**SIGNALING MOLECULES IN THE HYDRA HEAD ORGANIZER AND THE EVOLUTION OF AXIS FORMATION**

Bert Hobmayer¹, Fabian Rentzsch², Ulrich Technau³, and Thomas W. Holstein³

¹ Institut für Zoologie und Limnologie, Universität Innsbruck, Innsbruck, Austria
² Max-Planck-Institut für Immunbiologie, Freiburg, Germany
³ Abteilung Molekulare Zellbiologie, Technische Universität, Darmstadt, Germany

**ABSTRACT**

The molecular nature of signaling centers (organizers) plays a pivotal role in the formation of body axes in multicellular animals. Organizers secrete growth factors which act as long range regulators in axis formation and cell differentiation. To analyze the evolutionary origin of organizers, we studied Wnt and TGFβ/Bmp signaling pathways in Hydra, a member of the primitive animal phylum Cnidaria. Molecules of the Wnt pathway and the TGFβ/Bmp antagonist Chordin are expressed in the Hydra head organizer. They are transcriptionally upregulated early during asexual bud formation and head regeneration, and also define head organizers created by de novo pattern formation in aggregates. Thus, the Hydra head organizer exhibits astonishing similarities to organizers in vertebrates. Our results suggest that Wnt and TGFβ/Bmp signaling is involved in axis formation in Hydra, and support the idea that these signalling pathways played a key role in the evolution of axial differentiation in the earliest multicellular animals.

**INTRODUCTION**

Throughout embryonic development, inductive signals provide cells with positional information to regulate their differentiation behaviour and specification into different cell types. This finally leads to the emergence of a highly organized, multicellular animal. Signaling pathways which exhibit long-range action over several cell diameters are particularly interesting, as they are generally involved in the formation of the major three-dimensional coordinates during early embryogenesis. In recent years, functional screenings and the completion of three whole-genome sequencing projects in Caenorhabditis elegans, Drosophila melanogaster, and Homo sapiens demonstrated that only a surprisingly limited set of conserved signaling pathways acts in early embryos in bilateral metazoans, i.e. Wnt, TGFβ/Bmp, Hedgehog, FGF, Notch, and EGF pathways. Among these, primarily members of the Wnt and TGFβ/Bmp signaling pathways provide axial polarity, act in early axis determination, and are major building units for organizing centers like the Spemann organizer in the amphibian gastrula.

The evolutionary origin of Wnt and TGFβ/Bmp signaling pathways and signaling centers in general is unclear at present. Since detailed information comes presently only from bilateral, higher metazoans, it is crucial to analyze developmental signaling in extant, basal metazoans, whose divergence predates the Urbilateria - the common ancestor of all bilateral protostomes and deuterostomes (for review see Knoll and Carroll, 1999). In this review evidence is summarized for the action of Wnt and Bmp signaling pathways in the freshwater polyp Hydra, a member of the old, diploblastic metazoan phylum Cnidaria (Fig. 1A).

Hydra exhibits a simple body plan with an apical head and a basal foot separated by a gastric region along one major body axis. It is generally assumed that this oral-aboral body axis represents a basal character which gave rise to the anterior-posterior and dorsal-ventral body axes of bilaterians. A small number of about 10 different cell types is organized in two germ layers (ectoderm and endoderm). Hydra reproduces sexually and asexually and its tissue is in a dynamic state of permanent turnover. Stem cells in the gastric region divide continuously, and the newly produced cells create a tissue movement towards the ends and into the asexually forming bud. This situation requires that pattern forming processes are permanently active in an adult body (Bode and Bode, 1984). It probably also enables the polyps to regenerate any lost body part. Even more, polyp tissue can be dissociated into single cell suspensions, and these cells reaggregate and form intact polyps within several days (Fig. 1B), making Hydra one of the few animal systems that allow experimental analysis of self-organization (Gierer et al., 1972). In addition, a large number of classic transplantation experiments have demonstrated that Hydra tissue is capable of head induction and the formation of an ectopic body axis (Browne, 1909; Wolpert et al., 1972; MacWilliams, 1983; Meinhardt, 1993). A recent study has confirmed that this capacity resides primarily in the head (hypostome), and that the hypostomal tissue has characteristics similar to those of organizing centers in vertebrates (Broun and Bode, 2002).

**A CANONICAL WNT SIGNALING PATHWAY IN HYDRA**

Although a large number of developmental genes known from bilaterians have been identified in Hydra by recombinant DNA methods (for review see Galliot, 2000), genome sequence data are not accessible for cnidarians. Therefore, a PCR approach was used to identify Wnt signaling molecules in Hydra. We identified a Wnt ligand (HyWnt) and the cytoplasmic mediators Dishevelled (HyDsh), GSK3 (HyGSK3), and β-Catenin. **Gravitational and Space Biology Bulletin 17(2) June 2004 107**
(Hyβ-Cat). By using a two-hybrid screening with a Hydra β-Catenin bait, we also characterized the transcriptional co-activator Tcf (HyTcf) (Hobmayer et al., 2000). Recently, a Hydra member of the family of Frizzled receptors was published (Minobe et al., 2000). The predicted protein structure of HyWnt shows a Wnt-specific pattern of 22 highly conserved cysteines and a signal peptide sequence indicating that HyWnt is secreted, while amino acid identity of the whole open reading frame is relatively low as compared with other Wnt factors (Fig. 2A). The cytoplasmic mediators, however, are highly conserved. Their overall domain structure is typical for the corresponding protein family, and the known protein-protein interaction domains (shaded grey) show particularly high amino acid identities of about 80% compared to those of their human homologues (Fig. 2A). All in all, these structural data suggest that at least a core canonical Wnt signaling pathway is present in Hydra. Thus, Wnt signaling clearly originated ancestral to the common ancestor of diploblastic cnidarians and triploblastic bilateral metazoans.

To show that Wnt signaling acts in axial patterning in Hydra, we analyzed mRNA expression of HyWnt and HyTcf by in situ hybridization. HyWnt is expressed in a small number of about 50 epithelial cells in the apical tip of the hypostome which represents the Hydra head organizer. HyTcf expression is also restricted to the hypostome of the polyp, but the HyTcf-positive domain is broader than the HyWnt spot comprising the entire hypostome, and thereby possibly demarcates the range of action of the HyWnt ligand (Hobmayer et al., 2000).

To confirm that HyWnt signaling plays an instructive role in head induction, expression of HyWnt was studied in the two morphogenetic mutant strains mh-1 and reg-16. Polyps of the multiheaded strain mh-1 produce ectopic heads by a budding-like evagination process along the body axis. These ectopic heads remain attached to the mother polyp resulting in abundantly multiheaded bodies (Sugiyama, 1982). Under these conditions, HyWnt is expressed in the hypostome of the mother polyp and in the apical tip of the growing ectopic heads at any stage (Fig. 2B). Interestingly, spots of HyWnt expressing cells can be observed in the body column of the mother polyp without any visible sign of tissue evagination (arrows in Fig. 2B). We speculate that these HyWnt-positive spots define positions where the next ectopic heads will be forming.

In contrast, polyps of strain reg-16 have a severely reduced capacity for head regeneration. After removal of the original head, most polyps either completely fail to develop a head, or head development is strongly delayed (Achermann and Sugiyama, 1985). While the wild type regenerates activate HyWnt transcription in their regenerating tip very early after decapitation (details see below), completely inhibited reg-16 regenerates never express HyWnt. Reg-16 regenerates which show delayed formation of head structures also show a corresponding delay in HyWnt expression (Hobmayer et al., 2000). Thus, activation of Wnt signaling is tightly correlated with head induction under modified conditions of ectopic head formation and head regeneration deficiency.

**EXPRESSION DYNAMICS OF WNT SIGNALING MOLECULES DURING BUD FORMATION AND HEAD REGENERATION**

_Hydra_ continuously produce new polyps by an asexual budding process in a well-defined area of the lower body column – the budding zone. This zone has been previously regarded to represent a distinct field of enhanced morphogenetic activity, where the new body axis of the daughter polyp is initiated (Bode and Bode, 1984). HyWnt pathway activation occurs in two steps during budding. First, transcriptional upregulation of Hyß-Cat and HyTcf, the two transcription factors of the Wnt pathway, transiently defines the budding zone before tissue evagination starts. This represents the first detectable sign of bud initiation at a molecular level (Fig. 3A; Hobmayer et al., 2000). Upstream factors activating the Hyß-Cat and HyTcf genes are unknown at present. High expression levels are maintained in the evaginating bud until shortly before bud detachment (Fig. 3B). Then,
upregulated expression becomes restricted to the hypostome of the new daughter polyp (Fig. 3C). Temporal and spatial synexpression of \( \text{Hy} \beta\text{-Cat} \) and \( \text{HyTcf} \) supports the view that both act together as transcriptional regulators.

**Figure 2.** Structural conservation and function of molecules of a \textit{Hydra} Wnt signaling pathway. (A) Schematic representation of the predicted \textit{HyWnt}, \textit{HyDsh}, \textit{HyGSK3}, \textit{Hy}\beta-Cat, and \textit{HyTcf} proteins based on cDNA sequences. Numbers at the ends represent the predicted number of amino acids. Shaded areas represent protein-protein interaction domains. Numbers in parenthesis show amino acid identity with corresponding human homologues throughout the open reading frame or in protein-protein interaction domains. (B) \textit{HyWnt} in situ hybridization during ectopic head formation in the multi-headed mutant \textit{mh-1}. Arrows indicate \textit{HyWnt} expression domains before the onset of tissue evagination.

Second, \textit{HyWnt} expression starts in a few cells in the budding zone at the position where tissue evagination starts (Fig. 3D). This \textit{HyWnt}-positive spot then increases in size during bud growth until it resembles the hypostomal expression domain of a fully developed polyp (Fig. 3E,F; Hobmayer et al., 2000). Thus, once local transcription of \textit{HyWnt} is activated, the position of evagination seems to be determined and a new head organizer being established.

**Figure 3.** Expression dynamics of \textit{Hydra} Wnt signaling molecules during formation of a head organizer visualized by \textit{in situ} hybridization. (A-C) Expression of \textit{HyTcf} in the budding region immediately before tissue evagination (A) and during bud development (B,C). (D-F) Expression of \textit{HyWnt} at the onset of bud evagination (D) and during bud development (E,F).

Formation of a head organizer is also induced by head regeneration. Decapitation leads to wound healing within one hour, to a local increase in the competence for head induction in the distal tissue of the remaining body column within about 8-10 hr, and to the reappearance of tentacles and hypostomal structures within 30-36 hr. \textit{In situ} hybridizations carried out during this process show that \( \text{Hy} \beta\text{-Cat}, \text{HyTcf}, \) and \( \text{HyWnt} \) are significantly upregulated in the regenerating tips of the regenerates (Hobmayer et al., 2000). Similar to the budding process, \textit{HyWnt} pathway activation again happens in two steps. As an immediate response (about 30 min) upon head removal, \( \text{Hy} \beta\text{-Cat} \) and \( \text{HyTcf} \) are expressed. \( \text{HyWnt} \) is expressed about 2 hr later. Since establishment of organizer activity covers the first 8-10 hr of this process (MacWilliams, 1983), activation of the Wnt pathway is a very early event. In the case of \( \text{Hy} \beta\text{-Cat} \) and \( \text{HyTcf} \), it even starts during the first phase of wound healing. Again, factors regulating activation of these genes are not known, but we would speculate that time is too limited for the action of independent signaling pathways. More likely, proteins already present in the cytoplasm or nucleus might respond to head removal and mediate Wnt pathway-specific gene activation. Consistent with this idea, activation of \( \text{HyWnt} \) transcription is not sensitive to inhibition of protein synthesis by the addition of cycloheximide during early head regeneration (unpublished results).

**ACTIVATION OF HEAD ORGANIZER GENES DURING REAGGREGATION**

In bud formation and head regeneration, establishment of the head organizer starts from an established body axis of
the mother polyp, and one has to be cautious about the instructive role of the HyWnt pathway. Therefore, formation of the head organizer and HyWnt activation were studied in dissociation-reaggregation experiments, where the original positional information is completely lost. Here, head organizers form de novo and induce the surrounding tissue to develop into completely normal polyps (Gierer et al., 1972). The classical aggregation procedure was modified by manually introducing small clusters of vitally labeled cells with an elevated capacity for head induction into carrier tissue taken from the gastric region with low capacity for head induction. The labeled cell clusters were produced by isolating regenerating tips 12 hr after head removal, when the tips have gained maximal competence for head induction. The tips were dissociated into single cells, aggregated in rotary culture, and the resulting cell clusters were selected by size (Technau et al., 2000).

Figure 4. Community effect and expression dynamics of HyWnt and HyBra1 during head organizer formation in Hydras. (A) Efficiency of cell clusters to induce head formation during reaggregation is size dependent. Cell clusters were derived either from regenerating tips (closed triangles) or from carrier tissue (open symbols) as a control. (B-E) Expression of the head organizer genes HyWnt (B,C) and HyBra1 (D,E) in small domains after 24 hr (B,D), which then increase in size and co-localize with developing heads after 96 hr (C,E) of aggregation.

The labeled cell clusters retain their high capacity for head induction throughout dissociation-reaggregation and use it to induce head organizers under conditions of de novo pattern formation. They appear with non-random, very high frequency in the newly developing heads in the aggregates. Their ability to induce head organizers depends, however, on cluster size (Technau et al., 2000). Clusters of about 30 µm diameter representing one or few epithelial cells have no elevated frequency of induction, while clusters of about 90 µm diameter containing 10-20 epithelial cells show maximum induction (Fig. 4A). These data demonstrate that a relatively small number of cells is necessary and sufficient to act as a head organizer. They also suggest that a community effect between these cells is essential to maintain their inductive capacity.

In situ hybridizations during the process of reaggregation show that small spots of HyWnt-positive cells appear after about 20-24 hr (Fig. 4B). At this time, epithelial cells have completely sorted into ectodermal and endodermal layers (Gierer et al., 1972), indicating that HyWnt activation can occur only in intact tissue with bilayered epithelial organization. All HyWnt-positive spots then colocalize with developing heads (Fig. 4C), and in quantitative terms, every HyWnt expressing domain finally forms a head (Technau et al., 2000). It should be added that the first HyWnt-positive spots have a size equivalent to the size of cell clusters capable of head induction. Thus, HyWnt expression seems to define early head organizers created by de novo pattern formation.

Recent work has emphasized that individual signaling pathways are embedded in more complex signaling networks with multiple positive and negative feedback loops providing precision, stabilization and robustness, but also flexibility to the signaling system (Freeman, 2000; Davidson, 2002). Is there evidence for feedback control on the HyWnt pathway? Although we have no conclusive functional proof, preliminary data allow us to propose two feedback mechanisms. First, HyWnt might activate and stabilize its own expression directly via its transcriptional mediators Hyβ-Cat and HyTcf (Fig. 5). During bud formation and head regeneration, Hyβ-Cat and HyTcf are upregulated earlier than HyWnt. Also during reaggregation, Hyβ-Cat and HyTcf are expressed uniformly before HyWnt-positive spots appear. This is consistent with the idea that HyWnt is a direct target gene of an active Hyβ-Cat/HyTcf complex. In Drosophila, autocatalytic self-activation of Wg (the Drosophila Wnt1 homologue) and a functional Tcf-binding site in the Wg promotor have been demonstrated (van de Wetering et al., 1997; Lessing and Nusse, 1998).

Second, HyWnt might also be coupled by positive feedback with HyBra1, the Hydra homologue of the T-box gene Brachyury (Technau and Bode, 1999). Size and time of appearance of small HyBra1-positive spots during aggregation are equivalent to the HyWnt expression dynamics (Fig. 4D,E; Technau et al., 2000). HyBra1 also shows synexpression with HyWnt during budding and head regeneration as well as in adult polyps, although the HyBra1-positive domain in the steady state hypostome is broader than the HyWnt-positive domain (Technau and
In agreement with synexpression, a putative Tcf-binding site has been recently identified in the HyBra1 promoter (unpublished results). In mouse embryos and mouse cell lines, Brachyury is a direct target gene of Wnt3a signaling (Liu et al., 1999; Galceran et al., 2001), and Brachyury itself again activates transcription of Wnt11 in Xenopus (Tada and Smith, 2000). Here, it is interesting to add that phylogenetic sequence analysis puts HyWnt into the vertebrate Wnt3 cluster (unpublished results; Schubert et al., 2001). Taken together, several pieces of indirect evidence suggest a positive feedback loop between HyWnt and HyBra1 (Fig. 5). Direct experimental proof for such a feedback loop is of particular importance.

**PRELIMINARY EVIDENCE FOR BMP SIGNALING IN HYDRA**

The second major signaling system involved in establishing early embryonic polarities and mediating dorsoventral axis formation in bilaterians is represented by TGFβ/Bmp signaling pathways (DeRobertis and Sasai, 1996). In Xenopus and Drosophila, two evolutionary conserved mechanisms of TGFβ/Bmp modulation have been described. First, secreted TGFβ/Bmp antagonists, i.e. Chordin and Noggin, interact with extracellular Bmp ligands at the protein level and thereby block their signaling activity (for review see DeRobertis and Bouwmeester, 2001). Second, Wnt signaling inhibits transcription of Bmp mRNA via specific transcriptional regulators, i.e. Siamois (Carnac et al., 1996), and conversely, Bmp signaling inhibits Wnt transcription via its own transcriptional mediator Smad (Wiersdorff et al., 1996). In the Xenopus Spemann organizer, the best studied signaling center, both mechanisms seem to be active. Chordin and later Wnt are locally expressed in the organizer and presumably suppress any Bmp signaling or transcription (Fig. 6; for review see Harland and Gerhart, 1997).

Recently, genes of members of the TGFβ/Bmp signaling system have been identified in cnidarians in several laboratories, clearly indicating that TGFβ/Bmp signaling is present in basal metazoans (Samuel et al., 2001; Lelong et al., 2001; Hobmayer et al., 2001). In Hydra, two Bmp5-8 ligands (Reinhardt et al., 2004), a highly conserved receptor-regulated Smad1 homologue (Hobmayer et al., 2001), and the Bmp antagonist Chordin (unpublished results) have been identified. Up to now, in situ hybridization data are only available for Hydra. HyBMP5-8b acts in tentacle formation and patterning of the foot (Reinhardt et al., 2004). HySmad1 is expressed throughout the body column, but absent in the ectoderm of the head and in the lower foot (Hobmayer et al., 2001). Chordin mRNA is present in the head in intact polyps, and is strongly upregulated throughout bud formation, in regenerating heads and also in regenerating feet (unpublished results). These preliminary results are consistent with a hypothesis that – analogous to the situation in higher metazoans - Hydra Bmp signaling is suppressed by Chordin in developing organizer tissues.

However, the analysis has not been carried out in sufficient detail to give a conclusive answer about the spatial pattern of Bmp activity in Hydra. Particularly, Hydra Bmp2-4 orthologues have yet not been found. Once Bmp ligands bind to their receptors at the cell surface, cytoplasmic Smad proteins are recruited to the ligand-receptor complex, phosphorylated, and thereby activated to move into the nucleus to regulate target genes (for review see Massague et al., 2000). Visualization of these phosphorylated Smad factors by specific antibodies

**Figure 5. Putative positive feedback in Hydra Wnt signaling.** Preliminary evidence and comparison with higher metazoans support the view that direct, autocatalytic self-activation and indirect feedback between HyWnt and the transcription factor HyBra1 are involved in establishment and maintenance of HyWnt signaling.

**Figure 6. Model for similarities between the Xenopus Spemann organizer and the developing Hydra head organizer.** Based on the expression domains of Wnt, Chordin, and Bmp molecules in the Spemann organizer and on preliminary evidence for the expression of Wnt, Chordin, and Smad1 during Hydra head formation, we speculate that Chordin-Bmp and/or Wnt-Bmp antagonisms might have been present in early metazoan signaling centers.
is the most direct and convincing tool to show territories of Bmp activity. Such antibodies have been produced in *Drosophila* and vertebrates (Faure et al., 2000; Tanimoto et al., 2000), but are not available for phosphorylated HySmad1 at present.

CONCLUSION AND PERSPECTIVES

The results reviewed here suggest that *Hydra* has a signaling center regulating the establishment of its major body axis. This points towards an origin of signaling centers in the earliest multicellular animals. Based on the signaling molecules identified up to now and their putative feedback control, we speculate that a core network of molecular interactions constituting an organizer might be conserved throughout the metazoa (Fig. 6). However, here one has to be careful, since it theoretically cannot be excluded that feedback interactions might be the result of convergent evolution rather than sharing common descent.

To provide more convincing evidence, additional results are required at two levels: (1) Signaling factors activate transcriptional regulators, which again regulate signalling factors. This relationship has to be characterized at the genomic level to provide a more comprehensive view on the set of molecules acting in an organizer and on their feedback interactions in a gene regulatory network, as recently proposed (Davidson et al., 2002). (2) The spatial pattern of signaling factor activity seems to be generally controlled by complex (usually antagonistic) extracellular protein-protein interactions. Thus, we also need to characterize the complete set of secreted molecules involved in establishing an organizer and to define their biochemical interactions in the extracellular space.

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