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Contents, Volume 5, 1991

Number 1

Number 2

Number 3

Special Issue on Immunopathology
Edited by G.R.F. Krueger

Number 4

Number 5
Special Issue on Recent Trends in Development
Edited by P.A. Tsonis

Number 6

Special Issue on Immunotherapy of Cancer
Edited by C.J. Ioannides and W. Den Otter

Errata

Page 108: Figure 2 should appear with the right side up.

Page 180, left column, line 10 should read:

similar conditions. A spectral width of ± 2000 Hz and 2048 data points

Page 180, left column, line 35 should read:

$pH = pK + \log[\delta_{\alpha\text{-}Pi} - \delta(H_2PO_4^-)]/[\delta(H_2PO_4^-) - \delta_{\alpha\text{-}Pi}]$

Page 180, left column, line 47 should read:

$[Mg^{++}] = K_d((\delta_{A\text{-}TP}-\delta_{\alpha\text{-}\beta})/(\delta_{\alpha\text{-}\beta}-\delta_{MgA\text{-}TP}))$

Page 197: Photograph was printed inverted.

Volume 4 (1990): Page 341, right column, line 6 should read:

Furthermore, populations of metaphase chromosomes treated

Volume 4 (1990): Page 342, right column, line 8 should read:

interior of the chromosomes (117). *In vitro* exposure to

Volume 4 (1990): Page 347, left column, line 43 should read:

preparations are small and compact compared to the long thin

Subject Index

Author Index

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Decision Making in Interstitial Stem Cells of Hydra

THOMAS C.G. BOSCH and CHARLES N. DAVID

[1]*Zoological Institute, University of Munich, Luisenstr. 14, 8000 Munich 2, F.R.G.*

Abstact. *Intersitial stem cells in* Hydra *are a continuously proliferating and differentiating cell population. They represent a useful model system for studying mechanisms controlling stem cell differentiation. Here we review our current knowledge of the differentiation potential of these cells. Interstitial stem cells are multipotent and able to differentiate into several different cell types. The differentiation decisions appear to be controlled by positional signals and by the composition of the cellular environment. Since interstitial stem cells can be cultured in an* in vivo *environment and appear to be accessible to experimental manipulation by a range of new molecular techniques, an* in vivo *analysis of the molecular mechanisms underlying stem cell decision making can now be approached.*

Introduction

The freshwater polyp *Hydra* is a phylogenetically ancient multicellular organism with a relatively simple tissue structure. During asexual growth of the polyp its nerve cells, gland cells and nematocytes arise from a population of interstitial stem cells. Since these stem cells are a continuously proliferating and differentiating cell population which can be cultured and analyzed in an *in vivo* environment, they represent a useful model system for analysing the mechanisms controlling stem cell differentiation. In this review we discuss the differentiation behavior of these cells and the factors influencing it. We demonstrate that the interstitial stem cells are multipotent in the sense that they are able to differentiate into somatic cells (nematocytes, nerve cells and gland cells) as well as into germ line cells (sperm and eggs). The differentiation decisions appear to be controlled by morphogenetic signals associated with the position of stem cells within the body column as well as by the composition of the cellular environment and the presence of neuropeptides. We conclude by showing that hydra stem cells appear to be accessible to experimental manipulation by a range of new molecular techniques so that now an *in vivo* analysis of the molecular mechanisms underlying stem cell decision making can be approached.

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Key Words: Hydra, interstital stem cells, differentiation, morphogenetic signals.

In vivo cloning of interstitial stem cells in hydra

The main evidence for interstitial stem cells in *Hydra* comes from *in vivo* cloning experiments (1, 2). To prepare interstitial stem cell clones, small numbers of dissociated donor cells were mixed with large numbers of host cells and centrifuged to form aggregates which then regenerated normal polyps. In such regenerated polyps of *Hydra magnipapillata* all stem cell clones were capable of differentiating somatic cells, *i.e.* nerve cells, nematocytes and gland cells (2). Since 87 out of 92 clones could also give rise to male and female gametes, we concluded that *H. magnipapillata* contains multipotent stem cells capable of germ line and somatic differentiation. Recently, evidence for such a multipotent stem cell population has also been found in a second species of hydra, *H. oligactis* (3).

In numerous cloning experiments no stem cell clones were found containing only one differentiated type of somatic cells. Thus, there is no evidence for extensively proliferating subpopulations of somatic intermediates (e.g., nerve precursors) in hydra. This is supported by the observation that in tissue which displays extensive nerve cell differentiation (the peduncle region of *H. oligactis* polyps) only stem cells and postmitotic nerve cell precursors are present (4). Thus, signals inducing nerve cell differentiation appear to affect the multipotent stem cells themselves.

In the gamete differentiation pathway, however, unipotent subpopulations of interstitial cells have been isolated. They are capable of extensive proliferation but commited to either spermatogenesis (5) or oogenesis (3). These unipotent stem cells are present in asexually proliferating polyps in low numbers and dividing at a slower rate than their multipotent

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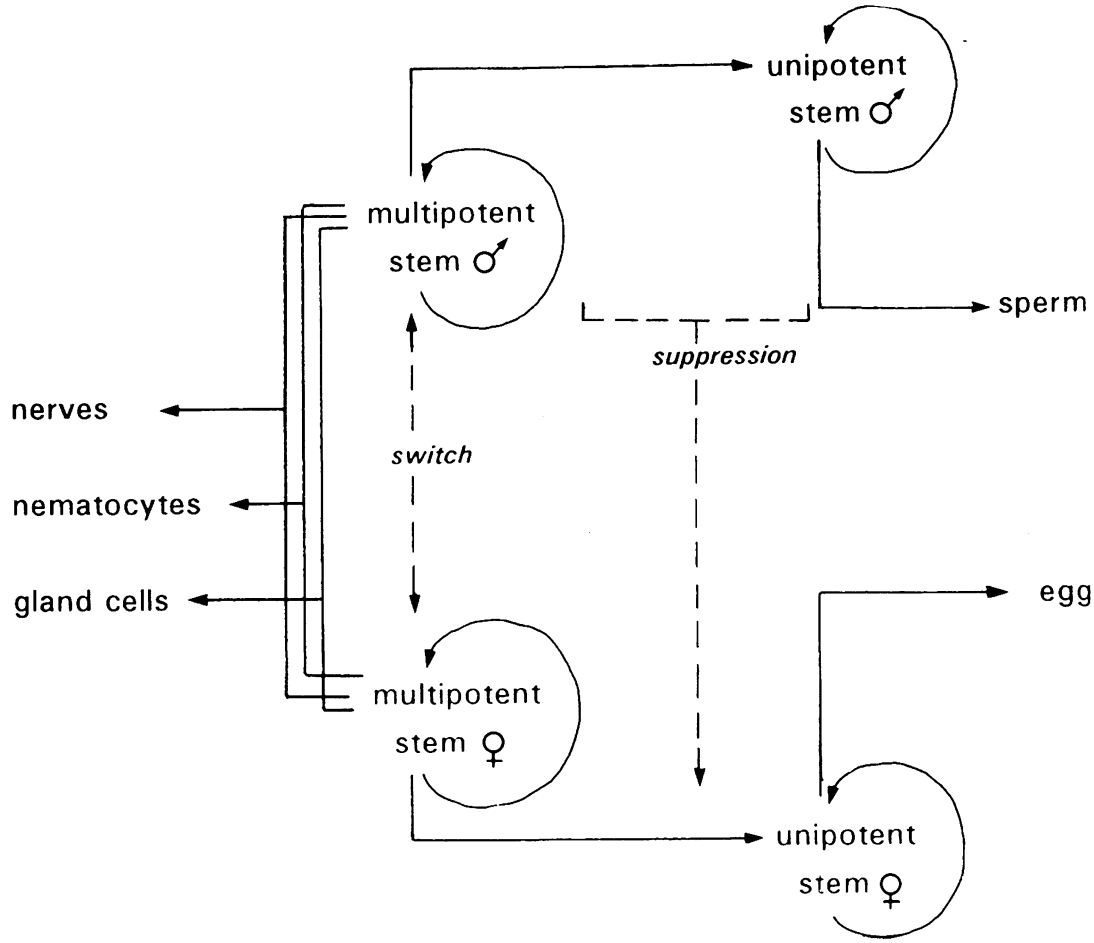

Figure 1. *Schematic diagram of the interstitial stem cells generating somatic and germ cells (modified from Bosch and David, 1986).*

precusor cells (6). In response to environmental stimuli such as low temperature, these cells start to proliferate rapidly and differentiate into gametes. Whether the environmental stimulus serves directly as a signal triggering these cells to gamete differentiation or whether the cells are able to complete their differentiation program only under certain environmental conditions remains to be shown.

When cloning stem cells from male polyps of *H. magnipapillata* we found, to our surprise, clones producing both male gametes and clones producing female gametes (2). Clones from female polyps, by comparison, differentiated female gametes only. The observation of male and female gametes in male polyps raised the possibility that male stem cells could switch their sexual phenotype to female. To test this idea we recloned the stem cells from a male clone (7). After about 200 days in culture we found 5 out of 15 male polyps containing both male and female stem cells. Thus, stem cells from male polyps indeed occasionally switch the sexual phenotype. Stem cells from female polyps, by comparison, were found to be stable in their sexual differentiation capacity after recloning.

Two more features of hydra interstital stem cells are of interest. First, the interstitial stem cells are not only the precursors for germ cells but also themselves responsible for sex determination (8, 9). Somatic components (e.g., epithelial cells) do not play a role in determining the sexual phenotype. Second, male interstitial cells of hydra suppress the ability of female stem cells to differentiate eggs thereby causing «masculinization» of females. They, however, do not interfere with the ability of female stem cells to proliferate and produce somatic cells. The suppression of female gamete differentiation by male interstitial cells was demonstrated experimentally by introducing male stem cells into female tissue by grafting (10, 11). The observed inhibition of female differentiation by male cells also explains the failure of female stem cells in «male» polyps to differentiate. The molecular nature of this suppression is completely unknown. Since a few male interstitial cells are sufficient to masculinize female tissue (10), direct cell-cell interaction between male and female cells probably is not involved. It appears more likely that male stem cells produce a humoral suppressor molecule. Its molecular characterization promises interesting insights into how different cell populations communicate with each other in these ancient metazoa. Figure 1 summarizes these findings and illustrates our current view of the dif-

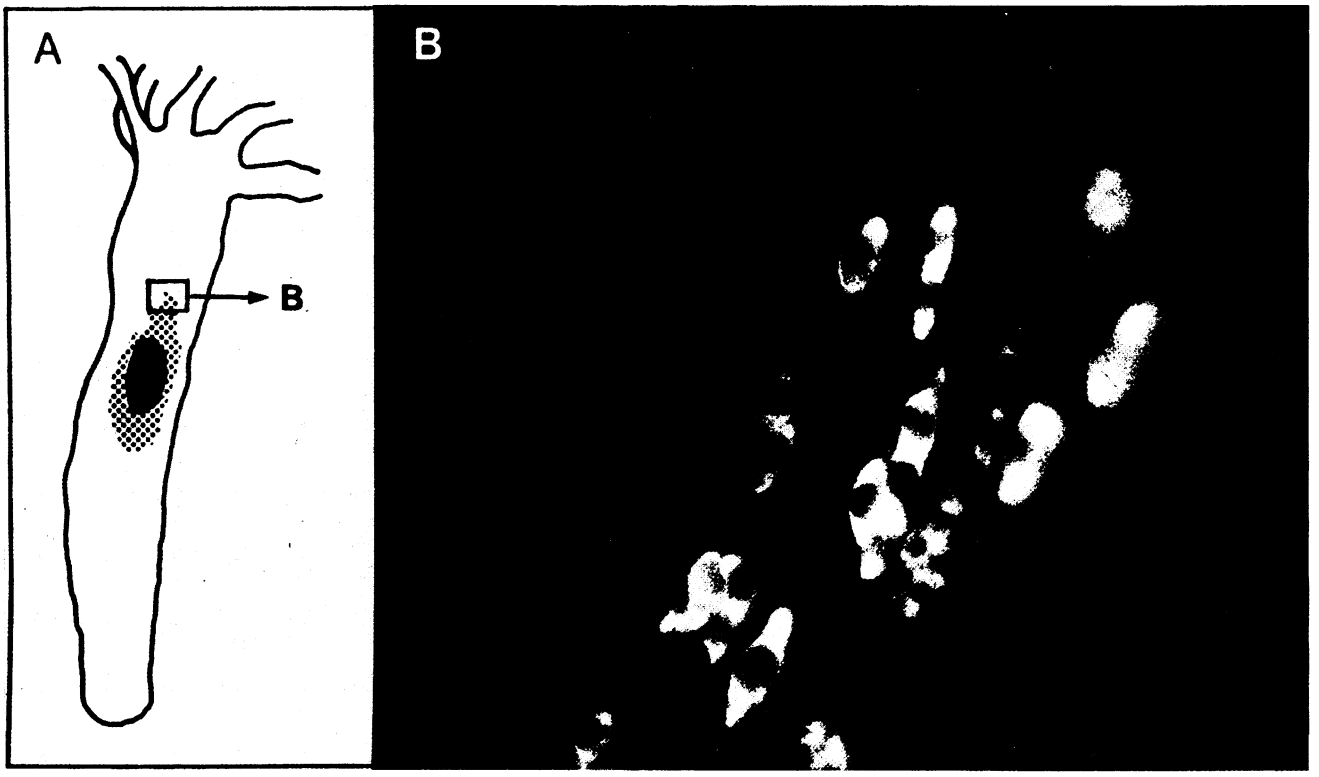

Figure 2. *In vivo localization of interstitial stem cells of Hydra magnipapillata in epithelial host tissue. The interstitial cells were introduced by implantation of a small piece (about 300 epithelial cells) of donor tissue into epithelial host tissue. In this nerve free environment stem cells do not proliferate but differentiate into nerve cells and nematocytes (19). (A) Camera lucida drawing showing localization of donor tissue (black), presence of donor interstitial cells 48 hr after implantation (grey) and area depicted in B (square). (B) Visualization of donor interstitial cells by monoclonal antibody C41.*

ferentiation and proliferation potential of hydra interstitial stem cells.

In vivo localization of interstitial stem cells

Interstitial stem cells in hydra are located primarily in the ectoderm throughout the gastric region (12). Although there have been numerous reports of interstitial cell migration in hydra (13, 14, 15), recent experiments with genetically marked interstitial cell clones indicated that stem cells grow as contiguous patches of cells (16). The finding of patches of clonally derived interstitial cells implys that the extent of migration of interstitial stem cells *in vivo* is limited. The earlier observation of migration in transplantation experiments could be explained by the finding that wounding, i.e. grafting itself, stimulates migration (17). In undisturbed tissue, however, interstitial stem cells appear not to crawl around extensively.

The growth of interstitial cells in clonal patches has important consequences for the distribution of stem cells to daughter polyps. For example, if in a male polyp (which contains male and female stem cells, see above) a clonal patch of female stem cells occurs close to the budding region, this bud will obtain only female cells and therefore become phenotypically female. Thus, due to the growth of stem cells in clonal patches male polyps can occasionally give rise to female polyps, as is indeed observed experimentally (ref. in 7).

Role of the cellular environment in stem cell differentiation

Interstitial cells grow and differentiate in the interstices between ectodermal epithelial cells. In addition to epithelial cells, the interstitial space can have contact to the mesoglea, a thin acellular basement membrane which is separating endoderm from ectoderm. Thus, the major cellular components of the microenvironment in which interstitial stem cells grow and make their decisions are epithelial cells and cells of the interstitial cell lineage themselves, i.e. nerve cells and nematoblasts. The mesoglea may represent an important acellular component of the microenvironment. To study the role of the cellular environment in stem cell proliferation and differentiation, stem cells have been introduced into a variety of different host tissues by transplantation as illustrated in Figure 2. The results of a number of experiments have documented two environmental parameters as particularly important. The first parameter – nerve cell density in host tissue – positively influences proliferation. The second parameter – interstitial cell density – negatively influences proliferation.

Nerve cells present in the head were shown to stimulate stem cell proliferation (18). When stem cells were introduced into host tissue with progressively increased numbers of nerve cells the growth rate of interstitial cells was positively correlated to the nerve cell density (19). Hosts with low nerve cell density inhibit stem cell proliferation; hosts with normal high nerve cell densities support rapid stem cell proliferation.

The growth rate of stem cells is also negatively correlated to the level of interstitial cells present: growth of interstitial cells is faster in tissue with reduced interstitial cell numbers than in normal tissue (6, 20, 21, 22). This finding indicates that stem cell proliferation is controlled by a feed back signal from interstitial cells and their derivatives: decreasing the number of stem cells causes an increase in the self-renewal probability (P_s) and leads to recovery of normal stem cell levels (22). Conversely, increasing the number of stem cells decreases the self-renewal probability.

The nature of the signal(s) by which interstitial cells measure their density is unknown. There is evidence suggesting that the feedback signal is of short range (19, 22, 23). When tissue containing a low density of stem cells is grafted to tissue with high interstitial cell density, the stem cells at low density continue to proliferate rapidly and are not affected by the stem cells growing at high density. Whether the signal is a diffusible molecule produced by stem cells or whether density measurement is mediated directly by cell-cell contact has not yet been determined. Feedback regulation by stem cell density does not, however, occur after grafting tissue between genetically unrelated strains (24). Under these conditions the stem cells behave as if the genetically distinct cells are not present.

Factors influencing stem cell differentiation *in vivo*

The differentiation pattern of interstitial stem cells exhibits a strong dependence on position along the body axis. Interstitial stem cells are found throughout the gastric region; they are absent, however, in the head and foot region (12). Nematocyte differentiation occurs exclusively in the gastric region with stenoteles produced more proximally and desmonemes produced more distally (25). Thus, positional signals are involved in the decision for nerve and nematocyte differentiation. What molecules in the environment serve interstitial cells as positional cues in their decision making?

Much attention has concentrated on the role of the neuropeptide «head activator» (HA) in specifying positional information in hydra (27). The 11 amino acid peptide (Glu-Pro-Pro-Gly-Gly-Ser-Lys-Val-Ile-Leu-Phe; purified by Schaller and Bodenmüller, (28) is capable of causing nerve cell commitment in all parts of hydra tissue (29) in the concentration range of 0.1 - 10 pM. Up to 50% of stem cells differentiate to nerve cells under such conditions compared to 10% in control tissue (29). HA was found to stimulate nerve commitment if present during S phase (29, 30) implying that responses in target cells depend on their position in the cell cycle. After commitment nerve precursors are arrested in G2 and require a second signal to differentiate into mature neurons. This differentiation inducing signal can be released by wounding (31) and can be replaced by HA (32).

Do other neuropeptides in hydra also affect stem cell decisions? Neuropeptides with the carboxyterminus Arg-Phe-NH_2 (RFamide) are ubiquitous in coelenterates (for review see 33). Antisera raised against RFamide stain a population of immunoreactive sensory neurons around the mouth of hydra and a cluster of neurons in the peduncle (34). In ultrastructural studies RFamide like peptides were localized in dense-cored vesicles (35). They are thought to be released by exocytosis and act as hormones or neurotransmitters. In the marine hydrozoan *Hydractinia* the presence of RFamide$^+$ neurons is correlated with the capacity to metamorphose (36) implying that RFamide may serve as a signal molecule during development. In an attempt to examine these neuropeptides for their influence on interstitial cell differentiation, we have analyzed on hydra the effect of Pol-RFaminde I, a 7 amino acid peptide (Glu-Leu-Leu-Gly-Gly-Arg-Phe-NH_2) isolated from *Polyorchis* medusae (37). Treatment of *Hydra magnipapillata* polyps with 10^{-12} M Pol-RFamide caused a twofold increase in the nerve cell density in isolated gastric tissue (Bosch, unpublished observation). Thus, at least two neuropeptides (head activator and Pol-RFamide) have been shown to affect interstitial stem cell behavior. Whether these neuropeptides directly affect stem cells or function indirectly, e.g., by stimulating the release of other biologically active peptide factors, remains to be shown.

Several more signal molecules stimulating or inhibiting the decisions of stem cells await their characterization. Their existence is evident from experiments with crude and partially purified extract. For example, Berking (38) demonstrated that hydra crude extract contains an inhibitor which causes neuronal precursor cells in S phase to remain precusors and prevents them from differentiating to nerve cells. Similarily, Fujisawa (39) found in crude extract an endogeneous factor preventing precursor cells from differentiating into stenoteles.

Stem cell differentiation in hydra - molecular prospects

We have reviewed the progress which has been made in the last ten years in understanding the differentiation potential of interstitial stem cells in hydra. Now a new set of questions is raised concerning the molecular mechanisms underlying stem cell differentiation.

(1) What genes are involved in regulation of stem cell differentiation? Which cis-acting elements mediate position dependent differentiation within the body column? To approach these questions, several groups now are identifying hydra genes expressed in specific regions and genes expressed solely in certain pathways of somatic and germ line differentiation. Progress is also being made toward establishing a system for manipulating genes *in vivo* by introducting gene constructs into intact polyps *via* electroporation (Bosch, Bode, David and Steele, unpublished results). This will

enable identification of DNA sequences controlling cell type specific or region specific gene expression and genes involved in proliferation control. In addition, a gene transfer system will allow introduction of markers into stem cells and thus permit a rigorous *in vivo* examination of the stem cell lineage outlined in Figure 1.

(2) The neuropeptides HA and RFamide apparently affect stem cell behavior. How do they do so? Which cell populations are the target cells? Which genes are activated? Using subtractive hybridization it should be feasible to isolate cDNAs for mRNAs made in response to such factor treatment. Equally important for understanding the *in vivo* function of these signal molecules will be the characterization of the receptors for the particular ligands. Evidence for two types of receptors for HA was presented recently by C. Schaller's group (40) and it was proposed that different functions of the HA molecule are mediated by different receptor types.

(3) «Stem cell systems in hydra and vertebrates strongly suggest the importance of spatial organization and thus extrinsic influences» (41). Can we define these extrinsic influences in molecular terms? What components of the environment (signal molecules, neuropeptides, growth factors) are involved in the decision making of stem cells? The search for such molecules would clearly be facilitated if signal molecules known to play crucial roles in regulation of differentiation and development in higher organisms are also used in hydra. It is thus encouranging that the first membrane associated protein tyrosine kinases and receptors cloned from hydra have been found highly homologous to vertebrate genes (42, 43, Steele, pers. commun.).

(4) There is a growing realization of the importance of the extracellular matrix in cell differentiation and morphogenesis (44). Strong links are emerging between growth factors and the ability of cell adhesion molecules and extracellular matrix molecules to participate in, and potentially regulate, morphogenesis and differentiation (45). It is tempting to speculate, therefore, that in hydra the mesoglea serves as an affinity matrix presenting growth and differentiation factors along the body axis. By specific release of these factors the mesoglea could specify positional information used in the spatial regulation of differentiation (46).

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