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Asymmetric expression of the BMP antagonists *chordin* and *gremlin* in the sea anemone *Nematostella vectensis*: Implications for the evolution of axial patterning

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Abstract

The evolutionary origin of the anterior–posterior and the dorsoventral body axes of Bilateria is a long-standing question. It is unclear how the main body axis of Cnidaria, the sister group to the Bilateria, is related to the two body axes of Bilateria. The conserved antagonism between two secreted factors, BMP2/4 (Dpp in *Drosophila*) and its antagonist Chordin (Short gastrulation in *Drosophila*) is a crucial component in the establishment of the dorsoventral body axis of Bilateria and could therefore provide important insight into the evolutionary origin of bilaterian axes. Here, we cloned and characterized two BMP ligands, *dpp* and *GDF5*-like as well as two secreted antagonists, *chordin* and *gremlin*, from the basal cnidarian *Nematostella vectensis*. Injection experiments in zebrafish show that the ventralizing activity of *NvDpp* mRNA is counteracted by *NvGremlin* and *NvChordin*, suggesting that Gremlin and Chordin proteins can function as endogenous antagonists of NvDpp. Expression analysis during embryonic and larval development of *Nematostella* reveals asymmetric expression of all four genes along both the oral–aboral body axis and along an axis perpendicular to this one, the directive axis. Unexpectedly, *NvDpp* and *NvChordin* show complex and overlapping expression on the same side of the embryo, whereas *NvGDF5*-like and *NvGremlin* are both expressed on the opposite side. Yet, the two pairs of ligands and antagonists only partially overlap, suggesting complex gradients of BMP activity along the directive axis but also along the oral–aboral axis. We conclude that a molecular interaction between BMP-like molecules and their secreted antagonists was already employed in the common ancestor of Cnidaria and Bilateria to create axial asymmetries, but that there is no simple relationship between the oral–aboral body axis of *Nematostella* and one particular body axis of Bilateria.
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Introduction

Comparative analysis of conserved molecular systems that govern the establishment of the body plan of diverse bilaterally symmetric animals has lead to the reconstruction of a hypothetical ancestor of all Bilateria, the "Urbilateria" (De Robertis and Sasai, 1996; Knoll and Carroll, 1999). Essential features of this bilaterian ancestor are the use of a cluster of homeobox transcription factors (the *Hox* genes) to pattern the anterior–posterior axis and an antagonism between the secreted TGFbeta superfamily growth factor Decapentaplegic (Dpp, BMP2/4 in vertebrates) and its inhibitor Short gastrulation (Sog, Chordin in vertebrates) for patterning of the dorsal–ventral axis. However, Bilateria are generally thought to have originated from radially symmetric animals, and this transition from radial to bilateral symmetry represents a major transition in the evolution of multicellular animals. How the single body axis of the Radiata is related to the two axes of Bilateria is still unclear.

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Both *hox*/*parahox* genes and components of a *dpp* signal transduction pathway have been identified in Cnidaria (Finnerty et al., 2004; Gauchat et al., 2000; Hayward et al., 2002; Hobmayer et al., 2001; Samuel et al., 2001; Shenk et al., 1993; reviewed by Darling et al., 2005; Ball et al., 2004; Martindale, 2005), an evolutionary old phylum regarded as a sister group to the Bilateria. A possible scenario for the origin of bilateral symmetry was provided by a recent study that analyzed the expression patterns of *hox* genes and a *dpp* homolog in the sea anemone *Nematostella vectensis*. While several *NvHox* genes occupy at least three different expression domains along the oral–aboral axis, *NvDpp* expression during early development is restricted to one side of the oral end, thus defining a second, or directive axis, perpendicular to the oral–aboral axis (Finnerty et al., 2004). An asymmetric expression of dpp during gastrulation of an anthozoan has previously also been shown in the coral *Acropora millepora* (Hayward et al., 2002). Since *Nematostella* and other cnidarians also show morphological features of bilaterality, one interpretation of these findings is that Cnidaria in fact possess two axes like Bilateria: the oral–aboral axis patterned by the anterior–posterior patterning system of the *hox* genes, and a directive axis patterned by graded activity of the dorsoventral patterning factor Dpp. However, in *Nematostella*, *hox* genes as well as *dpp* display a strikingly asymmetric expression with respect to both axes: *NvDpp* is only expressed at the oral pole during gastrulation, while several *NvHox* genes are expressed only on one side along the oral–aboral axis of the embryo, complicating a straightforward assignment of the axes in *Nematostella* to those of Bilateria.

A crucial part in the argument about a conserved molecular mechanism to pattern the dorsoventral axis in Bilateria is the complementary expression and antagonistic action of the secreted molecules BMP2/4 and Chordin in vertebrates and their homologs Dpp and Sog in *Drosophila*, respectively (reviewed by De Robertis and Sasai, 1996; Arendt and Nubler-Jung, 1997). In brief, Chordin determines the dorsal and therefore neural side of the vertebrate body, while BMP2/4 specifies ventral territories (Holley et al., 1995). In an attempt to better understand the role of BMP/Dpp signaling during *Nematostella* axis formation, we isolated two BMP/Dpp antagonists, *NvChordin* and *NvGremlin*, and show that they indeed function as BMP/Dpp antagonists in zebrafish microinjection experiments. We find that *NvChd* and *NvGrm* are expressed asymmetrically with respect to the directive axis but also along the oral–aboral axis. Our findings indicate that NvDpp activity gradients exist in two perpendicular directions in the *Nematostella* embryo, thereby refuting a simple relationship of the Cnidarian axes with those of Bilateria.

Materials and methods

Nematostella culture

The *Nematostella* culture (Hand and Uhlinger, 1992) and induction of spawning was carried out as described (Fritzenwanker and Technau, 2002). Synchronized embryos were obtained from in vitro fertilized eggs, which continued to develop at 23°C.

Identification and cloning of chordin, gremlin, dpp and GDF5-like homologs from Nematostella

Based on EST and shotgun genome sequence information, a 2568-bp cDNA fragment of *NvChd* was amplified using Pfu DNA polymerase (Promega, USA), primers ATG TTG GCC AAA TTC TTT GTT TTC G (forward) and TTA CCC CGA GCA AGG AAC ACA GC (reverse), and cloned into vector pCS2+ via ClaI and XhoI adaptors (*pCS2-NvChd*). To confirm that this fragment contains the complete open-reading frame of *NvChd*, 5′RACE was performed with reverse primers GCA AAG GAC ACA GCG CTG AAT ACC G, and CTC CTA TCT TGT AGT GGC GTA GTC G (nested), using embryonic cDNA prepared with the GeneRacer Kit (Invitrogen, USA). Obtained fragments were cloned into pGEM-T (Promega, USA). Genbank accession number for NvChd is DQ286294.

A full length *NvGrm* clone was identified in our EST library (Technau et al., 2005), amplified with primers CGC GCA CGC CTG ACC GCT TC (forward) and TAT AAA CGC CAC TCC GGG CGC AAT (reverse), and cloned via StuI and XbaI restriction sites into pCS2+ (*pCS2-NvGrm*). Genbank accession number is DQ471325.

A reconstruction of the genomic organization of the chordin and gremlin genes was carried out by local walks and assemblies using the publicly available genome shotgun data set. A partial *NvDpp* sequence was obtained by degenerate PCR using primers GAY TGG ATH GTI GCI CC (forward) and CAY GCI ATH GTI CAR ACI ST (reverse), followed by nested PCR with primers GGI GAR TGY CCI TWY CC (forward) and YTG RTA RTT YTT IAR IAC IAC (reverse). 5′RACE was performed with primers GAC TTT ATC GAA TTC GTT TAG GGA G and CGC GTG ATT TGT AGC GTT CAA GTG CTT TGC (nested). Primers for 3′RACE were GCA AAG CAC TTG AAC GCT ACA AAT C and TTG AAC GCT ACA AAT CAC GCG ATT G (nested). All fragments were cloned into pGEM-T. Sequence information from these clones was used to amplify full length *NvDpp* with primers TTG TTA CTC CTC TGA G (forward) and CTT TTA ATC GGC TAT TTT CC (reverse), which was then cloned via ClaI and XbaI restriction sites into pCS2+ (*pCS2-NvDpp*). Genbank accession number is AY363391.

A *NvGdf5*-like fragment was initially cloned by degenerate PCR with primers ATH GGN TGG AAY GAY GG (forward), CAN CCR CAY TCN TCN AC (reverse), and GAY TGG ATH GCN CC (nested forward). 5′RACE primers were ACA ACC ATG TCC TTG TAG TTC and CAA GAT ACT TAT AGG GCT TAG TTC (nested), primers for 3′RACE: GTA TGA CGC GTA CTT ATG CGA AG and GGG AGT GTA AAT ATC CGA GTT GAG (nested). The open-reading frame of *NvGdf5*-like was amplified with primers ATG GTT ACA GCA TTT TTT CAA G (forward) and CTT AAT TTA TTG ACA ACC GCA G (reverse) and cloned into pCS2+ utilizing BamHI and EcoRI restriction sites (*pCS2-NvGdf5*). Genbank accession number is AY496945.

Phylogenetic sequence analysis

Alignments of protein sequences were generated by ClustalW and then analyzed by maximum likelihood method (Schmidt et al., 2002; TreePuzzle 5.2), using the following parameters: model of substitution JTT, uniform rate of heterogeneity, 10.000 puzzling steps. Graphic illustrations were generated by TreeView (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

In situ hybridization

In situ hybridization in *Nematostella* was essentially done as described (Scholz and Technau, 2003) with the following modifications: embryos were fixed in cold 4% paraformaldehyde or 4% paraformaldehyde/0.0625% glutaraldehyde in MEM buffer for 1–3 h (Fritzenwanker and Technau, 2002; Kusserow et al., 2005). Hybridizations were carried out at 60°C, for posthybridization washes serial dilutions (75%, 50%, 25%) of hybridization solution with 2× SSCT were used, stringency washes were done with 0.2× SSCT for 3 × 20 min. Washing after anti-DIG or anti-FITC-AP incubation was done with PBS + 0.1% Tween20.

Fluorescent double in situ hybridizations were modified after a *Xenopus* protocol (http://www.xenbase.org/). Digoxygenin or FITC-labeled RNA probes (Roche, Switzerland) were visualized consecutively using HRP-coupled

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