STEM CELL GROWTH AND DIFFERENTIATION IN *HYDRA ATTENUATA*

II. REGULATION OF NERVE AND NEMATOCYTE DIFFERENTIATION IN MULTICLONE AGGREGATES

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SUMMARY

The differentiation of nerve cells and nematocytes from interstitial stem cells in *Hydra* has been investigated under conditions of changing stem cell density. Interstitial stem cells were cultured in a feeder layer system consisting of aggregates of nitrogen mustard-inactivated tissue. The aggregates were seeded with varying numbers of stem cells from 10 to 400 per aggregate; between 4 and 7 days later the rates of nerve and nematocyte differentiation were measured. Nerve differentiation was scored by labelling the stem cell population with [^{3}H]-thymidine and counting labelled nerve cells after 48 h. Nematocyte differentiation was scored by counting nests of 4 proliferating nematoblasts. In both cases the numbers of differentiating cells were normalized to the size of the stem cell population.

The results indicate that the rate of nematocyte differentiation increases as the concentration of stem cells increases in aggregates; under the same conditions the rate of nerve differentiation remains essentially constant. To calculate the numbers of stem cells entering each pathway per generation, a computer was programmed to simulate the growth and differentiation of interstitial stem cells. Standard curves were prepared from the simulations relating the rates of nerve and nematocyte differentiation to the fraction of stem cells committed to each pathway per generation. The rates of nerve and nematocyte commitment were then estimated from the experimentally observed rates of differentiation using the standard curves. The results indicate that nerve commitment remains constant at about 0·13 stem cells per generation over a wide range of stem cell concentration. Nematocyte commitment, by comparison, increases from 0·15 to 0·21 stem cells per generation as stem cell concentration increases in aggregates. The fact that the ratio of nerve to nematocyte commitment changes under our conditions suggests that stem cell commitment is not a stochastic process but subject to control by environmental stimuli.

INTRODUCTION

In *Hydra* differentiating nematocytes and nerve cells arise continuously from a population of interstitial stem cells (for review, see Bode & David, 1978). In rapidly growing animals, 40% of stem cell daughters differentiate in each cell generation. These cells are partitioned 3 : 1 to the nematocyte and nerve pathways on average over the whole animal (David & Gierer, 1974), although there are significant deviations

from this average behaviour in localized regions of the animal: in the hypostome and basal disk nerve differentiation predominates; in the gastric region nematocyte differentiation predominates. Although these results suggest that the ratio of nerve to nematocyte commitment can change, they do not prove this point since little is known about the possible migration and concentration of committed precursors in localized regions of *Hydra* tissue. In order to determine if the ratio of nerve to nematocyte commitment is a fixed parameter of *Hydra* stem cells or subject to regulation by environmental stimuli we have taken an alternative approach in the experiments described below.

In the preceding report (Sproull & David, 1979) and elsewhere (David & MacWilliams, 1978) we have demonstrated that the self-renewal probability (P_s) can be increased by culturing stem cells at lower than normal densities in a feeder layer culture system consisting of aggregates of nitrogen mustard (NM)-inactivated *Hydra* tissue. An increase in self-renewal implies a corresponding decrease in the number of stem cells available for differentiation. It is, therefore, possible to ask whether these additional stem cells are withheld from both differentiation pathways proportionately or whether one pathway is preferentially turned off in favour of stem cell self-renewal. The first result would be expected if the proportions of nematocyte and nerve differentiation were regulated by a stochastic process independent of the environment. The second result would imply that the proportions of nematocyte and nerve differentiation were subject to environmental control.

In the present experiments we have varied the self-renewal probability by varying stem cell density in NM aggregates and measured the rates of nerve and nematocyte differentiation. We have used computer simulations of the stem cell system in order to determine the behaviour of the stem cell population from the rates of nerve and nematocyte differentiation. The results indicate that almost all cells leaving the stem cell pool can be accounted for. To a first approximation, nematocyte differentiation varies inversely with P_s while nerve differentiation remains constant. Since the ratio of nematocyte to nerve differentiation changes with increasing P_s, the results suggest that nerve and nematocyte commitment are not regulated by a stochastic process but are subject to environmental control.

METHODS

Methods for the culture of stem cells in aggregates of nitrogen mustard (NM)-treated *Hydra* cells and the quantitation of stem cells and differentiated cells have been described in the preceding report (Sproull & David, 1979). Details of the computer simulation of stem cell growth and differentiation are also presented in that report. Classification of cells and terminology are the same as the preceding report.

Assay for nematocyte differentiation

NM aggregates were seeded with 10, 30, 100, 200, 300, and 400 clone-forming units (CFU) per aggregate. The aggregates used in these experiments are the same ones used to determine the growth rate of stem cells in the preceding report. Aggregates were macerated on days 6 and 7, and scored for 1s + 2s and 4s. 4s are the first cell type unique to the nematocyte differentiation pathway; the ratio of 4s/(1s + 2s) was used as an index of the rate of nematocyte differentiation.

Assay for nerve differentiation

NM aggregates were prepared with 30 or 400 CFU/aggregate. On day 4 aggregates were injected with [³H]thymidine (0·1 μl/aggregate; 100 μCi/ml; 30 Ci/mmol) at 2 times separated by a 12-h interval. This procedure labels essentially all stem cells since the *S*-phase of interstitial cells is 12 h and the cell cycle ~24 h. On day 6 (48 h after the first [³H]thymidine injection) aggregates were macerated, spread on slides, and autoradiographed. The preparations were scored for labelled nerve cells (Nv^*) and total 1s+2s. The ratio of $Nv^*/(1s+2s)$ was used as an index of nerve differentiation.

RESULTS

Assays for nerve and nematocyte differentiation in NM aggregates

Nerve and nematocyte differentiation in *Hydra* occur continuously from interstitial stem cells. The kinetics of differentiation and intermediates in the pathways have been described previously (David & Gierer, 1974). For nerve differentiation a committed precursor leaves the stem cell pool and divides; the daughter cells differentiate nerves in about 6 h. The time from the end of *S*-phase of the precursor stem cell to the appearance of differentiated nerves is about 18 h. The time of commitment is not precisely known but appears to be in *S*-phase of the precursor stem cell (Schaller, 1976). Thus, the delay between commitment and completed nerve differentiation is about 1 day.

Nematocyte differentiation starts when a committed precursor leaves the stem cell pool and proliferates to form a cluster of nematoblasts. The cell cycle of the proliferating nematoblasts is about 18 h (Campbell & David, 1974). Nests of nematoblasts are held together by cytoplasmic bridges (Slauterback & Fawcett, 1959) which are not broken by the maceration procedure used to analyse cells. Nests of 4 nematoblasts (4s) are the first cell type unique to the nematocyte pathway (2s are a mixture of stem cells and committed precursors). It is not known whether a single stem cell or a pair of stem cells undergoes commitment to nematocyte differentiation (David & Gierer, 1974). Thus, it is uncertain whether 4s are 1 or 2 generations post commitment. This uncertainty, however, does not affect the present experiments.

The purpose of the present experiments is to assay nerve and nematocyte differentiation in aggregates under different levels of self-renewal. Because early aggregates contain differentiating cells brought in with the stem cells (Gierer *et al.* 1972), it is necessary to delay assaying differentiated cells long enough for all committed precursors to complete differentiation. After this point, the only differentiation in aggregates occurs from the stem population. To be safe, we have chosen to assay differentiation on days 4–7. For aggregates at this time we have estimates of P_s (Sproull & David, 1979).

We assay nerve differentiation by injecting aggregates with [³H]thymidine on day 4 and scoring labelled nerve cells (Nv^*) on day 6; [³H]thymidine labelling is necessary in order to distinguish a particular cohort of newly differentiated nerve cells from residual (unlabelled) nerves in the host tissue. We assay nematocyte differentiation by scoring 4s on days 6 and 7. Since 4s are a rapidly turning over population, the number of 4s is a sensitive indicator of the rate of nematocyte differentiation; radio-
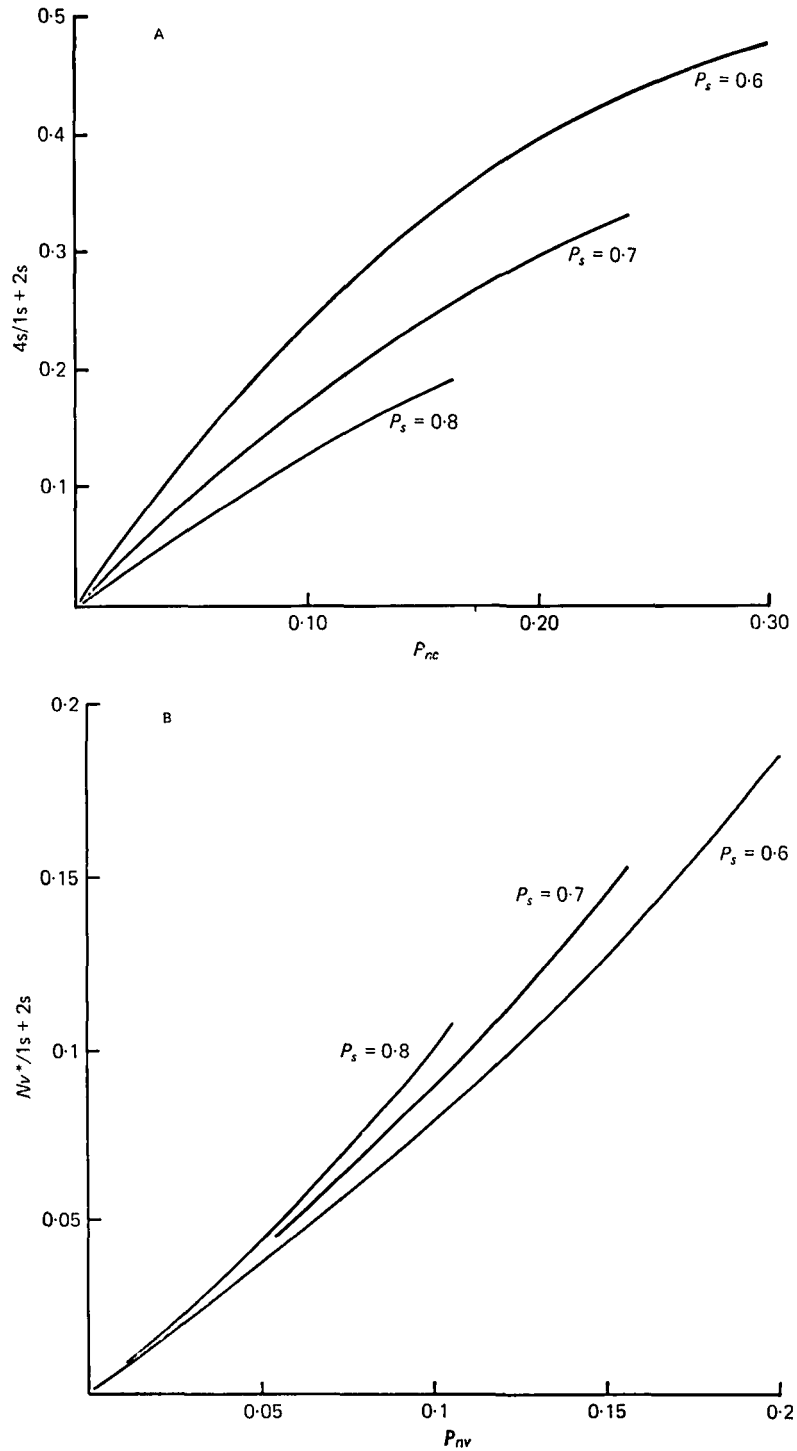
Fig. 1. Standard curves showing the relationship between: (A), the ratio of $4s/1s+2s$ and the probability of nematocyte commitment (P_{nc}), and (B), the ratio of $Nv^*/1s+2s$ and the probability of nerve commitment (P_{nv}). Curves were generated from computer simulations of growth and differentiation of the *Hydra* stem cell system (David & Gierer, 1974). Details of the simulation are presented in the preceding paper (Sproull & David, 1979). The probabilities of nerve and nematocyte commitment were varied in the simulations for given values of the self-renewal probability (P_s) and the ratio of $4s/1s+2s$ and $Nv^*/1s+2s$ respectively computed.

active labelling is unnecessary. The time of commitment for both assays falls within the early part of the 4–7 day period. Thus we are assaying commitment events occurring at the same time at which P_s was determined (Sproull & David, 1979). For both nerve and nematocyte differentiation we normalize the observed levels of 4s and labelled nerves (Nv^*) to the size of the stem cell population (1s + 2s).
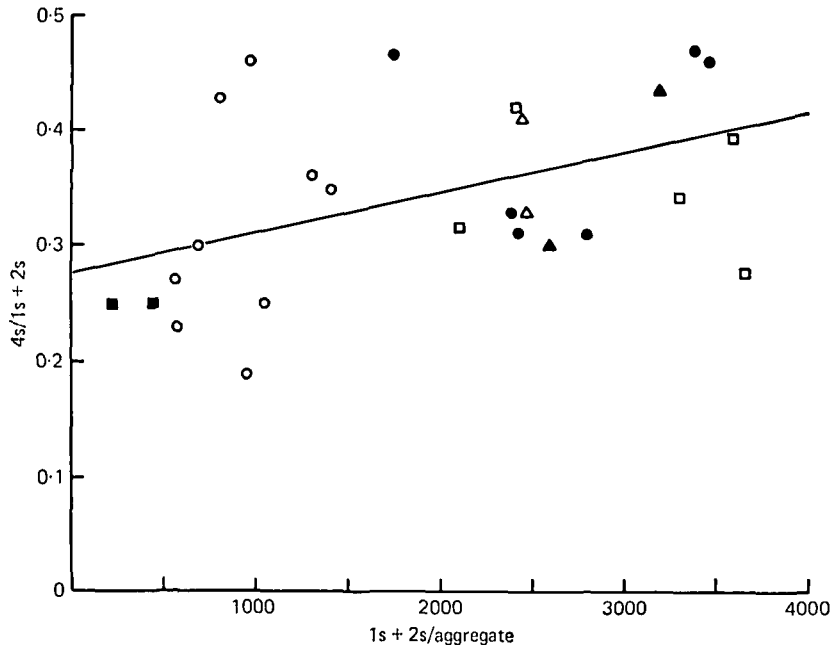

Fig. 2. Change in the ratio of 4s/1s + 2s with increasing stem cell density (expressed as 1s + 2s) in NM aggregates. Aggregates were seeded with varying numbers of CFU. After 6 and 7 days of growth 5 aggregates were macerated and the total number of 1s + 2s/aggregate and the ratio of 4s/1s + 2s determined. Different symbols indicate aggregates seeded with 10 (■), 30 (○), 100 (△), 200 (●), 300 (▲), and 400 CFU (□).

We have used computer simulation of stem cell growth and differentiation (see Sproull & David, 1979) to prepare standard curves relating the rates of nerve and nematocyte differentiation to the fraction of stem cells committed to nerve and nematocyte differentiation in each generation. The number of stem cells entering the nematocyte pathway is expressed as the fraction of total stem cells in that generation, P_{nc}. Similarly the fraction of stem cells entering the nerve pathway in each generation is given by P_{nv}. $P_{nv} + P_{nc} = 1 - P_s$, where P_s is the fraction of stem cells which self-renew (Sproull & David, 1979).

Fig. 1A, B show standard curves derived from computer simulations in which P_{nc} and P_{nv} were varied and the ratio of 4s/1s + 2s and Nv^*/1s + 2s were calculated. As P_{nc} and P_{nv} increase there is an increase in the ratio of 4s/1s + 2s and Nv^*/1s + 2s. In addition the ratios are affected by the value of P_s because it changes the size of the population of 1s + 2s and the proportion of stem cells and early committed cells in that population. In the present experiments, we have compared experimental

measurements of the rate of nerve and nematocyte differentiation to these standard curves in order to estimate the rate of stem cell commitment (P_{nc} and P_{nv}) to each pathway.

Rates of nematocyte and nerve differentiation in NM aggregates

Increasing the number of clones in NM aggregates causes a decrease in P_s (Sproull & David, 1979) and an increase in the number of cells available for differentiation. If the proportions of differentiating nematocytes and nerves remain constant, we

Table 1. *Nerve differentiation in multiclone aggregates*

CFU/aggregate	Nv^*/1s+2s	P_{nv}
	Experiment I	
30	0·13	0·14
200	0·09	0·11
	Experiment II	
30	0·15	0·15
400	0·11	0·13

NM aggregates were seeded with different numbers of CFU. Aggregates were labelled twice with [³H]thymidine on day 4 and macerated on day 6. Following autoradiography, the macerations were scored for labelled nerve cells (Nv^*) and 1s+2s. P_{nv} was calculated from the ratio of Nv^*/1s+2s using the standard curves in Fig. 1B. In estimating P_{nv} we assumed $P_s = 0·7$ for 30 CFU aggregates and $P_s = 0·6$ for 200 and 400 CFU aggregates.

expect an increase in the rates of nematocyte and nerve differentiation. We have assayed nematocyte differentiation in NM aggregates by scoring the ratio of 4s/ (1s+2s). Fig. 2 shows the results on days 6 and 7 from a number of experiments in which aggregates were seeded with 10–400 CFU. The experimental ratios of 4s/1s+2s scatter widely but generally increase as the number of 1s+2s per aggregate increases; a least-squares regression line through the experimental points indicates about a 1·5-fold increase in nematocyte differentiation over this range of stem cell density. Aggregates seeded with 10 and 30 CFU contain 200–1400 1s+2s on days 6 and 7; the ratio of 4s/1s+2s in such aggregates is about 0·2–0·3. Aggregates seeded with 100–400 CFU contain 1800–3500 1s+2s on days 6 and 7. About half these aggregates have ratios of 4s/1s+2s of 0·4–0·5; the remaining aggregates have ratios of 0·3–0·4 indicating somewhat lower levels of nematocyte differentiation.

To assay nerve differentiation, we have scored the appearance of labelled nerve cells following pulse labelling of the stem cell population with [³H]thymidine. Table 1 shows the ratio of Nv^*/1s+2s on day 6 in 2 independent experiments in which aggregates were seeded with 30 and 200 CFU or 30 and 400 CFU respectively. Both experiments yielded very similar results. They indicate that there is very little change in the rate of nerve differentiation as the number of CFU per aggregate increases 10-fold.

Calculation of stem cell commitment to nematocyte and nerve pathways

Increasing the clone density in NM aggregates causes an increase in the level of proliferating nematoblasts (Fig. 2). Since the cell cycles of 4s and stem cells remain constant under these conditions (Sproull & David, 1979; unpublished observations), we conclude that this observed increase in 4s represents a higher proportion of stem cells undergoing commitment to the nematocyte pathway. Concurrently, the rate of nerve differentiation remains almost constant (Table 1) suggesting that no change in nerve commitment occurs as a result of changing clone density in NM aggregates.

To determine quantitatively the proportions of stem cells entering each differentia-

Table 2. *Summary of P_s, P_{nv}, and P_{nc} in multiclone aggregates*

CFU/aggregate	P_s*	P_{nv}†	P_{nc}‡	$P_s + P_{nv} + P_{nc}$
30	0·7	0·15	0·15	1·0
200–400	0·6	0·12	0·21	0·93

* Results from Fig. 4 in preceding paper (Sproull & David, 1979).
† Table 1.
‡ P_{nc} was calculated from the ratio of 4s/1s + 2s (Fig. 2) using the standard curves in Fig. 1A.

tion pathway as P_s changes, we have compared the experimental results in Fig. 2 and Table 1 with the expected levels of differentiation using computer simulations of the cell flow model (Fig. 1). Ten and 30 clone aggregates have $P_s \cong 0·7$ on day 5·5 in NM aggregates (Sproull & David, 1979). The ratio of 4s/1s + 2s in most of the aggregates is 0·2–0·3 indicating $P_{nc} = 0·15$. The ratio of Nv*/1s + 2s in these aggregates is 0·13–0·15 indicating $P_{nv} = 0·14$–0·15. Aggregates with clone densities between 100 and 400 per aggregate have $P_s \cong 0·6$ on day 5·5 (Sproull & David, 1979). The ratio of 4s/1s + 2s in these aggregates is 0·35–0·45 indicating $P_{nc} = 0·21$. The ratio of Nv*/1s + 2s in these aggregates is 0·09–0·11 indicating $P_{nv} = 0·12$.

These results are summarized in Table 2. They indicate that in low density aggregates all stem cells can be accounted for by the 3 pathways and that the ratio of nematocyte to nerve differentiation is 1 : 1. In high-density aggregates the ratio of nematocyte to nerve commitment has increased to almost 2 : 1 although the exact value is uncertain because a small fraction of the stem cell population has not been accounted for.

DISCUSSION

The principal experimental finding in this report is that the rate of nematocyte differentiation increases about 1·5-fold as the rate of self-renewal decreases from 0·7 to about 0·6 in NM aggregates (Fig. 2); at the same time, the rate of nerve differentiation remains essentially constant (Table 1). By comparing these results to computer simulations of stem cell growth and differentiation, we have estimated the proportions of stem cells entering each of the 3 pathways under the conditions of our

experiments. At low clone density where $P_s \cong 0.7$, $P_{nc} = 0.15$ and $P_{nv} = 0.15$; at high clone density where $P_s \cong 0.6$, $P_{nc} = 0.21$ and $P_{nv} = 0.12$ with about 0.07 of the stem cell population unaccounted for. To a first approximation, therefore, the change in P_s is the result of a change in nematocyte commitment; nerve commitment remains essentially constant.

In 10 and 30 clone aggregates, the sum of $P_s + P_{nv} + P_{nv} = 1$ indicating that all stem cells can be accounted for. In high-density aggregates, however, the sum of $P_s + P_{nv} + P_{nc} = 0.93$ indicating that a small fraction of stem cells have not appeared in either the self-renewal or differentiation pathways. Although it is possible that 0.93 is not significantly different from 1.0 given the errors in our techniques, it appears more likely to us that 0.93 is, in fact, too low probably due to cell death. Because high-density aggregates are very similar to normal *Hydra* in cell composition and morphological development, we would anticipate $P_{nc} = 0.3$ (David & Gierer, 1974) in these aggregates compared to $P_{nc} = 0.21$ which was observed. Thus, it appears likely that a selective loss of proliferating nematoblasts is responsible for the small fraction of the stem cell population which is unaccounted for in our high-density aggregates.

The principal findings reported here have been arrived at independently by Yaross & Bode (1978) using different experimental conditions. These investigators varied stem cell density in normal *Hydra* by feeding regimes or partial killing with hydroxyurea and scored nerve and nematocyte commitment. Their results demonstrate an increase in nematocyte commitment with increasing density of stem cells similar to Fig. 2. Furthermore, they also observed considerable scatter in the ratio of 4s/1s + 2s between experiments. The origin of this scatter in both experiments is not presently known; it is, however, not characteristic of the 4s/1s + 2s ratio in normal *Hydra*.

There is one discrepancy between the results of Yaross & Bode (1978) and our own. Our results in NM aggregates indicate that, even at very low clone densities, there is extensive nematocyte differentiation. In Fig. 2 there is no evidence that the ratio of 4s/1s + 2s approaches zero; it is also not zero in single clones where the density of stem cells is initially as low as 1 per aggregate (David & Murphy, 1977). By comparison the results of Yaross & Bode (see fig. 13 of their paper) suggest that nematocyte differentiation can approach values of zero at low stem cell density in hydroxyurea-treated *Hydra*. At present there is no explanation for this difference except the difference in host tissue: NM- versus hydroxyurea-treated.

An interesting feature of our results and those of Yaross & Bode (1978) is that the rate of nematocyte differentiation appears to be controlled by the density of stem cells in NM aggregates. Furthermore, changes in self-renewal appear to be coupled almost quantitatively to changes in nematocyte differentiation: when P_s increases from 0.6 to 0.7, nematocyte commitment decreases from 0.21 to 0.15. There is accumulating evidence that P_s is controlled by negative feedback between stem cells: increasing stem cell density, decreases P_s (Bode, Flick & Smith, 1976; David & MacWilliams, 1978; Sproull & David, 1979). It is, therefore, possible that a positive stimulus for nematocyte differentiation in fact mediates the negative control over self-renewal

exercised by stem cell density. A similar suggestion has been made by Yaross & Bode (1978).

The observation that the ratio of nematocyte to nerve differentiation changes from 1 : 1 to 2 : 1 as P_s changes from 0·7 to 0·6 suggests that the proportions of nematocyte and nerve differentiation are not regulated by a stochastic process. It is necessary to be cautious about this conclusion if only the results in Table 2 are considered since not all stem cells were accounted for in the high-density aggregates. However, comparison of the nematocyte to nerve ratio in low density aggregates (Table 2) with normal *Hydra* significantly strengthens the argument. In normal *Hydra* the ratio of nematocyte to nerve commitment is 3 : 1 (David & Gierer, 1974) under conditions where all stem cells can be accounted for. This is clearly different from the nematocyte to nerve commitment ratio of 1 : 1 in low-density aggregates (Table 2). We can, therefore, conclude that the ratio of nematocyte to nerve commitment is not a fixed parameter of interstitial stem cell physiology. Rather our results support the idea that the proportions of stem cells entering the nematocyte and nerve pathways can be independently regulated probably by stimuli in the host environment.

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