Developmental Biology 414 (2016) 108–120

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Evolution of developmental control mechanisms

Glypican1/2/4/6 and sulfated glycosaminoglycans regulate the patterning of the primary body axis in the cnidarian *Nematostella vectensis*

Markus Bause, Roddy van der Horst, Fabian Rentzsch *

Sars Centre for Marine Molecular Biology, University of Bergen, Thormøhlensgt 55, 5008 Bergen, Norway

ARTICLE INFO

Article history:
Received 14 November 2015
Received in revised form
22 March 2016
Accepted 13 April 2016
Available online 14 April 2016

Keywords:
Glycoproteins
Proteoglycans
HSPGs
Wnt-pathway
FGF
Anterior-posterior axis
Apical sensory organ
Apical tuft
Endosulfatase
Frizzled5/8

ABSTRACT

Glypicans are members of the heparan sulfate (HS) subfamily of proteoglycans that can function in cell adhesion, cell crosstalk and as modulators of the major developmental signalling pathways in bilaterians. The evolutionary origin of these multiple functions is not well understood. In this study we investigate the role of glypicans in the embryonic and larval development of the sea anemone *Nematostella vectensis*, a member of the non-bilaterian clade Cnidaria. *Nematostella* has two *glypican* (*gpc*) genes that are expressed in mutually exclusive ectodermal domains, *NvGpc1/2/4/6* in a broad aboral domain, and *NvGpc3/5* in narrow oral territory. The endosulfatase *NvSulf* (an extracellular modifier of HS chains) is expressed in a broad oral domain, partially overlapping with both *glypicans*. Morpholino-mediated knockdown of *NvGpc1/2/4/6* leads to an expansion of the expression domains of aboral marker genes and a reduction of oral markers at gastrula stage, strikingly similar to knockdown of the Wnt receptor *NvFrizzled5/8*. We further show that treatment with sodium chlorate, an inhibitor of glycosaminoglycan (GAG) sulfation, phenocopies knockdown of *NvGpc1/2/4/6* at gastrula stage. At planula stage, knockdown of *NvGpc1/2/4/6* and sodium chlorate treatment result in alterations in aboral marker gene expression that suggest additional roles in the fine-tuning of patterning within the aboral domain. These results reveal a role for *NvGpc1/2/4/6* and sulfated GAGs in the patterning of the primary body axis in *Nematostella* and suggest an ancient function in regulating Frizzled-mediated Wnt signalling.

1. Introduction

The patterning of the anterior-posterior (A-P) body axis is a fundamental step in animal embryogenesis. In bilaterians, several conserved molecular systems have been identified that regulate the development of particular regions along the A-P axis. Wnt/β-catenin signalling specifies the site of gastrulation; this is considered to be the posterior pole. The expression of Wnts at the gastrulation site can then establish graded Wnt/β-catenin signalling activity and provide positional information with maximal levels at the posterior pole (Martin and Kimelman, 2009; Niehrs, 2010; Petersen and Reddien, 2009). *Hox* genes determine the identity of consecutive domains along the A-P axis (Akam et al., 1994; Gellon and McGinnis, 1998; Krumlauf, 1994; Mallo et al., 2010) except for the anterior-most regions, which develop under the control of several transcription factors with *six3* as a key regulator of the anterior patterning program (Lagutin et al., 2003; Posnien et al., 2011; Steinmetz et al., 2010; Wei et al., 2009). While the molecular basis of A-P patterning has been studied in detail in some bilaterian model organisms, the early evolution of axial patterning mechanisms is less well understood. Cnidarians are the sister group of bilaterians (Hejnol et al., 2009; Philippe et al., 2011, 2009; Ryan et al., 2013) and thus occupy an important phylogenetic position for comparative studies on embryonic development. Here we use the sea anemone *Nematostella vectensis* to analyse the molecular regulation of the patterning of the primary body axis in a non-bilaterian model system.

Nematostella embryogenesis encompasses a hollow blastula stage, gastrulation by invagination and the formation of a free-swimming planula larva before the animals become sessile polyps with a single body opening that is surrounded by a ring of tentacles (Hand and Uhlinger, 1992; Kraus and Technau, 2006; Magie et al., 2007). Gastrulation occurs in the domain that is derived from the animal pole of the oocyte and the blastopore becomes the single body opening (Fritzenwanker et al., 2007; Lee et al., 2007). This opening is traditionally described as oral, despite its three functions for the ingestion and egestion of food and as a gonopore. As in bilaterians, Wnt/β-catenin signalling is a key regulator of the patterning of the oral-aboral

* Corresponding author.
E-mail address: fabian.rentzsch@uib.no (F. Rentzsch).

http://dx.doi.org/10.1016/j.ydbio.2016.04.011

axis (Kumburegama et al., 2011; Kusserow et al., 2005; Leclère et al., 2016; Marlow et al., 2013; Röttinger et al., 2012; Sinigaglia et al., 2013; Wikramanayake et al., 2003). While gastrulation and endoderm formation occur at the site of high NvWnt/Nvβ-catenin signalling at the future oral pole (Lee et al., 2007; Wikramanayake et al., 2003), Nvβ-catenin is in addition required for establishing a regulatory system in the aboral half of the blastula, with *NvSix3/6* acting as a key positive regulator of aboral domain development (Leclère et al., 2016; Sinigaglia et al., 2013). Importantly, the Wnt receptor *NvFrizzled 5/8* (*NvFz5/8*) is expressed in the aboral domain from mid-blastula stage on, and serves to restrict the size of the aboral domain by negatively regulating *NvSix3/6* (Kumburegama et al., 2011; Leclère et al., 2016; Sinigaglia et al., 2015). The expression patterns of *Hox* genes do not suggest that they act in a bilaterian-like manner to determine the identity of adjacent territories along the oral-aboral axis (Finnerty et al., 2004; Layden et al., 2016; Manuel, 2009; Ryan and Baxevanis, 2007). After gastrulation, a long ciliary tuft develops at the aboral pole. This is believed to be part of a larval sense organ, the apical organ (Richter et al., 2010) although no functional or developmental connection to the nervous system has been shown yet in *Nematostella* (Marlow et al., 2014; Nakanishi et al., 2012; Richards and Rentzsch, 2014). The development of the apical organ is under the control of two Fibroblast Growth Factors (FGFs) with opposing functions: Signalling by NvFGFa1 via the FGF receptor NvFGFRa is required for the formation of the apical organ, whereas NvFGFa2 functions to limit its size (Rentzsch et al., 2008). Thus, Wnt and FGF signalling play prominent roles in the development of the aboral domain of *Nematostella* and these observations prompted us to analyse the function of potential co-regulators of these pathways, the glypicans.

Glypicans are extracellular proteoglycans that are connected to the cell membrane via a GPI (glycosylphosphatidylinositol) anchor. Proteoglycans are molecules in which one or more glycosaminoglycans (GAGs, long unbranched sugar chains consisting of disaccharide repeats) are covalently linked to a core protein (Esko et al., 2009). The GAG that is linked to glypicans is heparan sulfate and accordingly glypicans belong to the heparan sulfate subfamily of proteoglycans (HSPGs, Sarrazin et al., 2011). In bilaterians, glypicans regulate several developmental processes by their ability to modulate the activity of the Wnt, Hedgehog, BMP and FGF signal transduction pathways (Bishop et al., 2007; Fico et al., 2011; Filmus et al., 2008; Hacker et al., 2005; Lin, 2004). While in some cases the binding of the glypican core proteins to signalling ligands is required for their function (Kirkpatrick et al., 2006; Yan et al., 2009), the developmental roles of glypicans in most cases depend on the interaction of the heparan sulfate (HS) chains with ligands and/or receptors (e.g. Fico et al., 2011).

The covalent linkage of HS chains to the core protein and HS chain elongation occur in the Golgi. The growing HS chains are subject to several modifications, most prominently N-and O-sulfation, which are carried out by NDSTs (N-deacetylase/N-sulfotransferases) and HS-OSTs (HS O-sulfotransferases), respectively. O-sulfation can occur at different positions in the sugar molecules and is catalyzed by position-specific enzymes (HS2OST, HS3OST and HS6OST; reviewed in: Bulow and Hobert (2006) and Esko and Selleck (2002)). Sulfs (6-O endosulfatases) are unique among the HS modifying enzymes in that they also act in the extracellular space to remove sulfation (a modification that cannot be reversed by the only Golgi-localized HS-6OST) (Ai et al., 2003). Different studies have shown that the sulfation or desulfation of HS (by HS-OSTs and Sulfs, respectively) significantly affect the specificity of ligand binding in both the FGF and the Wnt pathways in a cell autonomous manner (Ai et al., 2003; Dhoot et al., 2001; Kleinschmit et al., 2013; Venero Galanternik et al., 2015; Wang et al., 2004).

Several mechanisms have been described by which glypicans can affect the activity of signalling molecules, including the promotion of ligand-receptor interactions, the sequestration of ligands and the enhanced or reduced mobility of ligands in the extracellular space. These mechanisms and their net effects on signalling activity can differ for individual glypican-ligand combinations and between different tissues or cell types (Fico et al., 2011; Matsuo and Kimura-Yoshida, 2013, 2014; Sarrazin et al., 2011; Yan et al., 2009). The variety of these interactions, including cell autonomous and long-range types of fine-tuning, enable glypicans to be multifaceted modulators of signalling pathways.

A previous study showed that the enzymes required for HS biosynthesis and modifications are present in *Nematostella* and that the HS chains in these animals have an unusual disaccharide composition (Feta et al., 2009). Here we show that *NvGlypican1/2/4/6* (*NvGpc1/2/4/6*) and the sulfation of HS chains are involved in the patterning of the oral-aboral axis and the proper development of the apical organ of *Nematostella*.

2. Materials and methods

2.1. Nematostella culture

Animals were maintained in 1/3 filtered seawater (*Nematostella* medium (NM)) and induced to spawn as described previously (Fritzenwanker and Technau, 2002). Fertilised egg packages were incubated in 3% cysteine/NM for 25 min on a rotary shaker to dissolve the jelly mass surrounding the eggs. Embryos were raised at 21 °C until 24 hpf (hours post-fertilisation) for gastrula stages, 48 hpf for mid-planula, and 72 hpf for late planula stages.

2.2. Identification of the sequences

Gene models for *NvGlypican1/2/4/6* and *NvGlypican3/5* (protein ID 247677 and 134347, respectively at http://genome.jgi.doe.gov/Nemve1/Nemve1.home.html) were used to design RACE (Rapid Amplification of CDNA Ends) primers. Standard methods for 3′ and 5′RACE were carried out with cDNA templates from mixed *Nematostella vectensis* developmental stages (SMARTer™ RACE cDNA Amplification Kit, Clontech Laboratories, Mountain View, CA, USA) The obtained full length sequences were cloned into pGemT vector (Promega, Madison, WI, USA) and sequenced.

2.3. Phylogeny

We used the MEGA6 software to align selected sequences and to carry out phylogenetic analyses, as described (Hall, 2013). The region selected for the alignment was the conserved glypican domain of ca. 390 amino acids, which contains 14 cysteine residues (Fico et al., 2011). We used Maximum Likelihood analysis with a LG+G+I model with partial deletion for all trees shown. Robustness was tested using the bootstrap method with 500 replicates. We used glypican orthologues of the following species for our analysis: Aq (*Amphimedon queenslandica*); Dm (*Drosophila melanogaster*); Hm (*Hydra magnipapillata*); Hs (*Homo sapiens*); Nv (*Nematostella vectensis*) Rn (*Rattus norvegicus*); Sp (*Strongylocentrotus purpuratus*. Accession numbers: AqGpcA: XP_003386976; AqGpcB: XP_003386974; DmDallyLike: AAS65005.1; DmDally: AAA97401.1; HmGpc4like: XP_002157574; HsGpc1: AAH51279.1; HsGpc2: AAH27972.1; HsGpc3: AAH35972.1; HsGpc4: AAH17166.1; HsGpc5: AAH39730.1; HsGpc6: CAC21820.2; RnGpc1: AAA41251.1; RnGpc2: NP 612520.1; RnGpc3: AAH85756.1; RnGpc4: AAH81962.1; RnGpc5: NP 1100755.2; RnGpc6: EDM02516.1; SpGpc5: NP 1138966.1; SpGpc6: NP 1138965.1.