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Review

Immortality and the base of multicellular life: Lessons from cnidarian stem cells

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ABSTRACT

Cnidarians are phylogenetically basal members of the animal kingdom (>600 million years old). Together with plants they share some remarkable features that cannot be found in higher animals. Cnidarians and plants exhibit an almost unlimited regeneration capacity and immortality. Immortality can be ascribed to the asexual mode of reproduction that requires cells with an unlimited self-renewal capacity. We propose that the basic properties of animal stem cells are tightly linked to this archaic mode of reproduction. The cnidarian stem cells can give rise to a number of differentiated cell types including neuronal and germ cells. The genomes of *Hydra* and *Nematostella*, representatives of two major cnidarian classes indicate a surprising complexity of both genomes, which is in the range of vertebrates. Recent work indicates that highly conserved signalling pathways control *Hydra* stem cell differentiation. Furthermore, the availability of genomic resources and novel technologies provide approaches to analyse these cells in vivo. Studies of stem cells in cnidarians will therefore open important insights into the basic mechanisms of stem cell biology. Their critical phylogenetic position at the base of the metazoan branch in the tree of life makes them an important link in unravelling the common mechanisms of stem cell biology between animals and plants.

Contents

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1. Introduction

Stem cells have been described for animals (Metazoa), fungi and plants and are probably a basic feature of all multicellular organisms. All stem cell systems share one universal property in that they continuously reproduce themselves and generate progeny of differentiated cells. During evolution, only stem cells have retained the capacity to give rise to differentiated cells, which in turn have lost their totipotency or pluripotency. Therefore, the capacity to remain in an undifferentiated state and to be able to generate one or more differentiated cell types is central for stem cell function in the various multicellular organisms [1]. In fungi and in sponges (Parazoa), cells of the mature organism have almost completely retained their totipotency suggesting that all cells may function as "stem cells" [2,3]. In plants, stem cell populations that are located at the opposite ends of an embryo in specialized meristems are used to generate the adult structures [1]. In higher metazoans, stem cell populations are used to build up and replenish various organ systems, such as the gonads, gut, the hematopoietic and nervous systems.

At present, it is an important question to what extent these different and highly evolved multicellular systems share common molecular mechanisms to regulate stem cell maintenance and differentiation. Comparative genomics and new functional approaches have unravelled molecular and functional similarities of stem cell biology not only within the animal kingdom, but also across the kingdoms. Although the picture is far from being complete, it is obvious that stem cell systems at the base of metazoan evolution are important to identify the basic mechanisms of stem cell biology in the animal and plant kingdom. The so far best understood stem cell system at the base of metazoan evolution is that of the freshwater polyp *Hydra*.

2. *Hydra*: The everlasting polyp

The freshwater polyp *Hydra* is a member of the >600 million years old phylum cnidaria that comprise the oldest living metazoans with a nervous system. Molecular phylogenetic studies clearly indicate that cnidarians diverged prior to the appearance of the bilaterians [4–6]. Two cnidarian genome projects have been recently performed, one on the anthozoan polyp *Nematostella vectensis* [7] and one the hydrozoan polyp *Hydra magnipapillata*, which started in 2004 [8] and was just finished (http://Hydrazome.metazome.net/index.php).

Among all cnidarians, the freshwater polyp *Hydra* has been the most intensively studied animal, both at the cellular and molecular level [9]. The polyp has a long history as an experimental system. Van Leeuwenhoek first classified the polyp in 1702 as a plant. Abraham Trembley (1744) described in his famous book that *Hydra* is a small animal with distinct patterns of behaviour and a remarkable regeneration capacity (Fig. 1). Since that time, *Hydra* was studied to understand the mechanism of regeneration, pattern formation and stem cells [9–11].

Adult polyps of *Hydra* exhibit the classical cnidarian body plan [12]. Two epithelial cell layers, an outer ectoderm and an inner endoderm are separated by a acellular mesogloea and form a tube-like body enclosing a gastric cavity, which is open at the oral end (mouth). The mouth is located on top of a dome-like hypostome, which is surrounded by a ring of tentacles. The aboral end forms a peduncle and a foot that contains a disk of epithelial cells (basal disc) that secrete a sticky mucous by which the animals adhere to the substratum. The oral end is frequently called the "head", which is a misleading term since it corresponds to the blastopore of the embryo at the gastrula stage corresponding to the posterior end in bilaterians; the bilaterian head forms at the anterior end [13,14].

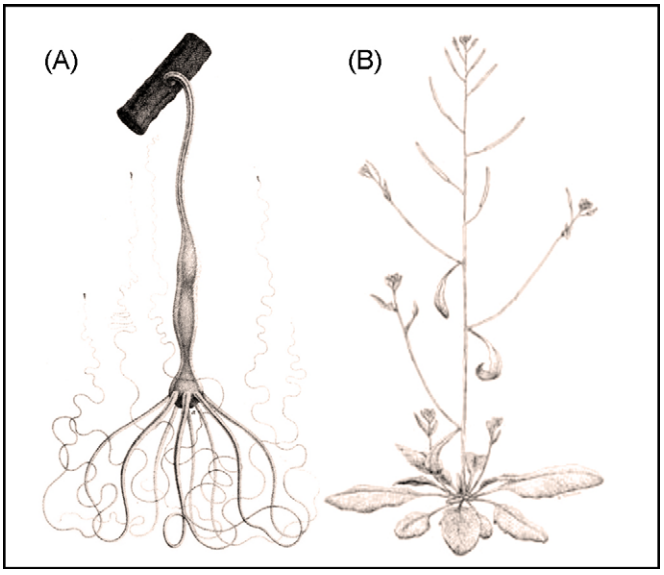

Fig. 1. *Hydra* and Plants. (A) *Hydra* attached to a small twig with its oral end pointing down (image from Trembley, 1744) [202]. (B) *Arabidopsis taleana* (image from Kirsten Bomblies).

Hydra can propagate sexually and asexually (Fig. 2A). The asexual mode of reproduction is budding. During bud formation, a region of the parent body becomes remodelled into a small but complete animal [15]. Budding is accompanied by a significant increase in the mitosis [16] and only depends on the supply of sufficient food. A well-fed population [17,18] grows logarithmically with a doubling time of about 3–4 days [19].

Not only a number of different *Hydra* strains, e.g. the *Hydra magnipapillata* 105 wild type strain [20], but also many *Hydra magnipapillata* mutant strains affecting pattern formation and stem cell differentiation [20–24] have been cultured for many years without any sexual reproduction. This unlimited growth indicates that *Hydra* lacks aging and by analysing the reproductive rates of individual budding polyps for four years Martinez found no evidence for a decline in the reproductive rates of individual *Hydra vulgaris* polyps [25]. Thus it is highly probable that *Hydra* does not undergo senescence and is biologically immortal [10,25].

However, not all cnidarians are immortal. Medusae, which represent the sexual form of many hydrozoans and scyphozoans, are also known to die at the end of gametogenesis. Under certain conditions, some *Hydra* species can undergo depression that was interpreted as senescence. In *Hydra oligactis* lowering the temperature induces sexual reproduction, which in turn results in an almost irreversible depression. This phenomenon has been described by Paul Brien as *crise gametogenique* [26] and was recently confirmed [27]. It will be a challenge for future research to unravel the molecular mechanism causing aging and senescence in these closely related *Hydra* species.

3. Stem cell populations

What is the cellular and molecular base for *Hydra's* almost unlimited life span? Similar to plants, there are populations of stem cells that are constantly dividing and used to generate the adult structures of the polyps. Three stem cell lines can be distinguished (Fig. 2B): the (i) ectodermal epithelial, (ii) endodermal epithelial and (iii) interstitial stem cell lineage. Transgenic animals using GFP-labelled cell lines [28] that these three stem cell lineages cannot interconvert in the adult polyp and therefore represent independent lineages show [9,29]. The three stem cell lines have the capacity for constant renewal, which is the

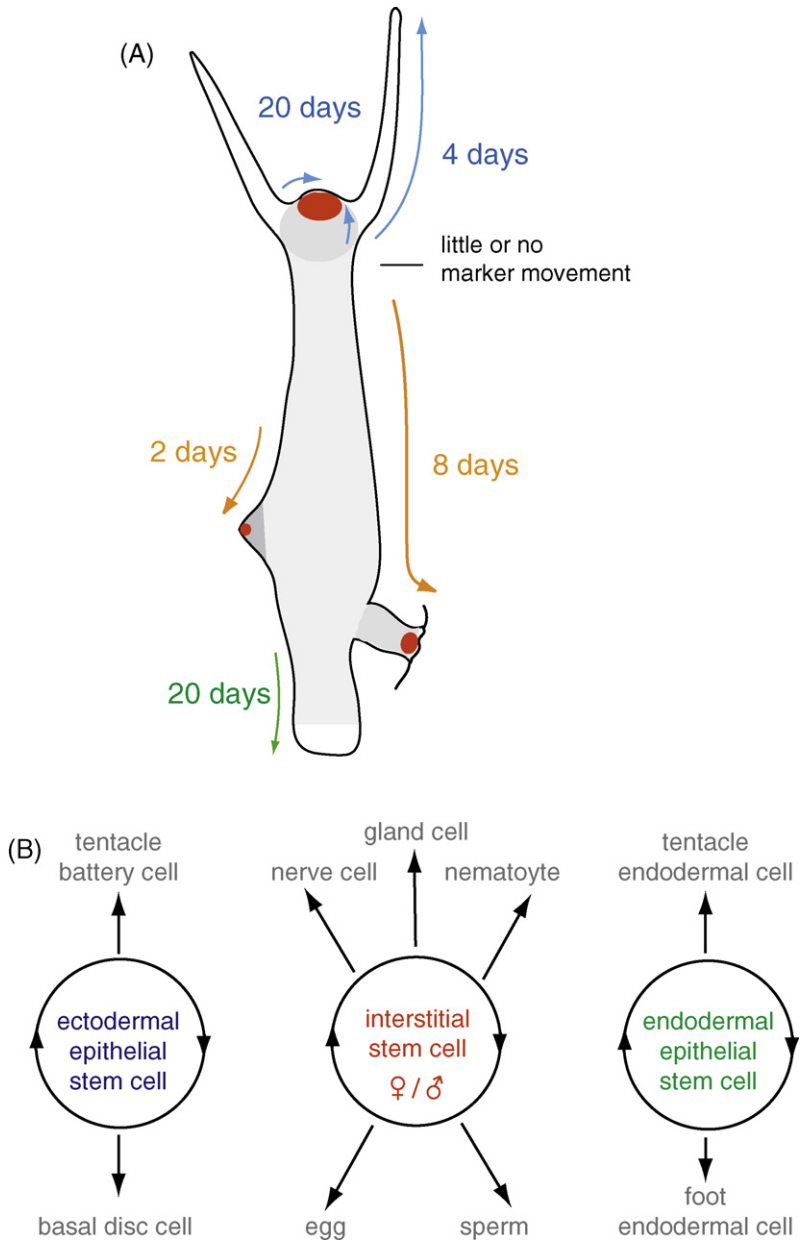

Fig. 2. Growth patterns, tissue dynamics and stem cells in the adult *Hydra* polyp. This figure is adapted from [9,33] and shows in (A) the tissue movements followed by marking the polyp with methylene blue or grafted tentacles. The arrows indicate the starting and ending positions of the marked tissue, and the number of days required for transit of the marked tissue are indicated. The shaded portion of the polyp is the region in which epithelial cells are dividing [16,31]; darker shading indicates shorter cell cycles in the developing bud and in the head region. In the unshaded (white) tentacle and basal disc regions epithelial cells are arrested in G2 of the cell cycle [32,99]. The red regions indicate the high point of *wnt* expression and canonical Wnt signalling [16,134]. (B) Cell lineages in *Hydra* (adopted from [9]. The epithelial cells of the body column consist of ectodermal and endodermal epithelial stem cells that divide and terminally differentiate into the epithelial cells of the foot or the tentacles following displacement from the body column as shown in (A). Dividing committed cells of the nerve, nematocyte, and secretory cell pathways [39] are not shown.

main reason behind the claim that *Hydra* is potentially immortal [10,30].

3.1. Epithelial stem cells

Epithelial stem cells are mainly located in the gastric and hypostomal region of the polyp [16,31]. Those epithelial cells that are located at the tentacles and foot are arrested in the G2 phase of the cell cycle [29,32]. Since epithelial cells in the body column continuously divide there is a continuous increase in epithelial cells. The excess of cells gets continuously sloughed from the tentacles and from the foot [33,34]. Non-dividing differentiated epithelial cells are lost by displacement from the body column within 20 days [34]. The majority of new cells, however, bud off at the sides to form new polyps [9,35]. Epithelial stem cells in the budding and in the hypostomal region exhibit a higher proliferation rate thereby enhancing the effect of the tissue dynamics [16,33].

An important consequence of these tissue dynamics is that epithelial cells in the body column continuously change their axial position. An epithelial cell that is located in the body column can be displaced either to the oral or the aboral end. Here cells differentiate into a tentacle battery cell or a foot basal disk cell (Fig. 2B). Thus, the patterning system must continuously provide the positional information for the displaced cells and is therefore continuously active. Epithelial cells must in turn continuously sense their axial position to make cell fate decisions. In contrast to many plants, this continuous growth process does not result in an unlimited increase in the

polyp's size. Due to proportion-regulation mechanisms the adult animals maintain a maximal size [9,36,37].

Similar to plant epithelial stem cells, stem cells in *Hydra* can also carry out several physiological functions, which is different from epithelial stem cells of higher animals. Epithelial cells contain muscle fibres [38,39], form the mesogloea [40,41], and have a protective function in that they secrete antibacterial and fungicidal substances [42]. The endodermal stem cells are involved in the digestion of food in the gastric cavity. The fact that several functions are combined in one cell type is a feature of the ancestry of

Table 1
Cnidarian orthologs of signalling and transcription factors known to be stem cell-specific in bilaterians.

	Hydra	Expression	Nematostella	Expression
A. Somatic cell reprogramming factors				
Oct4	No	–	No	
SoxB1	HySoxB1a, 1b Hma2.207023, Hma2.209373; Holstein lab unpublished	Interstitial stem cell	NvSoxB1 [192]	Somatic cells and neuronal subsets
Nanog	no	–	No	
c-Myc	HyMyc1, 2, 3 Hma2.227429, Hma2.220288, Hma2.222803; Holstein lab, unpublished	n.d.	NvMyc1, 2, 3, 4 XP 001640859, XP 001640858, XP_001627619, XP_001627620; Holstein lab unpublished	n.d.
B. Stem cell enriched nuclear proteins				
MCM2	HyMCM2a, 2b Hma2.204653, Hma2.220032; Holstein lab unpublished	n.d.	NvMCM2 XP_001627160; Holstein lab unpublished	n.d.
PCNA	HyPCNA Hma2.225202; Holstein lab unpublished	n.d	NvPCNA Stellabase 23876; Holstein lab unpublished	n.d.
Pumilio	HyPumilio1, 2 Hma2.213208, Hma2.211467; Holstein lab unpublished	Interstitial stem cell	NvPumilio XP_001634870; Holstein lab unpublished	n.d.
Nucleostemin/GNL3L	HyNucleostemin Hma2. 208076; Holstein lab, unpublished	n.d.	NvNucleostemin XP_001638945; Holstein lab unpublished	n.d.
C. Germline factors				
Piwi	HyPiwi-like1, 2 Hma2.223971, Hma2.220868; Holstein lab, unpublished	n.d.	NvPiwi-like1, 2, NvArgonaute/Eif2c-like XP 001641994, XP 001626127, XP_001638494; [193], Holstein lab unpublished	n.d.
Nanos	CnNos1, 2 [194]	Interstitial stem cells, germ cells	NvNos1, 2 [195,196]	Germ cells and somatic cell types
Vasa	CnVas1, 2 [197]	Interstitial stem cells, germ cells	NvVas1, 2 [195]	Germ cells and somatic cell types during early embryogenesis
D. Neuron differentiation factors				
SoxB2	HySoxB2a, 2b, 2c, 2d Hma2.219830, Hma2.224265, Hma2.207485, Hma2.207564; Holstein lab, unpublished	n.d.	NvSoxB2a, 2b, 2c, 2d [192], Holstein lab, unpublished	Neurons?
SoxC	HySoxC Hma2.216120; Holstein lab, unpublished	n.d.	NvSoxC [198]	Sensory neurons
AS-C	CnAsh, LiAS, HyAS-C3 Hma2.233345; [70,71,199], Holstein lab, unpublished	Nematoblasts, neurons	NvAS-C1, 2, 3, 4 XP 001632756, XP 001622360, XP_001630355, XP_001623850; Simionato [199], Holstein lab, unpublished	Neurons
ARP	HyNgn, HyNeuroD, HyOligo, HyAtonal Hma2.227242, Hma2.230454, Hma2.215418, Hma2.221652; [199], Holstein lab, unpublished	n.d.	NvNgn1, 2, NvOligo1, 2, NvAtonal1, 2 XP 001628331, XP 001628328, XP_001622931, XP_001628350; [199], Holstein lab, unpublished	Neurons
Zic/odd-paired	HyZic1, 2, 3, 4 Hma2.210038, Hma2.205360, Hma2.224151; [72], Holstein lab, unpublished	Nematoblasts	NvZicA, B, C, D, E [200], Holstein lab, unpublished	n.d.
Six	HySixA, B [201], Holstein lab, unpublished	n.d.	NvSix1/2a, 1/2b, 1/2c, 3/6, 4a, 4b XP 001634996, XP 001626434, XP 001633591, XP 001625159, XP_001623134, XP_001625507; Holstein lab, unpublished	n.d.
Musashi	HyMusashi Hma2.213686; Holstein lab, unpublished	n.d.	NvMusashi1, 2 3 XP 001631942, XP 001641412, XP_001628585; [180] Holstein lab, unpublished	Neurons?
ELAV	HyELAV1, 2, 3, 4, 5 Hma2.213650, Hma2.206380, Hma2.220034, Hma2.210021, Hma2.215147; Holstein lab, unpublished	n.d.	NvELAV1, 2 XP_001626979, XP_001634736; [180], Holstein lab, unpublished	Neurons?

Hydra; in more derived and more complex animals these functions exist in separate cell types [39].

3.2. Interstitial stem cells

The interstitial stem cell lineage is embedded in the interstitial space of the ectodermal and endodermal epithelial cells. Interstitial stem cells have a shorter cell cycle than epithelial cells [19,43,44] and can be easily removed by drugs affecting the cell cycle such as colchicines [45,46] or hydroxyurea [47]. There are also mutant strains carrying temperature sensitive interstitial stem cells [22,48]. In all cases the rapidly proliferating interstitial cells and their progeny are lost. These epithelial animals loose their nerve cells and nematocytes. By force-feeding it is possible to culture these animals [48]. Such animals can still bud and regenerate indicating that epithelial cells but not interstitial cells are necessary for morphogenesis [49].

3.2.1. Multipotency

The potential of self-renewal and multipotency of interstitial stem cells has been demonstrated in statistical cloning experiments by Charles David [50–52]. He found that the interstitial cell lineage consists of multipotent interstitial stem cells that differentiate into germ, gland, mucus, and nerve cells including nematocytes (Fig. 2B, 3) (reviewed in [9,10,39,53]). Together with differentiating intermediates and product cells, the interstitial cell system comprises about 75% of all cells in rapidly growing asexually reproducing animals. Stem cells alone constitute about 4% of the total cell number (n = 100,000) in *Hydra* [53].

3.2.2. Gametogenesis

Of particular interest is the differentiation of interstitial stem cells into the germ cells. Interstitial stem cells are committed either for eggs or sperms [54–57]. When stem cells were re-cloned it was found that male stem cells could switch their sexual phenotype [58]. This is different from higher metazoans where the somatic tissue controls the sexual phenotype and similar to yeast where sex is controlled by an intrinsic mechanism. *Hydra* therefore appears to occupy an intermediate position between single-cell eukaryotes and higher metazoans [58].

The findings that pluripotent interstitial stem cells can differentiate into germ and somatic cells were interpreted as evidence for the hypothesis that cnidarians are lacking a germline [50]. The lack of a germline fits with evolutionary considerations according to which an asexual mode of reproduction requires the presence of an actively dividing multipotent cell line that is capable of differentiating into somatic as well as germ cells [59,60]. However, the situation appears to be more complex since subpopulations of interstitial stem cells have been identified that are restricted to the differentiation of only germ cells [61,62]. These cells are slowly proliferating interstitial cells [44,61] that only differentiate into germ cells. The formation of a second, long-lived germline-restricted cell lineage might therefore reflect an early step in the evolution of the germline, which has been postulated a hundred years ago by Weismann for marine hydroids[1] [63].

3.2.3. Neurogenesis

Neuronal cells comprise the main cell type produced by the pluripotent interstitial stem cells. About 70% of all differentiation products in the interstitial cell lineage are sensory stinging cells (nematocytes) and neuronal cells that form a simple nerve net [64]. This nerve net exhibits higher densities of neurons at the oral and aboral end and is composed of a variety of sensory and ganglionic nerve cells that express distinct neuronal genes in a position-dependent manner. Commitment of neuronal cells occurs in the body column and precursor cells can migrate to the sites of final differentiation [65–67]. The importance of cnidarians as a model for understanding the evolutionary origin of the nervous system in metazoan evolution has been recently revised elsewhere [9,68,69]).

On the molecular level, neurogenesis in cnidarians is barely understood. The available data indicate, however, that there are some similarities with vertebrates. From *Hydra,* several genes have been isolated that are involved in bilaterian neurogenesis (Table 1). The first were the bHLH transcription factor achaete–scute orthologs Cnash [70–72] and Hyzic, an ortholog of the Zn-finger transcription factor zic/odd-paired [72], which both act in vertebrate neurogenesis downstream of the Bmp antagonist Chordin (see also [68,69] for further details). Along with proneuronal and neuronal genes also a large number of neuropeptides were described in cnidarians, particularly in *Hydra* and *Hydractinia*. These neuropeptides have not only morphogenetic functions, but also a function in neurotransmission (reviewed in [73]).

3.2.4. Nematogenesis

The nematocytes are a unique and highly specialized neural cell type that is characteristic for all cnidarians. Each nematocyte has a secretory and sensory function. Upon stimulation, it releases a complex secretion product, the nematocyst [74–76], which is the product of a giant post-Golgi vesicle consisting of a complex mixture of different proteins [77–79]. Major constituents of this unique organelle have been identified and characterised, e.g. the unusually short collagens (minicollagens) [78,80–83], spinalin [84,85] and NOWA [86,87], and a proteome project defined >200 intrinsic protein constituents (Balasubramanian, Özbek, and Holstein; unpublished).

We propose that the nematocyte lineage represent a proper neural cell line. (i) Nematocytes are sensory cells that can sense chemo- and mechanoreceptive stimuli with a ciliary receptor that exhibits structural and functional similarities to those in sensory cells of insects and vertebrates [88,89]. (ii) The specification of interstitial stem cells towards the nematocyte and neuronal cell lineage is regulated by a common set of basic Helix–Loop–Helix (bHLH) and Zinc-finger transcription factors that are specific for neural differentiation in insects and vertebrates [70–72] (see also Table 1).

4. Tissue homeostasis and the stem cell niche

The intriguing feature of *Hydra* stem cell biology is a distinct stem cell pattern and the tissue dynamics. This has important consequences for the tissue homeostasis and the maintenance of stem cell niches.

The constant growth process requires a homeostasis within the interstitial cell compartment as well as between the two epithelial compartments. The communication between the cells of the three compartments and the underlying molecular mechanisms are only poorly understood at present. Dividing stem cells of the interstitial lineage have a cell cycle time of 18–30 h [43,44], while stem cells of the epithelial lineages have a cell cycle time of 3–4 days [19], which is only moderately affected by the feeding regime [16,90]. The fact that no tumour formation or other malignancies have been reported for *Hydra* so far, indicates that growth control and tissue homeostasis in normal *Hydra* polyps are very efficient.

[1] August Weismann coined the term *stem cells* for the germline-restricted cells. The cells he described were only in part germline-restricted cells. They constitute the third stem cell lineage in *Hydra* and other hydrozoans. The term *stem cells* was later reused by E.B. Wilson in his famous book *The Cell in Development and Inheritance* to characterize the pluripotent or totipotent stem cells of an organism.

It is also easy to repopulate *Hydra* that where experimentally depleted of the interstitial stem cell population by grafting a piece of donor tissue on top or below the depleted tissue. Under these conditions stem cells rapidly immigrate into the depleted tissue [91] indicating that migration of interstitial cells is a basic feature of interstitial stem cells ([9] for review). Lowering the interstitial stem cell density to about 25% causes an acceleration of the cell cycle of stem cells by 50% and an increased rate of stem cell self-renewal at the expense of neuronal differentiation [44].

On the molecular level, so far one well-documented example exists providing evidence how the homeostatic regulation between the interstitial and epithelial cell stem cells might work. Here epitheliopeptides and neuropeptides have an antagonistic function [92]. The neuropeptide (Hym-33H) inhibits and the epitheliopeptide (Hym-355) stimulates neuronal differentiation while a cotreatment with both peptides results in normal neuronal differentiation [94]. Coupling of the inhibitory and stimulatory effects of both peptides provides a potential mechanism for maintaining homeostasis in the nerve cell population of adult polyps [9].

Stem cell niches ensure the balance between stem cell self-renewal and differentiation to progenitor cells [94–97]. The distinct patterns in the localisation of the three stem cell lines also suggest the presence of corresponding stem cell niches in *Hydra*. Proliferating epithelial stem cells are localized in the gastric region and hypostomal region [16,98,99] (Fig. 2A). The interstitial stem cells and proliferating precursors in the nematocyte and nerve cell differentiation pathway are exclusively located in the gastric region with sharp boundaries towards the oral and aboral ends [86,100,101] (Fig. 3). Although stem cells can migrate, they do not exceed these boundaries in normal animals and it is completely unknown so far which positional cues are responsible for this behaviour. In higher metazoans cadherins, integrins and signalling factors such as Wnts, Bmps, Notch and Hedgehog specify stem cell niches. All these molecular components have been also identified in cnidarians (see below), but the analysis of the stem cell niche in *Hydra* is only in its beginning [10].

Another major and so far unanswered question is whether stem cells in *Hydra* exhibit any asymmetric division. The asymmetric cell division has been defined as any division that gives raise to two sister cells that have different fates—a feature that can be recognized by differences in size, morphology, gene expression pattern, or the number of subsequent cell divisions undergone by the two daughter cells [102,103]. Again, despite the importance of asymmetric cell division in animal stem cell systems virtually nothing is known about the dynamics of stem cell division in *Hydra*. The development of transgenic lines expressing specific GFP reporter genes [28,104] or GFP-fusion proteins under the control of specific promoters will help to unravel the molecular organization of the stem cell niches in *Hydra*. Of particular interest are also the functional organisation of the mesogloea and the identification of the release sites of morphogenetic active molecules (see below).

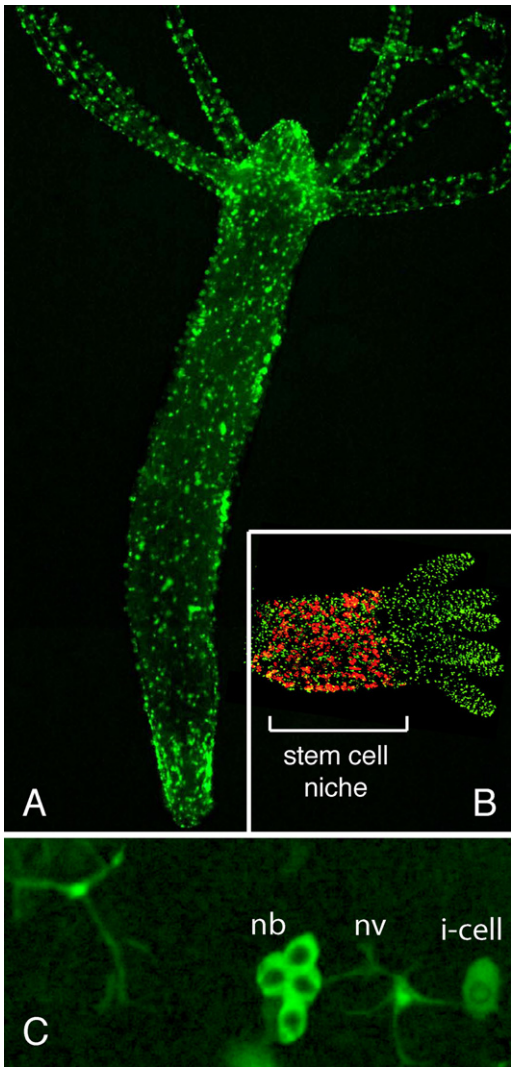

Fig. 3. Interstitial stem cell differentiation analysed by EGFP reporter transgenic cell lines. (A) Whole-mount fluorescence analysis of transgenic interstitial cells expressing the reporter gene eGFP. (B) Confocal analysis of nematogenesis with an antibody against minicollagen (red) staining differentiating nematocytes and with mAb H22 (green) staining mature nematocysts in a fully developed bud (from Engel [86]). (C) Whole-mount fluorescence image of differentiated interstitial stem cell differentiation products.

5. Signalling and nuclear factors

As outlined above, intrinsic and extrinsic cellular mechanisms control the self-renewal and differentiation capacity of stem cells resulting in asymmetric cell divisions. Although stem cell systems have been identified in all multicellular organisms so far virtually nothing is known about the molecular mechanisms of these stem cell systems in basal metazoan groups, i.e. planarians, cnidarians and sponges [3,105]. One central question is to clarify to what extent all stem cells in the animal kingdom follow the same rules [10]. The fact that the same signalling pathways act in a diversity of vertebrate and invertebrate stem cell systems suggests that the regulatory mechanisms in these stem cell systems have a common evolutionary origin. *Hydra* and other cnidarians are therefore ideal models to study the mechanisms of stem cell renewal and stem cell differentiation. Since all animals have a common ancestor in single-cell organisms it will be exciting to identify common principles in the regulatory mechanisms, on the level of signalling molecules as well as for the transcriptional and epigenetic machinery. These data will be helpful to identify common principles between the animal and plant kingdom. Table 1 summarizes current progress in the identification of cnidarian orthologs of nuclear factors involved in stem cell maintenance and differentiation known from bilaterians.

5.1. Transcription factors

On the level of the transcriptional machinery three genes have been shown to induce pluripotency when expressed in differentiated somatic cells of mammals: Nanog, Oct4, and Sox2 [106,107]. These genes are actively expressed in pluripotent stem cells of the inner cell mass of the mouse embryo. In addition Myc and Klf4 have an important function in stem cell maintenance. At present there

is no clear picture to what extent the regulatory transcriptional network to maintain stem cell function has been conserved during metazoan evolution. In the *Hydra* genome two Sox2 and three Myc orthologs are present (Table 1). In case of the other transcription factors only the larger gene family exists in cnidarians, the specific orthologs are missing. Nanog [108] is a homeobox transcription factor related to the NK-2 gene [109], Oct-4 a Pou-class5-homeobox protein [111], and Klf4 a member of the Krüppel-like family of zinc-finger transcription factors; all three classes were identified in *Hydra* or *Nematostella* [72,110,112]. Comparative genomic studies suggest that only the *Hydra* SoxB1 gene is homologous to the Sox2 gene that evolved in vertebrates from an ancient SoxB1 gene [112,113].

5.2. Argonaute proteins

Argonaute proteins represent a unique class of proteins that bind small non-coding RNAs, which have an important function in gene regulation in bacteria, archaea and eukaryotes [114]. Two major groups have been identified, the Argonaute-like proteins related to *Arabidopsis* AGO1, and the Piwi-like proteins in metazoans related to *Drosophila* Piwi [114]. Piwi proteins are a widely conserved stem cell and germ cell marker throughout higher animal systems including annelids [115], insects [116], sea urchins [117], and vertebrates [118]. They have been also identified in gametogenesis of cnidarians [119,120] and platyhelminths [121]. Additionally it has been recently also shown that Piwi-like proteins are associated with a new class of small, germline-specific RNAs called the Piwi-interacting (pi)RNAs [122,123] (Table 1). The fact that they are, similar to Ago1 in fission yeast, also involved in heterochromatin formation [114] suggests that they are important regulators of genome integrity [114] and stem cell function in both, the animal and plant kingdom.

5.3. Signalling pathways

Signalling factors are of crucial importance to maintain the integrity of a stem cell niche and to initiate the process of stem cell differentiation. All major signalling pathways have been found to be involved in these complex regulatory networks. Of crucial importance is the canonical β-Catenin/Wnt signalling pathway, which stimulates stem cell proliferation in the intestine, the hair follicle, and the hematopoietic stem cell system (HSC) of the bone marrow [124–126]. Also Bmp and Notch signalling are involved in these regulatory networks [127,128]. All major signalling pathways that are known to act in bilaterian stem cell differentiation have been identified in cnidarians [7,129]. The signal pathways acting in *Hydra* stem cell differentiation have been recently reviewed [9,69,130–132] and will be only shortly addressed here.

5.3.1. Wnt signalling

Wnt signalling is of fundamental importance for the patterning of *Hydra* epithelial stem cells and for axis formation (Fig. 4A). Wnt glycoproteins are specific for the animal kingdom. In cnidarians, they are expressed in the *Hydra* head organizer [38,133,134] and at the side of the blastopore in cnidarian embryos [135]. It was postulated that Wnts form gradients that induce transcription factors controlling stem cell fate [131] and represent the activating factor of an autocatalytic feedback loop of a short-range activator and a long range inhibitor predicted in reaction diffusion models [13,136].

In cnidarians, the Wnt/β-Catenin pathway was first detected in *Hydra* [134] and a comprehensive analysis of cnidarian *wnt* genes revealed that all bilaterian *wnt* gene subfamilies are present [38,131,133,135,137]. Since only six Frizzled (Fzd) receptors have been identified [131,137,138] in cnidarians, the evolution of *wnt* genes was probably accompanied by a diversification of Wnt receptors. New functional studies demonstrate that along with canonical Wnt/β-Catenin signalling, two *wnt* genes have a function in non-canonical Wnt/PCP signalling during the differentiation of epithelial stem cells at sites of tentacle and bud formation [38].

Extracellular factors that exhibit an antagonizing or modulating effect on Wnts like the family of secreted Frizzled related proteins, sFRP, the Wnt inhibitory factor WIF, Cerberus, and the Dickkopf proteins Dkk [139] have been identified from *Hydra* and *Nematostella* [131,137,140], and some of them (Dkk and Cerberus) are absent from the *Drosophila* and *Caenorhabditis elegans* genome databases. Wnt signalling has a major function in the continuous patterning of epithelial *Hydra* stem cells and in the regeneration process. Wnt signalling is also essential for early cnidarian embryogenesis [135,141–143]. There is indirect evidence that Wnt signalling is involved in the differentiation of interstitial stem cells. In *Hydra* and in the colonial hydroid *Hydractinia echinata*, treatment with alsterpaullone (AP), a drug inducing β-Catenin signalling by inhibiting GSK-3b results in an increase of nematocytes and nerve cells [104,144].

5.3.2. Notch signalling

Notch signalling has been shown recently to be involved not only in the control of stem cell self-renewal and multipotency [145], but also in human tumour progression [146]. In *Hydra*, Notch activity is required by differentiated nematocytes and appears to be a key component in the acquisition of nematocyte fate [10,147]. The nuclear translocation of the Notch intracellular domain (NID) can be inhibited by the synthetic γ-secretase inhibitor DAPT [147]. Inhibition of Notch signalling by DAPT treatment of *Hydra* polyps causes distinct differentiation defects in the interstitial stem cell lineage [147]. Nerve cell differentiation continues normally but post-mitotic nematocyte differentiation can be dramatically reduced. Interestingly also germ cell differentiation can be inhibited [147]. These data suggest that Notch signalling is required to control differentiation events in the interstitial stem cell lineage of *Hydra*.

5.3.3. Bone morphogenetic proteins (BMPs)

BMPs belong to the Transformation Growth Factor β (TGFβ®) superfamily of signalling proteins. In bilaterians, the signalling pathway is involved not only in axis formation and neuroectoderm specification during early embryogenesis [148], but also in cell proliferation, differentiation, and apoptosis [149]. The BMP antagonists such as Chordin and Noggin [150] inhibit BMP action during neural induction [148]. They are also critical for maintenance and self-renewal of mouse embryonic stem cells [151]. In cnidarians all major components of the Bmp signalling pathway have been identified including Bmps [152], Chordin/Noggin [14,129,153–155] and Smads [156,157]. Considering the distinct expression domains it was proposed that Chordin-BMP antagonism is active in *Hydra* axis formation and neurogenesis [14,129].

5.4. Peptides

Peptides were among the first factors that have been isolated by biochemical approaches in *Hydra*. The first characterized factor was the so-called head activator [158,159], a small peptide, which has been shown to increase the rate of head regeneration [160,161], bud formation [162], and neuronal differentiation [163]. However, so far no gene encoding a HA pre-pro-peptide was identified in the *Hydra* and *Nematostella* genome. In a second large screen 286 peptides have been isolated from *Hydra magnipapillata*, synthesized, and examined by differential display-PCR for their ability to affect gene expression [9,73,93]. Most peptides affected the physiology, but some of them inhibited or enhanced nerve cell differentiation (see above, Section 4).

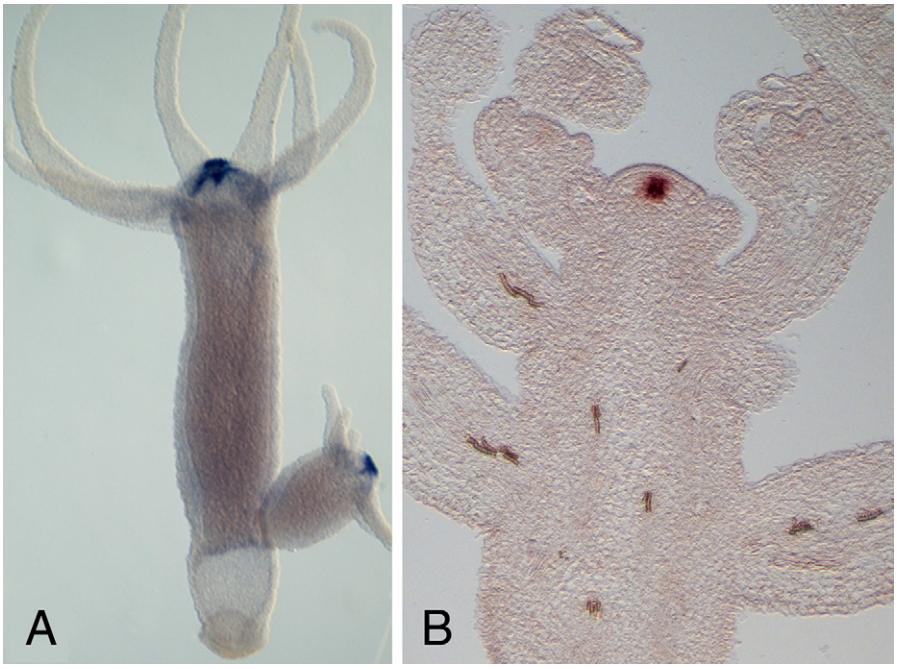

Fig. 4. Organizers in *Hydra* and *Arabidopsis*. (A) In situ hybridisation of a Wnt gene in the *Hydra* head organizer [133] and (B) of the Wuschel gene in *Arabidopsis* shoot meristems.

6. Stem cells and cnidarian regeneration

The exceptional regeneration capacity of cnidarians is highly reminiscent to that in plants. Its discovery goes back to an experiment by Abraham Trembley that was performed almost 270 years ago [164] (see Fig. 1). At that time regeneration was only attributed to plants. Trembley therefore tested whether the green polyps[2] he collected in a pond were plants or animals by cutting the polyps into two pieces. He found that each polyp perfectly regenerated a head[3]. In a number of further experiments Trembley carefully described the biology of these simple animals revealing also relatively complex patterns of their behaviour. His epochal observations on *Hydra* regeneration were followed by further studies demonstrating that regeneration was widely dispersed among the metazoans including vertebrates [165,166].

The ability to regenerate gets significantly reduced throughout metazoan evolution. In higher bilaterians the capacity to regenerate is limited to specific organs or tissues. The very basal metazoans exhibit an almost unlimited regeneration capacity [167–170]. In that respect *Hydra* regeneration shares important similarities with plants although multicellularity almost certainly evolved independently in animals and plants [171,172]. However, *Hydra* polyp can even regenerate from dissociated single cells and thereby serve as a paradigm for de novo pattern formation [173].

The regeneration stimulus starts the re-patterning of the tissue at the site of regeneration. Work of the last years in our laboratory has revealed that the Wnt-pathway plays a fundamental role in this process [38,131,133,135,174]. The *wnt* genes are the main constituents of the head organizer and blastoporal organizer formed during embryogenesis [133,135,175,176]. The kinetics of *wnt* gene expression during head regeneration suggests a cascade of consecutive *wnt* activation with *hywnt3* at the top of the cascade [38,133]. Accordingly, HyWnt3 protein rescues the head regeneration deficient mutant strain reg-16.

7. Evolution of cnidarian stem cell systems

Interstitial stem cells have been also described in several other marine hydrozoan species. These interstitial cells are similar to those in *Hydra*, in that they also differentiate into nematocytes and neurons (a solid proof with cloning experiments is still lacking).

In the hydrozoan jellyfish *Clytia hemisphaerica* interstitial stem cells differentiate into nematocytes within a special tissue at the bell margin of the medusa from which the tentacles emerge [120]. In tentacle bulbs clusters (nests) of nematoblasts are enriched and nematocyte differentiation progresses from the base to the tip of a bulb. Cells in the more distal region express nematocyst-specific differentiation markers, e.g. the *minicollagen* [78,83] or *nowa* gene [177] while cells at the base of the tentacle bulb express a Piwi ortholog [120]. Since this tissue was found to contain undifferentiated proliferating cells with a high nucleocytoplasmic ratio, it was proposed that this tissue comprises a population of interstitial stem cells. The Piwi gene was also identified in the hydrozoan jellyfish *Podocoryne carnea* [119] and in *Hydra* (Table 1), but it is expressed during gametogenesis (Ch. Fujisawa, personal communication).

Interstitial stem cells have also been studied in the colonial hydroid *Hydractinia echinata*. In *Hydractinia echinata*, interstitial stem cells are enriched in the stolon compartment of the colony [178]. In sharp contrast to the findings in *Hydra,* however, interstitial stem cells were described to differentiate into epithelial cells suggesting that these stem cells are indeed totipotent. In *Hydra* transgenic interstitial cells expressing the GFP or dsRED reporter

[2] Trembley used *Hydra viridissima* carrying endosymbiotic *Chlorella* algae in the endoderm.

[3] "... I speculated anew that perhaps these organisms were plants, and fortunately I did not reject this idea. I say fortunately because, although it was the less natural idea, it made me think of cutting up the polyps. I conjectured that if a polyp were cut in two and if each of the severed parts lived and became a complete polyp, it would be clear that these organisms were plants... On November 25, 1740 I sectioned a polyp for the first time... The two parts extended the same day that I separated them. They were quite easy to distinguish from one another because the first had its anterior end bedecked with those fine threads, which serve as the polyp's arms and legs, whereas the second had none at all... I assumed that the second part was only a kind of tail without the organs vital to the life of the animal... Who would have imagined that it would grow back a head! ... I had not the least expectation of being a spectator to this marvellous kind of reproduction" [165].

gene under control of the actin promoter clearly reveal the independence of the three stem cell lines in *Hydra* [28,104,179] and similar approaches can prove the totipotency of interstitial stem cells in marine hydrozoans unambiguously.

Multipotent interstitial stem cells are absent from the other major cnidarian clades, i.e. the cubozoans, scyphozoans, and anthozoans. Pulse chase experiments using polyps of the cubozoan *Carybdea marsupialis* and the anthozoan *Nematostella vectensis* clearly indicate that epithelial stem cells differentiate into neurons and nematocytes (Stangl and Holstein, unpublished; Berger, Özbek, and Holstein unpublished [180]). It will be important to analyse the role of asymmetric cell division in this process.

8. Common principles in cnidarian and plant stem cell systems?

Although different morphogenetic signals have evolved in plants and animals to pattern the multicellular organism there are clearly similarities in the basic principles of pattern formation shared between both kingdoms [166]. One example is the organizer formation in *Hydra* and *Arabidopsis* (Fig. 4). In the *Arabidopsis* shoot apical meristem (SAM) an organizing centre is formed in which the homeodomain transcription factor WUSCHEL (WUS) is specifically expressed and is tightly integrated with the more widely active signalling systems of auxin and cytokinin phytohormones [181] (Fig. 4B) Consistently, elevated levels of auxin and cytokinin can induce de novo and stem cell formation in vitro [182–184]. However, in contrast to *Hydra*, plant stem cells are localized in a restricted domain next to the organizer [185].

The regeneration process in *Hydra* and plants indicates further similarities between animal and plant during the process of regeneration. In both systems, the multicellularity gets disrupted upon injury and an intact tissue must regenerate at a completely unpredictable location in the organism. Animals and plants have developed self-organizing systems that can be induced after injury. When *Hydra* polyps are dissociated into suspensions of single cells so that the original polarity of the animal is completely disturbed [37,173,186] the cells rapidly regenerate an intact organism. Within 24 h ectoderm and endoderm form, and then new head organisers form de novo, organising the surrounding tissue into new body axes that separate into individual polyps [168,186]. Wnt and BMP signalling play a key role in re-establishing the symmetry, similar to the auxin distribution in plants [134,166,186]. These dynamics are highly reminiscent to CUC2 mRNA that gradually partitions into specific domains in the disorganized mass of callus cells during plant shoot regeneration [166].

It is unclear at present to what extent the molecular pathways involved in stem cell recruitment are conserved between the animal and plant kingdom. However, in all cases the key signal in the induction of the regeneration process is an injury stimulus that is probably linked with epigenetic and cell cycle changes at the site of cutting [166,187]. We found in *Hydra* a decrease in cell proliferation and an increase in hydkk1/2/4 expression at the site of regeneration [131]. We presume that epigenetic mechanisms can also trigger the onset of regeneration in *Hydra*.

Also on the cellular level, *Hydra* regeneration seems to share similarities with that in plants. In both systems, pluripotent stem cells become activated from pre-existing stem cell sources that are partially differentiated. During regeneration of bilaterian animals such as planarians or salamanders however, pluripotent stem cells either migrate to the site of regeneration [170,188] or they must be newly generated by complete dedifferentiation of the adult tissue [189,190]. However, recent findings in axolotl may qualify this notion since even the blastema was found to be a heterogeneous collection of restricted progenitor cells still retaining cell-type-specific properties [166,191]. We therefore presume that cnidarians will reveal important new information on the basic mechanisms underlying the unlimited regeneration capacity and stem cell recruitment they share with plants.

9. Future directions

Hydra and the cnidarian have a critical phylogenetic position at the base of the metazoan branch in the tree of life that makes them to an important link in unravelling the common mechanisms of stem cell biology between animals and plants. The generation of transgenic animals established in the laboratory of Thomas Bosch [8,28] was a major breakthrough in the field and has opened up new directions in the *in vivo* analysis of cnidarian stem cell population. The new approaches include transgenic lines expressing GFP-proteins under the control of constitutive (actin) promoters (Fig. 3), transgenic lines expressing a fusion protein of GFP and a protein of interest under the control of a stem cell-specific promoter, as well as gain-of-function and loss-of-function approaches. Furthermore, stem cell populations can be easily FACS sorted and characterized by functional and next generation sequencing approaches (Huang, Watanabe, Ho, and Holstein; unpublished). The combination of genomic and proteomic approaches and the availability of complete genome data from two major cnidarian species (*Hydra*, *Nematostella*) will not only stimulate evo-devo research, but will also provide answers to a major question in biology: What are the common molecular properties of stem cells in multicellular organisms. This knowledge can be exploited for biomedical research and open new perspectives for regenerative medicine.

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