partial secondary axis was observed in 100% of the embryos injected with hydra *noggin* mRNA, similar to that of *Xenopus noggin* (figure 3; Chandramore *et al.* 2010). Also, hydra *noggin* could induce the secondary axis at a similar concentration (50 pg) of mRNA as that of *Xenopus noggin*. These results indicate that hydra Noggin mimics actions of endogenous Noggin in *Xenopus* (Chandramore *et al.* 2010). Formation of the body axis of the *Xenopus* embryo can be abolished by UV-irradiation (De Robertis *et al.* 2000), and it can be completely rescued by overexpression of *noggin* through RNA microinjection (Smith *et al.* 1993). The post-fertilization exposure to UV resulted in severe ventralization in *Xenopus* embryos with no axis development. When hydra *noggin* mRNA (50 pg) was injected into UV-treated *Xenopus* embryos, most were rescued, albeit partially (Chandramore *et al.* 2010). Further, to illustrate the inhibitory effect of hydra Noggin on downstream targets of BMP signalling, animal cap assay was performed. Two-cell-stage *Xenopus* embryos were injected with *BMP4* mRNA, either alone or in combination with hydra *noggin* or *Xenopus noggin* mRNA. The inhibitory effect of hydra Noggin was analysed by checking the expression levels of *msx1* and *vent2*, which mediate the effect of BMP signalling during patterning of the ventral axis (Onichtchouk *et al.* 1998; Tríbulo *et al.* 2003). The expression level of *msx1* and *vent2*, downstream targets of BMP signalling, was studied using semi-quantitative RT-PCR. The expression levels of *msx1* and *vent2* were similar in animal caps of embryos injected with 2 pg of *BMP4* as that of whole embryos. In embryos injected with *BMP4* mRNA (20 pg) in combination with hydra *noggin* (2 ng), both *msx1* and *vent2* were absent. Also, embryos injected with hydra *noggin* alone showed no detectable *msx1* and *vent2*. Although higher concentrations of hydra *noggin* were required to block the BMP activity, the results were similar to those obtained with positive controls, i.e. embryos injected with *Xenopus noggin* mRNA (2 ng) alone or in combination with mRNA-encoding *BMP4* (20 pg). Absence of *Brachyury* expression in non-injected caps confirmed the mesoderm-free animal caps. Expression of *ornithine decarboxylase*, a control for internal normalization, was unaffected. The absence of downstream genes to BMP in embryos treated with hydra *noggin* either alone or in combination with *BMP4* showed that it could block the BMP signalling in *Xenopus*. Thus, these findings strongly suggest that hydra Noggin is a true homologue of vertebrate Noggin (Chandramore *et al.* 2010).

3.3 *Localization and possible function of Noggin in hydra*

The analysis of spatiotemporal pattern of Noggin in intact and regenerating hydra under normal and certain experimental conditions (*see* later) has been initiated with a view to elucidating its function. The preliminary data from *in situ* hybridization with riboprobes are briefly summarized here (K Chandramore and S Ghaskadbi, unpublished observations).

3.3.1 *Normal adult polyp:* Expression of *noggin* in the Indian species of hydra and *H. vulgaris* was studied using whole mount *in situ* hybridization with DIG-labelled hydra Noggin antisense riboprobes. In both these species, *noggin* was expressed uniformly throughout the body column and at the base of tentacles. Noggin transcripts were not detected in the foot region and tentacles. Very low levels of

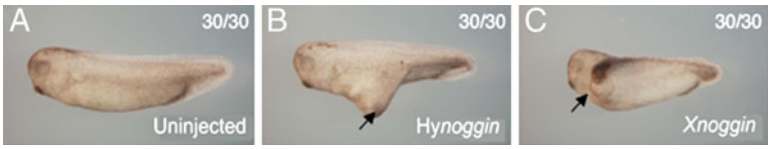

Figure 3. Heterologous expression of hydra *noggin* in *Xenopus laevis* embryos. (**A**) Uninjected embryos, (**B**) 50 pg Hydra *noggin* mRNA-injected embryos and (**C**) 50 pg *Xenopus noggin* mRNA-injected embryos. Overexpression of both *noggin* mRNAs induces the incomplete secondary axes (arrow). The number of surviving embryos per total number of injected embryos is indicated at the upper right of each panel. Reproduced from Chandramore *et al.* 2010 with permission.

transcripts were detected in the head region. Expression of *noggin* was prominent in endodermal layer although it did not appear to be confined to any specific cell type. In hydra, distinct expression of *noggin* was first observed in stage 2 bud exclusively at the tip of growing bud. In later stages of bud formation, the expression spread throughout the body column and resembled the adult pattern of expression.

3.3.2 *Expression of* noggin *in regenerating pieces of hydra treated with chemicals that perturb pattern formation:* Since Noggin plays an important role in axial patterning and neurogenesis in vertebrates (De Robertis *et al.* 2000), the expression profile of *noggin* was studied in hydra in which pattern formation was perturbed using lithium, UV or alsterpaullone (Hassel and Bieller 1996; Broun *et al.* 2005; Ghaskadbi *et al.* 2005).

3.3.2.1 Expression of noggin *in response to lithium chloride:* In regenerating hydra pretreated with 1 mM LiCl, only the number of Noggin transcripts seemed to decrease as compared with controls, without any significant change in the pattern of expression, whereas both the expression domain as well as the intensity appeared to change with 2 mM LiCl pretreatment.

3.3.2.2 Expression of noggin *in response to alsterpaullone:* The role of Wnt signalling in Organizer formation of hydra has been demonstrated through blocking of the Gsk-3 activity by alsterpaullone treatment (Broun *et al.* 2005). In vertebrates, Noggin is associated with Organizer function and plays an important role in neural and axial patterning (Smith *et al.* 1993). To study the possible functional synergism of Wnt and Noggin during axial patterning, expression of *noggin* was studied in hydra treated with alsterpaullone, which enhances Wnt signalling. Using semi-quantitative RT-PCR, the expression level of Noggin transcripts in alsterpaullone-treated hydra was assessed. In alsterpaullone-treated hydra, the expression level of Noggin was decreased by about 40% as compared with controls, indicating the inverse relationship between Noggin and Wnt signalling during the patterning in hydra.

3.3.2.3 Expression of noggin *in response to ultraviolet radiation:* Expression level of *noggin* in UV-treated hydra was studied using semi-quantitative RT-PCR. Noggin expression level was reduced by about 50% in UV-treated hydra as compared with controls. Since Noggin regulates the expression of BMP, to see whether UV-induced decrease in *noggin* results in a consequential increase in *BMP* transcripts, semi-quantitative RT-PCR for *BMP* was carried out. Surprisingly, *BMP* levels did not appear to be altered drastically in response to reduction in *noggin* expression.

4. Summary and conclusions

We have isolated and characterized the *noggin* gene from hydra for the first time. The coding DNA sequence of hydra *noggin* has been deposited in NCBI database as EU314928 (Chandramore *et al.* 2010). This finding is significant since Noggin is a key player governing various developmental processes of higher metazoan. Hydra *noggin* shows considerable similarity with its vertebrate orthologues especially at the amino acid level. We find that ectopically expressed hydra Noggin functions as the ventralizing and dorsalizing agent in *Drosophila* and *Xenopus* embryos, respectively. Further, hydra Noggin mRNA can effectively rescue UV-ventralized *Xenopus* embryos. This confirms the functional conservation of hydra *noggin* in higher metazoans.

Noggin is structurally and functionally conserved from hydra through vertebrates. In view of the absence of mesoderm and dorsoventral axis in hydra, the presence of *noggin* is interesting from evolutionary perspective. Elucidation of the function of Noggin in hydra may provide important clues regarding evolution of complex signalling mechanisms in neural induction and dorsoventral patterning. Work on hydra Noggin supports the recent findings that the regulation of development of the complex nervous system in vertebrates has its evolutionary origins in the simple organism like hydra. The characterization of Noggin in hydra thus provides a framework to investigate the extent to which patterning system is conserved across species.

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