Chapter 3
Stem Cells in Immortal *Hydra*

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Abstract *Hydra*'s potential immortality and extensive capacity to regenerate and self-renew is due to the presence of three distinct stem cell lineages: ectodermal and endodermal epithelial stem cells, and interstitial stem cells. Over the last few years, stem cells in *Hydra* became well-defined in cellular terms of their biology. More recently, efforts using the nearly unlimited potential for tissue manipulation combined with functional transgenesis have shed light on the molecular control mechanisms involved. Here I review those efforts in an attempt to give both a historical perspective and an update on the recent experimental highlights. In particular, I will focus on six aspects of stem cells in Hydra: (i) their continuous transition through the proliferation/differentiation switch; (ii) their rapid responses to signals from the cellular environment; (iii) the emerging importance of Wnt and Notch signaling in controlling stem cell behavior; (iv) the role of chromatin modification in terminal differentiation; (v) the observation of transdifferentiation in some of the stem cell progeny; and (vi) the implications for the evolution of germ cells, ageing and cancer. Together, these findings seem to indicate that *Hydra* not only provides insights into signalling pathways involved in stem cell differentiation in the Bilaterian ancestor; they also demonstrate that despite morphological and functional differences, and more than 500 million years of phylogenic separation between *Hydra* and human, common signaling pathways are responsible for stem cell maintenance, lineage determination, and differentiation.

Keywords Epithelial stem cell, evolution of development, Hydra, interstitial stem cell, Notch, senescence, Wnt, Weismann's doctrine

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3.1 Hydra, a Classical Model System in Developmental Biology

Cnidaria are sister group to the bilaterians (Collins, 1998; Philippe et al., 2005) and therefore provide information for reconstructing the early history of bilaterian developmental mechanisms. They consist of two epithelia, the ectoderm and the endoderm surrounding a gastric cavity; and they are the first in evolution that have a defined body plan, a nervous system, and a tissue layer construction. Cnidarians such as the freshwater polyp *Hydra* have a long history as model systems in developmental biology because of their remarkable capacity to regenerate. This ability for self-organization is at least partially due to the continuous presence of stem cells with high self-renewal capacity and high phenotypic plasticity in adult tissue. The capacity for constant renewal is also the main reason behind Hydra's potential immortality.

In molecular terms, *Hydra* as all other members of the phylum Cnidaria is astonishingly complex. The genomes in different *Hydra* species vary in size but in general are large with *H. vulgaris* having a genome of 1,250 Mbp (Zacharias et al., 2004). Moreover, Cnidaria not only have about the same number of genes as human and share most of their genes with human (Miller et al., 2005; Miller et al., 2007) but their protein sequences, surprisingly, are often more similar to mammalian sequences than to those from fly and worm (Kortschak et al., 2003). Thus, at the level of genomic complexity and gene complement, *Hydra* is much more complex than previously imagined. Novel computational tools and the development of genomic resources over the past few years have brought a new perspective on *Hydra* as a model organism. A National Science Foundation-funded large-scale *Hydra* EST Project (www. hydrabase.org) resulted in 170,000 ESTs. A National Human Genome Research Institute-funded *Hydra* genome project at the J. Craig Venter Institute currently provides 6x coverage of the *Hydra magnipapillata* genome with an assembled draft genome sequence appearing later in 2007. For database searches, Georg Hemmrich in my group has established a local Blast-platform, www.compagen.org, containing selected raw genomic (NCBI Trace archive) and EST (NCBI dbEST, JGI) sequence datasets from sponges and cnidarians up to the lower vertebrates (Hemmrich and Bosch, in prep.).

While there is no evidence that *Hydra* is simpler in molecular terms than vertebrates nor that *Hydra* cells are fundamentally different from those in mouse or human, there may be a profound difference in the differentiation potential and plasticity of the cells between *Hydra* and vertebrates. Vertebrates depend on specialized cells with limited differentiation potential to perform sophisticated functions. Cells in *Hydra*, in contrast, are capable to produce and receive positional signals continuously even in adult tissue and, therefore, have features which most cells in vertebrates have only during the short period of embryogenesis. It is this feature which makes adult *Hydra* tissue different from tissue of all other invertebrates and vertebrates.

3.2 Key Properties of Stem Cells in Hydra

Stem cells in *Hydra* represent one of the most ancient stem cell systems in the animal kingdom and, therefore, provide information for reconstructing the early history of stem cell control mechanisms. In *Hydra*, there are about 20 cell types distributed among three stem cell lineages. Each of the epithelial layers is made up of a stem cell lineage, while the remaining cells are part of the interstitial stem cell lineage which resides among the epithelial cells of both layers. Both the epithelial cells as well as the interstitial cells in the body column continuously undergo self-renewing mitotic divisions. As result of these tissue dynamics, cells in *Hydra* are constantly displaced either apically onto the head, or basally onto developing buds, or onto the foot (for recent review see Bosch, 2007a). Non-dividing differentiated cells of all three lineages are lost by displacement from the body column within 20 days (Campbell, 1967). Dividing stem cells of the interstitial lineage have a cell cycle time of 18–30 h, while stem cells of the epithelial lineages are proliferating with a doubling time of about 3.5 days (David and Campbell, 1972; Bosch and David, 1984) have a cell cycle time of 3–4 days. Hence, cells in *Hydra* are either constantly renewing by cell division, or they are lost from the animal in a relatively short period of time. An individual cell, therefore, does not exist long in a *Hydra* body.

3.2.1 Epithelial Stem Cells in Hydra: Unipotent Stem Cells with Remarkable Phenotypic Plasticity

Epithelial cells in the *Hydra* body column continuously undergo mitotic divisions (Dübel et al., 1987; reviewed in Bosch, 2007a). To prove that *Hydra* epithelial cells indeed have stem cell properties, we have made use of transgenic polyps and transplanted a single GFP-expressing endodermal epithelial cell into a nontransgenic polyp. By doing so we (Wittlieb et al., 2006) have generated polyps in which the entire ectodermal or endodermal epithelium contains the transgene. Thus, *Hydra* epithelial cells are capable, by successive divisions, both of indefinite self-renewal and of producing different types of specialised cells such as tentacle or foot specific epithelial cells. Since there is no evidence for subpopulations of epithelial cells which cannot repopulate the host tissue, all *Hydra* epithelial cells in the gastric region, therefore, must be considered as stem cells.

Supporting earlier observations, our *in vivo* tracking of GFP labelled epithelial cells also showed that there is continuous tissue displacement towards the extremities. Displacement of ectodermal epithelial cells into the tentacles results in differentiation of battery cells which contain cnidocytes. Displacement of epithelial cells towards the lower body regions results in differentiation of epithelial cells into basal disk cells which secret mucus. Other examples for epithelial cells with diverse architectural designs and physiology include the endodermal epithelial cells surrounding the gastric cavity, and the ectodermal epithelial cells encasing the

body, the tentacles, the testes, and the egg cup holding the developing oocyte. This remarkable morphological plasticity of epithelial cells in response to positional signals allows *Hydra* with only a limited number of cell types to generate structures that display a fascinating array of various cellular architectures, each of which are specifically tailored for distinct functions.

To execute these different programs of terminal differentiation, epithelial stem cells must be instructed by their microenvironment. Although the precise mediators for the different positional signals along the body column are not well defined yet, secreted Wnt ligands may be one of the first signals involved in this communication. In vertebrates, Wnt/β-catenin signalling pathways have been shown to control the specification, maintenance, and activation of epithelial stem cells (Reya and Clevers, 2005). Consistent with that, treatment of *Hydra* with alsterpaullone, which specifically blocks the activity of GSK-3β, induces rapid transformation of body column epithelial cells into an epithelial cell with morphology typical for the head region (Broun et al., 2005; Anton-Erxleben et al., in prep.). While these observations provide direct evidence for the involvement of the canonical Wnt pathway in controlling epithelial stem cell behavior, there is no clear understanding yet how β-catenin/Tcf signalling regulates epithelial stem cells in *Hydra*. Identification of the Wnt targets by transcriptional profiling might contribute to fully understand the effect and potency of β-catenin/Tcf activity in epithelial stem cells.

3.2.2 Hydra's Interstitial Stem Cells – Following the Hematopoietic Trend

Hematopoietic stem cells (HSC) and their progressively committed progeny have become the prototype examples of what might be expected of candidate stem cells in other organisms (Metcalf, 2007). Maintenance of the HSC is central to the lifelong production of blood cells by the hematopoietic system. Tests for proving the self-renewal and the plasticity of HSCs are based on injection into a mouse that has received a dose of irradiation sufficient to kill its own blood-producing cells. If the mouse recovers and all types of blood cells reappear (bearing a genetic marker from the donor animal), the transplanted cells are deemed to have included stem cells. These studies have revealed that there are two kinds of HSCs, long-term stem cells that are capable of self-renewal and short-term precursor cells capable of proliferating, but with a limited capacity to differentiate into more than one cell type. Another key feature of hematopoietic stem cells is their ability to migrate in a site-specific fashion and their interaction with their niche, a unique environment that is able to confer stem-like properties on occupying cells. The Wnt/β-catenin and Notch pathway are potent regulators of HSC function (Duncan et al., 2005; Trowbridge et al., 2006). *Hydra*'s interstitial stem cells are remarkable similar in many aspects to HSCs.

3.2.2.1 Multipotency of Interstitial Stem Cells

Interstitial stem cells are multipotent and able to differentiate into several different cell types (Fig. 3.1; Bosch, 2007b). The direct demonstration of the existence of multipotent interstitial stem cells in *Hydra* (David and Murphy, 1977) relied on the method of cloning HSCs in lethally irradiated mice (Till and McCulloch, 1961, 1963). In the original procedure (David and Murphy, 1977), *Hydra* were treated with nitrogen mustard which inactivates rapidly proliferating cells of the interstitial cell lineage and causes their elimination from the tissue. The remaining epithelial tissue was used as host for culturing added interstitial cells. Interstitial cells to be cultured were introduced into host tissue using a technique for dissociating and reaggregating *Hydra* cells (Gierer et al., 1972). In a later modification of this clonal assay (Bosch and David, 1987), elimination of host interstitial cells was achieved using a mutant strain (sf-1) which contains temperature-sensitive interstitial cells as host tissue. Temperature resistant donor cells were added in low numbers to sf-1 such that the added cells grew as clones. Subsequently host sf-1 interstitial cells were eliminated by a temperature shift. This technique made possible long-term clonal culture of *Hydra* stem cells. The results indicated that interstitial stem cells are multipotent in the sense that individual stem cells can differentiate into somatic cells as well as germ line cells (Bosch and David, 1987). The results provide no evidence for the existence of subpopulations of interstitial cells with restricted differentiation capacities (Bosch and David, 1987).

3.2.2.2 Migration of Cells of the Interstitial Cell Lineage

Similar to cells of the hematopoietic lineage, cells belonging to the *Hydra* interstitial stem cell lineage but most likely not the stem cells *per se* (see below) have an extensive capacity to migrate (Heimfeld and Bode, 1984; Fujisawa, 1989; Fujisawa et al., 1990; Teragawa and Bode, 1990, 1995). Following differentiation into nematoblasts and neuroblasts in the gastric region, these cells must traverse great distances to reach their final destination in the tentacles where most of them get incorporated in a “battery cell complex” (Bosch, 2007b). To determine the intrinsic migratory behavior and to examine whether all cells of the interstitial cell lineage display high motility or whether migration is restricted to certain subpopulations, we grafted transgenic tissue containing EGFP + interstitial cells to “naïve” tissue. Since polyps are essentially transparent, live imaging of eGFP + cells demonstrated that migration of nematoblasts and neuroblasts occurs as individual cells and never as cluster of cells (Khalturin et al., 2007). Surprisingly, migrating cells were capable of rather rapid (0.2 μm/min) motility *in vivo*. Interestingly, nearly all migrating cells could be classified as nematoblasts or neuroblasts whereas interstitial stem cells (large 1 + 2s) were mostly residing at the transplantation edge and were not actively motile. This *in vivo* observation is consistent with our previous view (Bosch and David, 1990; Fujisawa et al., 1990) that interstitial stem cells in *Hydra* in contrast to differentiating interstitial cells show little if any migratory activity.

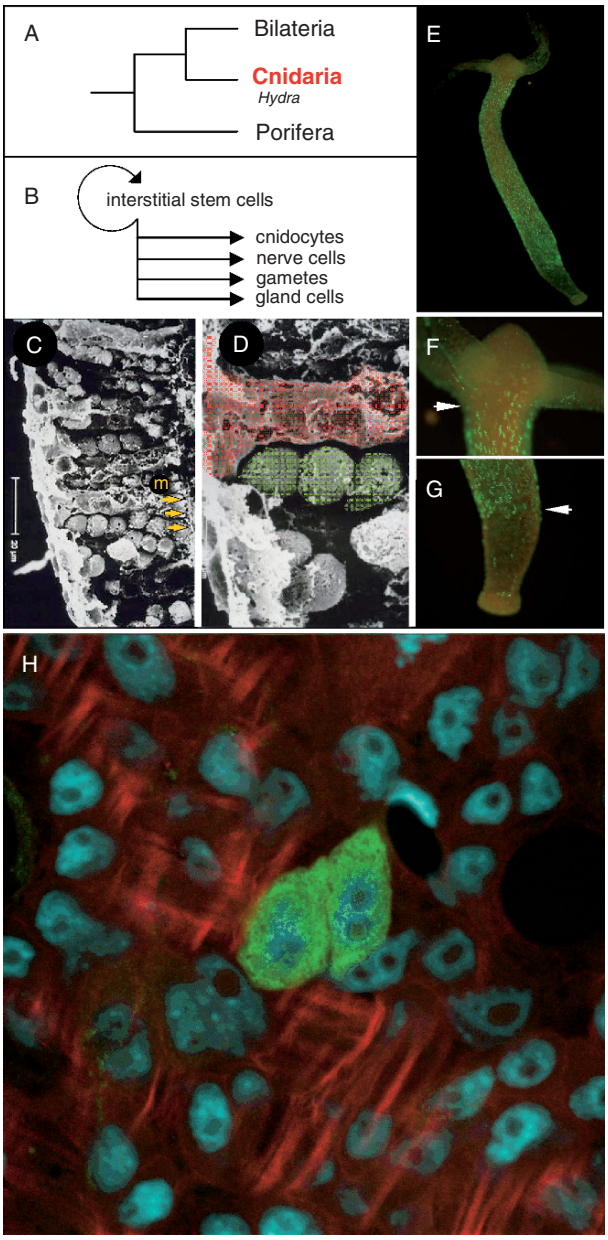

Fig. 3.1 Interstitial stem cells in *Hydra*. (A) Relationships at the base of animal evolution. Cnidaria are often regarded as the closest outgroup of the Bilateria. (B) The interstitial stem cell system in Hydra. (C) Scanning electron microscopic view of a longitudinal section through the ectoderm. m, mesoglea. Yellow arrows indicate the location of the mesoglea. (D) Scanning electron micrograph showing interstitial cells within their niche. Note the close contact of interstitial cells (green) to an ectodermal epithelial cell (red). (E) Distribution of interstitial cells in Hydra. Interstitial cells were stained with monoclonal antibody C41. (F–G) Interstitial cells (stained by monoclonal antibody C41) follow positional cues and are absent in head (F) and foot (G) tissue. Arrows indicate the border at which interstitial cells disappear. (H) A pair of transgenic interstitial cells expressing eGFP. (Modified from Khalturin et al., 2007)

3.2.2.3 The Interstitial Stem Cell Niche

Similar to the niche of HSC, *Hydra* has a distinct interstitial cell niche. Interstitial cells grow and differentiate in the interstices between ectodermal epithelial cells. To visualize their precise site of residence (referred to as niche) we performed scanning electron microscopy (Khalturin et al., 2007). Images of the ectodermal epithelium show (Fig. 3.1) chains of interstitial cells and their derivatives in close contact with the ectodermal epithelial cells. Since the mesoglea (extracellular matrix) may present an additional important acellular component of the microenvironment, the interstitial cell niche in *Hydra* appears to contain a high level of structural complexity (Khalturin et al., 2007).

3.2.2.4 The Cellular Environment Controls Interstitial Cell Behavior

Reflecting sophisticated and largely unknown signalling and growth requirements, interstitial stem cells and their derivatives display a striking position dependent distribution pattern. Interstitial stem cells are found throughout the gastric region; they are present at only very low numbers in the head and foot region (David and Plotnick, 1980). Cnidocyte differentiation occurs exclusively in the gastric region as a highly complex, multistep process (David and Challoner, 1974; David and Gierer, 1974; Shimizu and Bode, 1995; reviewed in Tardent, 1995; see also Nüchter et al., 2006). Neuron differentiation from multipotent interstitial stem cells also occurs exclusively in the gastric region (David and Gierer, 1974). After entering the neuron differentiation pathway, about a half of the neuron precursor cells migrate toward the head and foot (Heimfeld and Bode, 1984; Fujisawa, 1989; Teragawa and Bode, 1990; 1995; Technau and Holstein, 1996; Hager and David, 1997). The remaining half of the neuron precursors do not migrate, but complete differentiation and are integrated into the nerve net.

While these differentiation decisions appear to be controlled by the composition of the cellular environment, little is known about the molecular mechanisms by which the interstitial cell niche regulates interstitial stem cells. The current state of the art has been summarized recently (Bosch, 2007b). Briefly, there is accumulating evidence (Takahashi et al., 2000; Bosch and Fujisawa, 2001) that epithelial cells affect interstitial cell differentiation behavior by secreting epitheliopeptides. There is also experimental evidence that derivatives of the interstitial cell lineage such as neurons affect interstitial cell differentiation (Takahashi et al., 2000), and that nerve cell density influences interstitial cell proliferation (Bosch et al., 1991). Despite these observations, however, a direct evidence for a role of the interstitial cell niche in interstitial cell behavior and a spatio-temporal dialog between hydra interstitial stem cells and niche cells has not been demonstrated yet. Currently we use transgenesis to uncover niche characteristics by introducing alterations in the niche by gain-of-function and loss-of-function experiments. For example, does overexpression or silencing of epitheliopeptides in transgenic polyps affect nerve cell differentiation? Since it is likely that loss of neurons has broad effects on the microenvironment: what happens to interstitial cell differentiation in *Hydra* tissue

in which the number of neurons is severely reduced? Using such experimental approaches we expect to obtain direct in vivo evidence that the microenvironment plays a dominant role in interstitial cell behavior, self-renewal and multi-lineage differentiation.

3.2.3 Wnt and Notch Pathways Are Involved in Controlling Stem Cell Behavior in Hydra

To investigate the critical regulatory events that control the transformation of an interstitial stem cell into a differentiated cell, we produced transgenic polyps expressing eGFP specifically in the interstitial stem cell lineage. Because Wnt signals appear to constitute the principle driving force behind developmental processes in *Hydra* (Hobmayer et al., 2000; Broun et al., 2005), we hypothesized that alteration in Wnt signalling may specifically alter interstitial cell behavior. Wnt signalling was activated in transgenic polyps by the addition of alsterpaullone (ALP), a drug which specifically inhibits the cytoplasmic destruction complex causing degradation of β-catenin. Addition of 5 μM ALP had drastic effects on eGFP expressing interstitial cells and their derivatives (Khalturin et al., 2007). Within 48 h upon treatment, nests of differentiating nematoblasts broke up into single cells indicating terminal differentiation of all nematoblasts present in the gastric region (Khalturin et al., 2007). In untreated control polyps, nematoblast nests disaggregated progressively and new nests were produced continuously. ALP, therefore, seems to directly inhibit the differentiation of interstitial cells into nematoblasts by inducing ectopic terminal differentiation of nematoblasts in the gastric region. The expression of two genes, which are markers for nematocyte differentiation, supported this view (Khalturin et al., 2007). To determine whether the Wnt induced changes in interstitial cell behavior reflect cell-intrinsic activity or rather a response towards the changed microenvironment, we grafted tissue with eGFP + interstitial cells to unlabelled host tissue which had been treated with ALP for 48 h preceding transplantation. We observed normal migratory activity of interstitial cells and nematoblasts into ALP treated tissue indicating that ALP has no effect on the migratory activity of interstitial cells per se (Khalturin et al., 2007). In sum, extrinsic signals from the microenvironment play a major role in interstitial cell differentiation and migration, and may be mediated by the Wnt pathway. This pathway in adult Hydra obviously fulfils two functions, one in patterning (Hobmayer et al, 2000; Broun et al., 2005) and one in interstitial cell differentiation (Khalturin et al., 2007).

To further dissect the molecular mechanisms that are involved in interstitial cell differentiation, we investigated the function of the Notch pathway in vivo. We treated polyps for 48–96 h with DAPT which blocks Notch activity by inhibiting the γ-secretase-dependent cleavage that releases the Notch intracellular domain (Geling et al., 2002; James et al., 2004). A block in Notch signalling prevented nematoblast differentiation (Khalturin et al., 2007). Strikingly, nerve cell differentiation appeared

not to be affected at all. To analyze the inhibition of Notch signalling on interstitial cell differentiation at the molecular level, we studied the expression of several genes expressed in interstitial cells after entering either the nematoblast or the neuron differentiation pathway. We obtained evidence (Khalturin et al., 2007) that Notch activity is required by differentiated nematocytes, not just by the dividing cells of the nematoblast nests. Since no differences could be observed in control and treated Hydra tissue with regard to the number of neurons present, there seems to be no trade-off between nematoblast and nerve cell differentiation when Notch activity is blocked. Suppression of Notch signalling causes immediate death of differentiating nematoblasts. Inhibition of Notch does not affect neurons nor interstitial cells as the nematocyte population quickly recovers after termination of DAPT treatment. Notch, therefore, appears to be a permissive cue in nematoblast differentiation, rather than an instructive one. These observations supported a previous study (Käsbauer et al., 2007) and strongly implicate Notch signalling as a key component in the acquisition of nematocyte fate. They also point to Notch as an ancient molecular "switch" used already in early branching metazoans to distinguish different cell types. Taken together, since Wnt and Notch pathways are involved in controlling stem cell behavior in hydra, similar key signalling pathways appear to orchestrate stem cell behaviour throughout the animal kingdom from *Hydra* to man.

3.2.4 Hydra Stem Cells and the Evolution of Senescence

Senescence is generally defined as progressive declines of physiological functions, leading to an increase in the mortality rate as a function of time. Senescence has been found in all metazoans where careful studies have been carried out. Although it is often postulated that stem and progenitor cell depletion or dysfunction might contribute to senescence, the biochemical basis behind remained elusive. It came as a surprise, therefore, that Wnt proteins in stem cells were recently shown to play an instructive role in controlling the onset of senescence (Liu et al., 2007; Brack et al., 2007). Constitutive Wnt stimulation appears to contribute to stem cell depletion and aging as mice lacking a Wnt antagonist have a shortened life span and exhibit a number of age-related changes (Liu et al., 2007).

Hydra has been suggested not to undergo senescence, and being biologically immortal (Martinez, 1998; 2002). This potential immortality of Hydra has been a hotly disputed controversy over the last few decades. However, a careful and elegant study (Martinez, 1998) analyzing the mortality patterns and reproductive rates of four groups of individuals of *Hydra vulgaris* for a period of 4 years (Martinez, 1998) could find no evidence for aging in *Hydra* and no apparent signs of decline in reproductive rates. Extremely low mortality rates and lack of senescence appears to be due to the tissue dynamics and the fact that the body can be constantly renewed from populations of stem cells.

This view has been challenged recently by Yoshida et al (2006) who searched for signs of aging in sexually differentiated *Hydra oligactis*. Since after sexual reproduction Yoshida et al. (2006) found a significant decline in the capacities for food capture, contractile movements, and reproduction as well as an exponential increase in the mortality rate of the population, they proposed that the degenerative process in *H. oligactis* following sexual reproduction represents the aging process. These data, however, have to be taken *cum grano salis* for two reasons. First, these degeneration processes are observed only under laboratory conditions and might be simply the consequence of the excessive sexual production activity described by the late Pierre Tardent (1974): "Particularly in males gamete production is so intense that we can speak in terms of a "gametic crisis" ("crise gametique," Brien, 1966) leading to a complete exhaustion and death of the animals." Second, there are several species of closely related *Hydra*, e.g. *H. magnipapillata* and *H. vulgaris*, which do not undergo degeneration after sexual reproduction but continue to proliferate and grow indefinitely. Since it seems unlikely that there are immortal *Hydra* species and mortal ones, degeneration in *H. oligactis* might simply be based on the fact that in this species environmental signals cause multipotent stem cells to shift their differentiation program exclusively to germ cell differentiation.

Taken together, there is no convincing evidence for senescence in *Hydra* at the cellular and individual level. Intriguingly, this is also supported by recent observations at the molecular level in transgenic polyps with constitutive Wnt signaling in the epithelial stem cell lineage (Hartig et al., in prep). Transgenic *Hydra* that express β-catenin driven by the actin promoter have a characteristic phenotype with multiple axes as well as ectopic tentacles, supporting the view that the canonical Wnt pathway is involved in the activity of the head organizer (Hartig et al., in prep). These animals, however, continuously produce buds that subsequently develop into multiple axes animals. Epithelial stem cells in these animals, therefore, must continuously proliferate. Thus, constitutive Wnt signaling at least within the epithelial stem cell lineage in *Hydra* does not appear to lead to a rapid exhaustion of long-term repopulating stem cells. We might conclude, therefore, that the molecular language involved in control of senescence in vertebrates with the instructive role of Wnt in aging does not to work in immortal *Hydra*.

Hydra has chosen a life cycle in which proliferation occurs mostly asexual by budding. That requires that each bud obtains the complete cellular repertoire from the mother polyp. By giving all the epithelial cells in the budding region stem cell properties and by filling the interstitial space with multipotent interstitial stem cells with the potential to differentiate not only into somatic cells but also into gametes, buds obtain all what they need. Thus, it is the stem cellness of the tissue which allows *Hydra* its unique life cycle. It seems that this feature alone is sufficient to explain *Hydra*'s immortality. While young Achilles was dipped in the pool of immortality by his mother, natural selection has removed senescence from the life cycle of *Hydra*.

3.2.5 *Hydra Stem Cells Violate Weismann's Doctrine of the Continuity of the Germ Plasm*

About a century ago August Weismann observed that in hydrozoans germ cells are derivatives of "common embryonic tissue cells" found in a given part ("Keimstätte") of the tissue (1883). Based on this observation he concluded that only certain groups of predetermined cells can differentiate gametes and published his doctrine of "the continuity of the germ-line" (Weismann, 1892). Soon thereafter the idea of a complete separation in metazoans between an immortal germ line and a soma that would serve to transfer the germ products to the next generation and then senesce became very popular. In cnidarians, however, the existence of a germ line, despite Weismann's observation, has never been demonstrated experimentally. In contrast, clonal assays have shown (Bosch and David, 1987) that *Hydra* contain multipotent interstitial stem cells continuously capable of differentiating somatic as well as germ line cells suggesting a common origin of germ cells and somatic cells. No evidence was found for the existence of a particular group of cells with germ line-restricted differentiation capacities (Bosch and David, 1987). These results provide experimental support for a proposal made by Buss and Green (1985; see also Buss, 1987) that asexual proliferation by budding ("ramet production") requires the presence of an actively dividing multipotent cell line capable of somatic as well as germ line differentiation. For this reason, colonial organisms such as many cnidarians are expected to differentiate germ cells from a pool of multipotent stem cells. Comparative data from *Hydra*, flatworms and the annelid *Platynereis* (Rebscher et al., 2007; and references herein) indicate that a two-step mode in germ cell specification may be ancestral for metazoan germ line segregation.

The dual potential of interstitial stem cells is also reflected at the molecular level since multipotent stem cells as well as germ line cells both express *nanos (Cnnos)* and *vasa (Cnvas)* homologous genes (Mochizuki et al., 2000; Mochizuki et al., 2001). *Vasa* encodes an ATP-dependent RNA helicase belonging to the DEAD box protein family and is one of the most reliable markers for germline cells (Raz, 2000) throughout the animal kingdom. In *Hydra magnipapillata*, Cnvas1 and Cnvas2 both are expressed in multipotent stem cells as well as male and female germline cells (Mochizuki et al., 2001). A *Nanos* gene, encoding an RNA binding protein, was first identified as a maternal effect gene in *Drosophila* (Wang and Lehmann, 1991). To date, *nanos*-related genes have been cloned in several invertebrates and vertebrates and found to play a critical role in germ cell development. In *H. magnipapillata*, Cnnos1 is expressed in both multipotent stem cells and germ line cells while Cnnos 2 shows a less clear expression pattern (Mochizuki et al., 2000). Although the expression pattern of Cnos1 and Cnvas1 is suggestive, a function of these proteins in regulating germ cells has not been demonstrated yet.

In sum, as stated 2 decades ago (Bosch and David, 1987), *Hydra*'s pattern of germ cell development characterized by the absence of early terminal differentiation clearly violates Weismann's doctrine. Weisman held that there existed a "molecular

distinction" between the germ plasm and the soma, such that the soma was merely a mortal vessel upon which selection acts. Phylogenetic distribution of this trait, however, shows that early terminal differentiation is a character limited exclusively to some higher metazoan taxa. In *Hydra* and other early-branching metazoans, cells destined to become gametes derive from multipotent stem cells which display a dual potency. Unravelling how *Hydra* interstitial stem cells become determined to differentiate into gametes, and how the germ cells are specified remains one of the great challenges for the future.

3.2.6 Continuous Self-Renewal and the Risk of Developing Cancer

The evolution of multicellularity necessitated the development of strict controls to keep cellular proliferation in check. Stem cells possess an enormous developmental potential and have the unique ability to self-renew. These two features essential for their normal behaviour could make stem cells a major threat to the organism if the machinery that keeps them in check becomes defective. One interesting thought (Clarke and Fuller, 2006) is that long-lived organisms have developed a strategy to limit the number of long-lived cells with self-renewal capacity. Restricted long-term renewal of short-lived cell types may reduce the chance that a single cell with proliferative capacity will accumulate the mutations required for malignant transformation.

As described above, in *Hydra* every epithelial cell in the body column has stem cell feature and stem cells of the interstitial cell lineage are abundant throughout the tissue and continuously undergo self-renewing mitotic divisions. Despite this potential vulnerability to cancers, *Hydra* tissue, however, never shows signs of malignant transformation. Although up to now the regulatory mechanisms involved remain completely elusive, a plausible reason for the absence of malignant cells which have loosened themselves from the constraints on proliferation may be that all cells in *Hydra* including the stem cells are short-lived cells which rapidly get displaced towards the ends of the body axis due to the tissue dynamics. Thus, the chance that a single *Hydra* cell will accumulate the mutations required for malignant transformation is greatly reduced. While higher organisms may have reduced the number of continuously dividing cells and evolved small populations of stem cells as a protection against cancer (Clarke and Fuller, 2006), in *Hydra* there simply may be no need to limit the number of stem cells since due to the tissue dynamics all cells are short-lived. Another aspect contributing to the conspicuous absence of cancer in long-lived cnidarians may be their extensive repertoire of metabolic signalling (Blackstone, 2006) and the fact that redox signalling could activate signalling pathways involved in balancing cellular proliferation and quiescence (Blackstone, 2007).

Hydra tissue, however, is not completely free of the risk of hyperproliferation and developing tumors. In a culture of *H. oligactis*, a polyp with drastic alterations in the tissue composition was identified (Anokhin et al. in prep). Loss of anatomical

integrity of the ectodermal and endodermal epithelium resulted in tumor-formation and loss of capacity to bud. Longitudinal sectioning followed by regeneration allowed the production of a clonal culture of polyps which all displayed the same tumor-bearing phenotype. To get first insight in the cellular and molecular background for this phenotype, tumor-bearing polyps were analyzed using a variety of histological and molecular techniques. Since microscopic examination revealed large, pale multilobulated masses composed of interstitial cells, loss of tissue integrity appears to result from loss of cellular homeostasis and increased interstitial cell proliferation. In situ hybridization using the germ cell specific gene *nanos* as probe showed that the rapidly proliferating interstitial cells were primordial germ cells. Thus, these Hydra tumors have histologic and molecular features of ovarian germ cell tumors (dysgerminomas) as described in mammals. While germinal cell tumours represent 2–5% of all cancers of the ovary, ovarian tumors are uncommonly encountered outside mammals, and never observed so far in any invertebrate. This observation, therefore, is the first report of ovarian germ cell tumor in any early-branching metazoan species. Identifying the biochemical basis of tumor formation will undoubtedly contribute to our understanding of the mechanisms that normally control tissue homeostasis and cell proliferation in *Hydra*; and that allow these potentially immortal organisms to simultaneously escape both cancer and senescence. Moreover, as molecules and pathways involved in biological processes are evolutionary conserved and most vertebrate gene families have deep evolutionary roots (Kortschak et al., 2003, Miller et al., 2005; Technau et al., 2005), greater understanding of how germ cell differentiation is regulated in *Hydra* could also have clinical significance. Since the cause of ovarian cancer is unknown, *Hydra*'s position at the base of animal evolution might be advantageous and useful for the identification of genes controlling key steps in germ cell differentiation.

3.2.7 Stem Cells Are Not Enough to Keep Up with the Tissue Dynamics: Gland Cell Complexity in Hydra Is Maintained by Both Stem Cell Based Mechanisms and Transdifferentiation

Three of the interstitial cell differentiation products are gland cells found in the endoderm of the body column, and two types of mucous cells found in the endoderm of the hypostome, the apical part of the head. Little was known until recently about gland cell differentiation from interstitial stem cells (Schmidt and David, 1986; Bode et al., 1987). It was assumed but was not shown yet that the mucous cells in the head are also derived from the interstitial cells. We re-addressed the issue of gland cell differentiation by generating transgenic *Hydra* in which eGFP expression was under control of the promoter of a gland cell specific gene, HyDkk1/2/4 C (Siebert et al., 2008). The fact that transgenic *Hydra* recapitulated faithfully the previously described graded activation of HyDkk1/2/4 C expression along the body

column, indicated that the 1.027 bp promoter contains all elements essential for spatial and temporal control mechanisms. Tracing individually labelled zymogen gland cells (ZMGs) allowed us to show that continuous changes in position along the single body axis are accompanied by continuous changes in gene expression and morphology; and to define a distinct ancestor-descendant relationship between ZMGs in the gastric region and granular mucous gland cells (gMGC) in the head. In addition we observed that part of the mucous gland cell population in the head is directly derived from interstitial cells. The *in vivo* observations, therefore, show that in *Hydra* both stem cell-based mechanisms and transdifferentiation are required for maintaining a population of differentiated cells in the context of active tissue dynamics. The results demonstrate a remarkable plasticity in the differentiation capacity of cells in an organism which diverged before the origin of bilaterian animals. The studies reveal differentiation in *Hydra* to be a surprisingly dynamic process and, to our knowledge, provide the strongest evidence to date that transdifferentiation *in vivo* plays a major role in maintaining cell complexity.

3.2.8 Epigenetic Control of Interstitial Stem Cell Differentiation

Epigenetic genome modifications are important for specifying pluripotency and lineage commitment (Azuara et al., 2006; Spivakov and Fisher, 2007). Studies examining specific epigenetic features of human and mouse stem cells – such as the abundance of modified histones, Polycomb group (PcG) protein-binding patterns, and chromatin accessibility – have provided important insights into the unique properties of pluripotent stem cells. PcG proteins form multiple Polycomb Repressive Complexes (PRCs) and are epigenetic chromatin modifiers involved in maintenance of embryonic and adult stem cells (Plath et al., 2003; Valk-Lingbeek et al., 2004). The PRC2 complex, comprising embryonic ectoderm development (EED), Enhancer of zeste (EZH2), and additional components, initiates gene silencing and catalyzes histone H3 methylation on lysine 27 (H3K27) at target loci (Kirmizis et al., 2004; Kuzmichev et al., 2004). Embryonic stem cells appear to manage their pluripotent status by "keying up" important regulator genes for future expression, using a PcG-mediated repressive histone lock (Spivakov and Fisher, 2007). This prevents precocious expression of genes that drive the differentiation along specific differentiation pathways, but also allows the same genes to be primed for future expression. Thus, the default state appears to be "differentiation" while "stemness" must be actively maintained.

We previously have shown that in *Hydra* the gene encoding Polycomb protein HyEED is specifically expressed in interstitial cells and differentiating nematoblasts (Genikhovich et al., 2006). HyEED is not expressed at later stages of differentiation and, therefore, absent in the head and foot regions. In male polyps, HyEED is co-expressed with the *Hydra* homologue of EZH2 (Genikhovich et al., 2006). Since sperm precursors expressing HyEED show high levels of histone methylation (Genikhovich et al., 2006) and co-express HyEZH2, the PRC2

complex appears to be involved in remodeling and silencing sperm chromatin. To explore whether epigenetic histone modifications are important for differentiation of interstitial cells, we produced polyps which overexpress HyEED in the interstitial cell lineage under the control of the *Hydra* actin promoter (Khalturin et al., 2007). Unexpectedly, the localization of the fusion protein emulates the endogenous expression of HyEED. Confocal microscopy showed HyEED-eGFP expression in interstitial cells as well as developing nematoblasts. Other derivatives of the interstitial stem cells were not observed to express the fusion protein.

To examine whether the disappearance of the eGFP signal in the head and foot tissue is correlated with terminal differentiation, we monitored HyEED-eGFP positive cells located in the gastric region as well as during regeneration. HyEED-eGFP is actively degraded during the transition of a nematoblast into a mature nematocyte. Inhibition of the proteasome system by the MG132 membrane-permeable proteasome inhibitor (Nencioni et al., 2006) leads to presence of HyEED-eGFP + nematocytes in head and tentacle tissue whereas in control polyps such transgenic cells are never observed in these regions. Thus, the ubiquitin proteasome system is involved. We have proposed (Khalturin et al., 2007) that nematoblasts entering head or foot territory (the region of terminal differentiation) abruptly loose the HyEED-eGFP fusion protein by proteolytic degradation to facilitate terminal differentiation. Our overexpression construct seems to be unable to override this endogeneous control mechanism.

Taken together, the observations support the view that remodelling of chromatin structure is involved in interstitial cell differentiation and that – similar to cells in higher animals – HyEED is likely to actively suppress final differentiation steps in *Hydra* interstitial stem cells. Determining how these epigenetic features relate to the transcriptional signatures of stem cells, and whether they are also important in other types of stem cells in *Hydra*, is a key challenge for the future.

3.3 Problems and Prospects

Although substantial efforts have been made in recent years to identify the molecular regulators of stem cells in *Hydra*, we have as yet gained only a rough outline of the interplay of mechanisms that coordinate proliferation and differentiation of the three stem cell lineages. Figure 3.2 illustrates the current view of the differentiation and proliferation potential of interstitial stem cells in *Hydra*. Despite this progress, the information yielded from current studies only paints part of the picture, and many challenges for the future remain.

Most importantly, the fundamental question “What are the signals that control stem-cell proliferation and dictate whether a daughter of a stem cell shall remain a stem cell or become committed to differentiation?” is still unanswered. The control of stem cell numbers, their commitment, and progeny generation are biological questions of great importance in *Hydra* as in any other organism.

Much remains also to be learned about the transcription factors that regulate stem cell behavior in *Hydra*. Wnt signalling, for example, ultimately controls

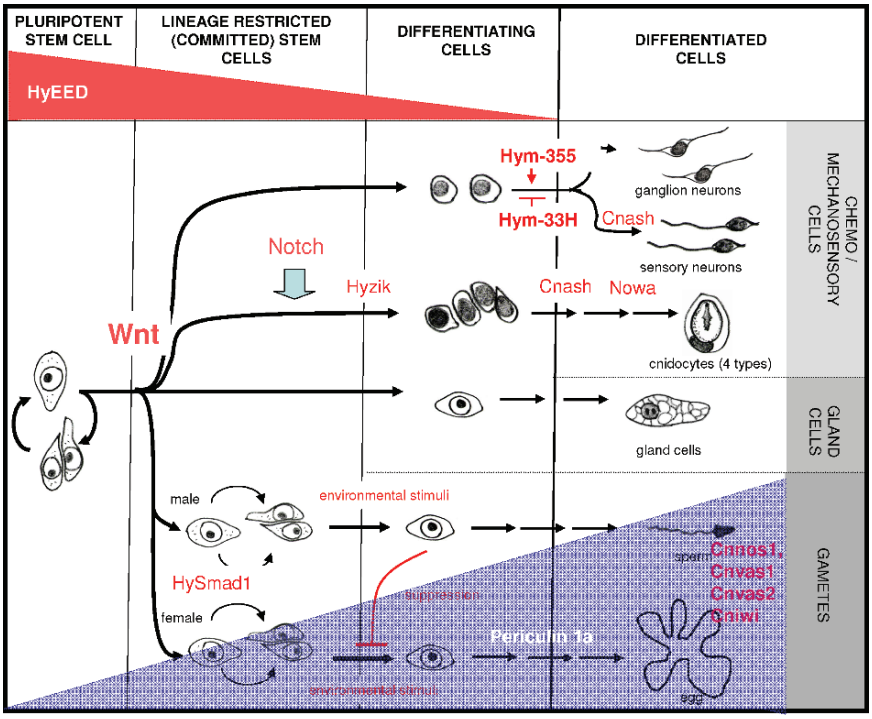

Fig. 3.2 A model for the differentiation of interstitial stem cells in Hydra. Factors affecting steps of differentiation are shown in red. For details and references see text. (Modified from Bosch, 2007b)

developmental fates through the transcription of cell-type specific programs of Tcf target genes. The gaps in our knowledge are due to the fact that conserved stem cell specific transcription factors such as OCT4, REX11, SOX2, and Nanog all are missing in the *Hydra* genome. We, therefore, must discover which genes lie upstream and are in control of pluripotency and self-renewal capacity. With advances in cell sorting and array-based technologies, we expect that the dissection of the gene programs in *Hydra* stem cells before and after differentiation decisions will provide a wealth of insight into the biology of these processes. At the end, these efforts should put one of the grails of stem cell biology in sight – reconstruction of the gene set controlling stem cell behavior in the common ancestor.

Furthermore, in *Hydra* three independent stem cell-lineages co-exist: (i) ecto – and (ii) endodermal epithelial stem cells as well as (iii) interstitial stem cells. In the long run, *Hydra*, therefore, appears to be an excellent model for studying how the divisions of three distinct types of stem cells within an organism are controlled – a question central to the understanding of tissue and cell type homeostasis.

Little is known about the epithelial stem cells in *Hydra*. As I have outlined elsewhere (Bosch, 2007a), epithelial stem cells in *Hydra* show features which can also be found in intestinal stem cells in the vertebrate gut (Bosch, 2007a). How

these epithelial stem cell are maintained, and which environmental components endow stem cell properties to them, are intriguing questions. Moreover, what features do epithelial stem cells and interstitial stem cells share? As characteristics of both epithelial and interstitial stem cells emerge, it will be interesting to ascertain what features they will share that might account for their common properties of self-renewal and repression of differentiation state. It will also be exciting to uncover the features that uniquely define them, such as their unipotency versus multipotency?

Resolving the function of the evolutionarily conserved PIWI/Argonaute family of proteins also seems fundamentally important in understanding of the intrinsic cellular processes serving as determinants of asymmetric-segregating cell fates (Bosch, 2004; Peters and Meister, 2007; Seto et al., 2007). Members of this protein family are found in plants, yeast, and throughout the animal kingdom; and define the first family of evolutionarily conserved genes that are essential for stem cell division in both animal and plant kingdoms.

Lastly, *Hydra* is the only cnidarian in which multipotent interstitial stem cells have been found so far and in which it was clearly shown that germ cells continuously differentiate from multipotent stem cells. The origin of germ cells in well-studied anthozoans such as corals or *Nematostella*, but also in scyphozoans such as *Aurelia* or other hydrozoans (*Podocoryne*) remains enigmatic. Where do they come from? Do these cnidarians lack the interstitial cell lineage and produce all their cell types from only two stem cell lineages, the ectodermal and endodermal epithelial cells? Does, therefore, development of the interstitial cell lineage in *Hydra* reflect an evolutionary relatively young event by which the population of endodermal epitheliomuscular cells segregated into a unipotent endodermal epithelial and a multipotent interstitial cell lineage?

Despite these problems and open questions, the imminent availability of methods for functional analyses of cnidarian genes and the massive advances in molecular technology that are presently taking place, make *Hydra* a powerful and also intellectually attractive system for studying stem cells since it allows easy access to combined genetic, cell biological, molecular and computational approaches. In particular, transgenic *Hydra* are paving the way for many applications including in vivo imaging to analyze stem cell behavior and niche function in an animal that diverged from the main line of metazoan evolution about 560 million years. Thus, since fundamental processes that are relevant for understanding asymmetric division and self-renewal are expected to be conserved in the animal kingdom, with the molecular dissectioning of the components controlling epithelial homeostasis and decision making in *Hydra*, the stage is set for lower metazoan biologists to uncover the mystery of "stemness" and deciphering the fundamental components controlling pluripotency and lineage commitment that underlie all stem cell systems. There is still a lot to learn.

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