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Male and female stem cells and sex reversal in *Hydra* polyps

(sex switch/female repression/model for sex determination)

THOMAS C. G. BOSCH AND CHARLES N. DAVID

Zoologisches Institut der Universität München, Luisenstrasse 14, D-8000 München 2, Federal Republic of Germany

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ABSTRACT **Single interstitial stem cells of male polyps of *Hydra magnipapillata* give rise to clones that differentiate either male or female gametes. To test the sexual stability of these clones, stem cells were recloned. The results indicate that stem cells from female clones are stable in their sexual differentiation capacity; male stem cells, by comparison, switch sexual phenotype at the rate of 10^{-2} per cell per generation. As a result, female polyps contain only female stem cells; male polyps contain a mixture of male and female stem cells. A model is presented in which the sexual phenotype of *Hydra* polyps is controlled by (*i*) the switching rate of male and female stem cells and (*ii*) the repression of female differentiation by male stem cells.**

In most metazoans the sexual phenotype is determined by somatic tissue or by environmental factors. Hydra are unusual in this regard in that their sexual phenotype is determined by the interstitial cell lineage, which itself gives rise to the gametes; epithelial tissue appears to have no influence on sex determination (1, 2). A further unusual feature of *Hydra* is the occurrence of sex reversal in adult polyps. Although in some species (*Hydra attenuata, Hydra carnea, Hydra magnipapillata*) spontaneous sex reversal is rather common, in other species (*Hydra oligactis*) only rare cases of spontaneous sex reversal are observed. A number of species are "simultaneous hermaphrodites" (*Hydra viridis, Hydra circumcincta*).

Although the cellular mechanism of sex reversal is not understood, it can be induced experimentally by grafting male to female tissue. In such heterosexual grafts conversion of females into males is observed. Such "masculinized" females behave as stable males. Masculinization is caused by migration of male interstitial cells into female tissue (3), suggesting that male interstitial cells suppress differentiation of female gametes in female tissue.

We have shown that stem cells of *H. magnipapillata* are multipotent in the sense that individual stem cells can differentiate somatic cells as well as germ-line cells (4). Surprisingly, however, most of the stem cell clones (about 70%) derived from male polyps differentiated female gametes (4).

One possible explanation for the occurrence of male and female clones is that individual stem cells can switch the sexual differentiation pathway. An alternative explanation is that stem cells are restricted in their sexual differentiation capacity and that polyps (in this case, male polyps) contain male and female stem cells. In such male polyps, female stem cells are prevented from egg differentiation (3); in the absence of male stem cells—e.g., under cloning conditions—female stem cells differentiate eggs.

To test which of these two alternatives is true, stem cells from polyps containing male or female clones were recloned. If stem cells can switch sex, the resulting clones should differentiate male or female gametes irrespective of the sex of the donor clone. However, if stem cells are restricted in their sexual differentiation capacity, the sex of the resulting clones should be of the donor type only.

The results provide evidence that female stem cells are restricted in their sexual differentiation capacity; female clones, when recloned, give rise to female polyps only. By comparison, male clones, when recloned, give rise to male and female polyps. Hence, male stem cells can occasionally switch sex.

These observations together with the results of other investigators support a model in which the sexual phenotype of *Hydra* polyps is controlled by the sex of stem cells and by cell–cell interactions between male and female stem cells. Sex reversal in polyps is due to sex switching events in individual stem cells.

MATERIALS AND METHODS

Strains and Animal Culture. Two mutant strains of *H. magnipapillata* were used. Strain ms-1 (male sterile 1), which has immotile sperm (5), was used as the interstitial cell donor; strain sf-1 (self-feeder 1), which has temperature-sensitive interstitial cells (6), was used as the host strain. Animals were cultured by using standard methods (7).

Preparation and Analysis of Clones. To prepare interstitial cell clones, small numbers of dissociated ms-1 cells were mixed with large numbers of dissociated sf-1 cells and centrifuged to form aggregates. Sets of 20–30 aggregates were prepared using dilutions of the ms-1 cell suspension such that aggregates contained 0.05–2 clones per aggregate (4). Aggregates regenerated normal polyps. After 6 days, interstitial cells of the host sf-1 were eliminated by culturing the aggregates at the nonpermissive temperature (24°C). Regenerated polyps were propagated clonally for 1–2 months. Aggregates containing stem cells were distinguished from aggregates without stem cells by their capacity for self-feeding. To analyze the sexual differentiation capacity of stem cell clones, regenerated polyps were induced to sexual differentiation as described (5).

RESULTS AND DISCUSSION

Stem cell clones derived from male polyps of strain ms-1 were used as donor tissue. The clones were obtained in a previous experiment (4) and have been shown to differentiate male or female gametes. Donor clones were 6–8 months old and had a high statistical probability of being derived from a single stem cell.

The results of the recloning experiments show that stem cells taken from female donor clones differentiated eggs only (Table 1). Even in classes that contain statistically more than one clone per aggregate, no differentiation of male gametes occurred. These results indicate that stem cells of female *Hydra* are limited to female differentiation.

Stem cells taken from male donor clones differentiated male gametes in most cases (Table 1). However, in five cases, stem cells from male clones differentiated eggs. One clone

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Table 1. Sexual differentiation capacity of stem cells derived from male and female clones

Donor clone		Stem cells,* no. per aggregation	♂	♀	Hermaphrodite†
Sex	Age, days				
♀	247, 210	0 –0.1	—	1	—
		0.1–0.2	—	3	—
		0.2–0.3	—	—	—
		0.3–0.4	—	9	—
		0.4–0.5	—	—	—
		0.5–1.0	—	8	—
		>1.0	—	19	—
		Total	—	40	—
♂	181, 261, 161	0 –0.1	—	—	—
		0.1–0.2	2	2	—
		0.2–0.3	2	—	—
		0.3–0.4	2	2	1
		0.4–0.5	—	—	—
		0.5–1.0	4	1	—
		>1.0	ND	ND	ND
		Total	10	5	1
♂	Mass culture of strain ms-1‡	0 –0.5	13	42	4
		0.5–1.0	10	16	2

ND, not determined.
*Calculated according to Poisson statistics from the nonfeeder/self-feeder ratio at each input concentration (see ref. 4).
†Polyps produced testes first and eggs later.
‡Results from previous experiments.

was found to differentiate sperm first and eggs later. Stem cells from three different male donor clones showed the same behavior (Table 1). By comparison, clones derived from male polyps in a mass culture had a significantly higher proportion of female stem cell clones (4).

Stem Cells Are Able to Switch Their Sexual Phenotype. The results of the recloning experiments (Table 1) showed that stem cells taken from "male" clones gave rise to clones that in some cases differentiated male gametes and in other cases differentiated female gametes. We interpret the occurrence of female and male clones as the result of a switch in the sex of male cells during proliferation of the original clone. The proportion of male to female clones was found to be about 2:1 among the recloned stem cells (Table 1). If male cells switch to female cells with a frequency of about 1% per cell per generation, then clones derived from single male cells will contain about 30% female cells after 50–100 stem cell generations. Since the original clones were cultured for about 200 days prior to recloning and since the stem cell generation time is about 1–2 days (8), this is consistent with our observations.

As shown below, stem cell switching from female to male has not been observed. Thus, the proportion of female cells within a male clone is expected to increase slowly with time. In fact, such a slow increase appears to be observed when one compares our recloning results (200-day-old clones) with animals taken from a mass culture of the male strain. Stem cell populations in such cultures have proliferated for longer periods of time than the clones in our experiments and indeed the proportion of female cells has risen drastically (Table 1). Such a continuous increase in female stem cells would appear to be unstable, leading ultimately to only female polyps. Since this is not the case in natural populations, male stem cells must have a slight growth advantage or female-to-male switching must indeed occur at low frequency.

Evidence for Repression of Female Differentiation by Male Germ Cells. The results in Table 1 indicate that male clones contain male and female stem cells. This finding agrees with the original cloning experiments on male polyps in which male and female clones were observed (4). *In vivo* observations by Tardent (9) also support the finding of mixed populations of male and female cells within individual male polyps: treatment of *H. attenuata* males with doses of x-rays sufficient to eliminate most interstitial cells led to the occurrence of 70–90% females among polyps that survived treatment.

The presence in male polyps of female stem cells that are unable to differentiate suggests that male stem cells repress the differentiation of female cells. This repression provides an explanation for the classical observation of "masculinization" of females following grafting to male tissue (ref. 10 and references therein). A particularly elegant experiment of this sort has been done in *H. magnipapillata* by Sugiyama and Sugimoto (3). They prepared a heterosexual chimera by grafting a female strain marked with a somatic mutation in nematocyte differentiation to a male strain containing a mutation in sperm differentiation. During 4 months of parabiosis, the female and male interstitial cells were found to proliferate and differentiate somatic cells (recognizable as a mixture of wild-type and mutant nematocytes in the tentacles of the chimera). Only male interstitial cells, however, differentiated gametes since all sperm exhibited the mutant character; female interstitial cells did not differentiate gametes.

In view of the observed repression of female differentiation by male stem cells, female polyps are expected to lack male stem cells. The results of our cloning experiments (Table 1) directly confirm this prediction. Stem cells taken from female clones give rise to clones that differentiate only female gametes.

Sex Determination and Sex Reversal in Hydra Polyps and its Relationship to the Presence of Male and Female Germ Cells. Based on our results and those of others, it is now possible to construct a model for the control of sex determination in hydra polyps. The model is shown schematically in Fig. 1. (*i*) Stem cells or their derivatives determine the sex of polyps; cells of the epithelial cell lineage appear to have no influence on the sexual phenotype (1, 2). (*ii*) Stem cells are restricted to male or female differentiation. (*iii*) Male stem cells can switch sex with a low frequency (Table 1). Female stem cells, at least in *H. magnipapillata*, do not switch sex. However, there is indirect evidence for female-to-male stem cell switching in *H. attenuata* (see below). (*iv*) Male stem cells and/or early intermediates in the spermatogenic pathway repress differentiation of female stem cells. This effect is most clearly seen as masculinization following grafting of male tissue to female tissue (references in refs. 3 and 10). The number of male stem cells required for effective repression appears to be low.

In terms of the model one can predict the stem cell composition and the sexual phenotype of hydra polyps. Female polyps contain only female stem cells since the presence of male stem cells represses the differentiation of female gametes. Male polyps contain a mixture of male and female stem cells due to sex switching during proliferation of male stem cells. Female-to-male switching appears not to occur in *H. magnipapillata* and *H. oligactis* and only occurs rarely in *H. attenuata*.

As shown in Fig. 2, sex reversal in hydra polyps occurs by two different mechanisms depending on the direction of the change. (*i*) Sex reversal of female polyps is caused by the introduction of male stem cells. This occurs when male tissue is grafted to female tissue (masculinization by grafting) or when a female stem cell switches to male. (*ii*) Sex reversal of male polyps is caused by any process that separates male from female stem cells. This occurs in stem cell cloning experiments or as a result of x-ray inactivation in cases in which female stem cells are more abundant than male stem

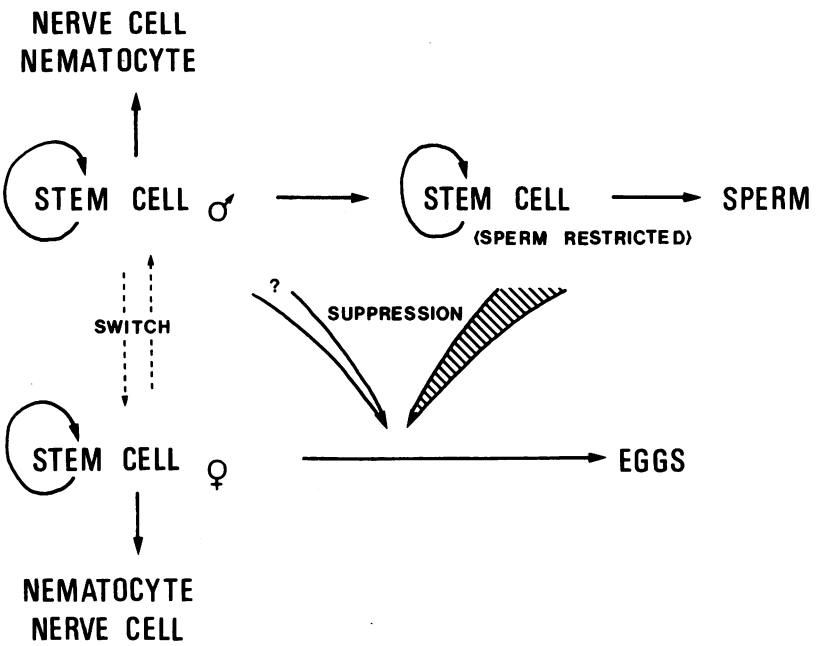

FIG. 1. Model for the control of sex determination in *Hydra* showing the interactions between male and female stem cells. The sperm-restricted stem cell was discovered by Littlefield (10) and is recognized by the monoclonal antibody AC2.

cells (as a result of extensive male-to-female stem cell switching). Finally, sex reversal of male polyps can occur by the process of bud formation in cases in which polyps contain only a few male stem cells. Since stem cells grow as clones and therefore are nonuniformly distributed in polyps (unpublished observations), budding occasionally gives rise to polyps lacking male stem cells. Such polyps are female.

Different species of *Hydra* exhibit various degrees of sex reversal and hermaphroditism (Table 2). These differences can be simply understood in terms of the model as changes in the rate at which stem cells switch their sexual phenotype.

In *H. magnipapillata* male stem cells switch to female stem cells at a frequency of 10^{-2} per cell per generation (Table 1). Female stem cells were never observed to switch to male. As a result of this difference in switching frequency, the proportion of male stem cells decreases during proliferation of a male population. Male polyps taken from a mass culture contained on average 30% male cells (4), although some individual polyps probably contained significantly fewer male stem cells. Due to the spatial localization of stem cell clones, such male polyps can, in rare cases, give rise to female polyps if buds containing only female cells are formed. The rare observation of female polyps in mass cultures of male strains of *H. magnipapillata* (unpublished observation; T. Sugiyama, personal communication) is probably the result of such an event. The observed stability of female polyps in

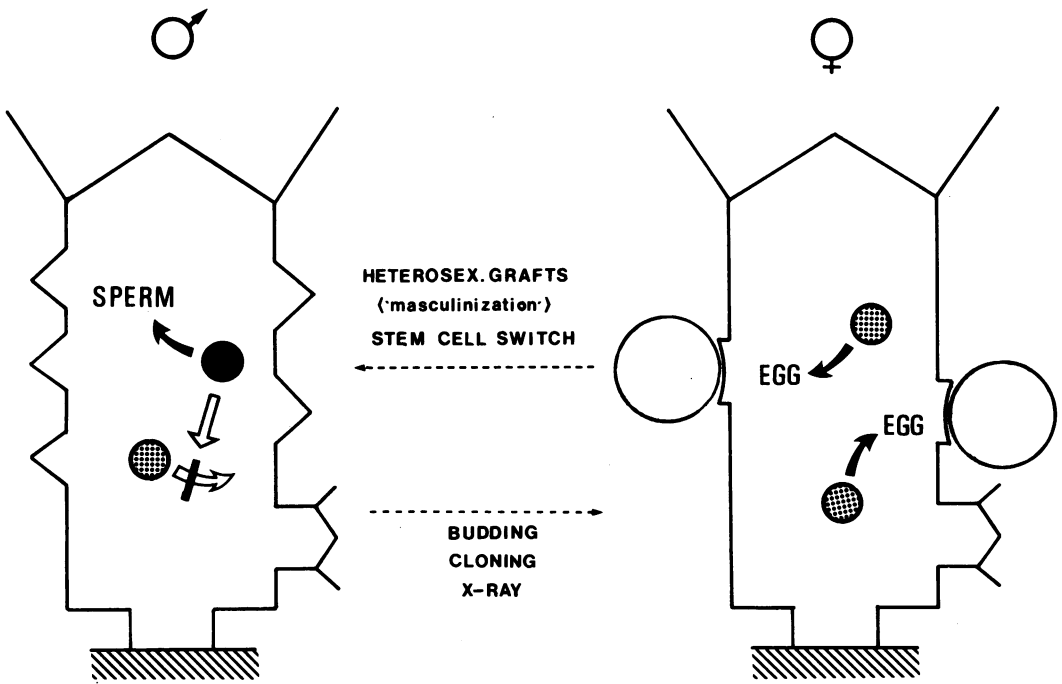

FIG. 2. Stem cells, sexual phenotype, and sex reversal in hydra. Male polyps contain male (●) and female (⊗) stem cells; female polyps contain only female (⊗) stem cells. Curved arrows indicate gamete differentiation. The straight open arrow indicates repression of female differentiation by male stem cells.

Table 2. Sex determination in *Hydra*

Hydra species	Stem cell sex		Masculinization in heterosexual grafts	Sex reversal in polyps		Sex switch in stem cells, rate*	
	In ♀ polyps	In ♂ polyps		♂ → ♀	♀ → ♂	♂ → ♀	♀ → ♂
oligactis	[♀]	Mostly ♂†	+†	–	–	[Low]	ND
magnipapillata	♀‡	70% ♀, 30% ♂‡	+§	Rare¶	–	$\approx 10^{-2}$‖	–‡
attenuata	[♀]	Mostly ♀ (90% ♀, 10% ♂)**	+††	+‡‡	+‡‡	[$>10^{-2}$]§§	[$\approx 10^{-4}$]§§
circumcincta	(♂/♀)		Simultaneous hermaphrodites			ND	ND
viridis							

ND, not determined. [ ], Conclusions drawn indirectly from other results in table.
*Switch per cell per generation.
†Ref. 10.
‡Table 1.
§Ref. 4.
¶T. Sugiyama, personal communication; unpublished observation.
‖Calculation based on Table 1.
**Ref. 9.
††References in ref. 10.
‡‡Refs. 9 and 11.
§§Estimated from ref. 11.

this species is consistent with the lack of female-to-male switches in cloned stem cells (Table 1).

In species such as *H. attenuata*, female polyps are rather unstable and frequently undergo sex reversal to male polyps (9, 11). Since masculinization is observed in *H. attenuata* (Table 2), it seems likely that female polyps contain only female stem cells. A female-to-male switching frequency of about 10^{-4} per cell per generation can be estimated since female polyps contain about 10^3 stem cells, the stem cell cycle is 1–2 days (8), and sex reversal occurs within 1–2 months. Male polyps of *H. attenuata* also undergo frequent sex reversal (9, 11). Such polyps appear to contain primarily female stem cells and only a few male stem cells (about 10%) since x-ray inactivation results in sex reversal in almost all surviving polyps (9). Such a high level of female cells in male polyps suggests that male-to-female switching is more frequent than in *H. magnipapillata*. Consistent with this lower number of male stem cells is the observation that male polyps of *H. attenuata* undergo sex reversal more frequently than male polyps of *H. magnipapillata* (Table 2).

In species such as *H. oligactis*, only very rare cases of spontaneous sex reversal have been observed (12), indicating a very low stem cell switching frequency. Consistent with this interpretation is the observation that male *H. oligactis* polyps exhibit a high proportion of male stem cells, in striking contrast to male polyps of *H. magnipapillata* and *H. attenuata*, which contain a majority of female stem cells.

Hermaphroditic species such as *H. viridis* and *H. circumcincta* are most easily understood as polyps in which male and female stem cells are present and in which the factor causing female suppression is absent. Thus, both kinds of gametes can differentiate simultaneously.

Recent work by Littlefield (10) has provided evidence for the existence of a stem cell capable of extensive self-renewal, restricted to spermatogenic differentiation and identifiable by a monoclonal antibody ("AC2"). Such cells appear to arise from multipotent stem cell precursors capable of somatic and sexual differentiation (10) and to be early intermediates in the spermatogenic pathway (Fig. 1). In addition, Littlefield (13) has shown that $AC2^+$ cells produce the factor that represses differentiation of female gametes in male polyps.

In our cloning experiments no stem cells restricted to spermatogenesis were observed (4), suggesting that they are a rare cell type. Nevertheless, such cells appear to exist in *H. magnipapillata* since a monoclonal antibody that stains AC2-like cells in asexual polyps has recently been found (T. Schmidt, personal communication). Whether only these intermediates or, in addition, the multipotent male stem cells cause inhibition of female differentiation cannot be determined in our experiments (Fig. 1).

It is tempting to extend the model to sex determination during embryogenesis. Under these conditions one might expect germ cells in the embryo to be male; female germ cells would be generated by the switching mechanism during ontogenesis. Nevertheless, this does not appear to be likely since primary polyps of hydra are either male or female (12). Such an observation is most consistent with a chromosomal mechanism of sex determination. Thus, the sex switching mechanism that we observed here would appear to be an alternative mechanism that permits the generation of both sexes during prolonged asexual growth. Such a mechanism might well be selected for in organisms that grow extensively by asexual ramet formation.

With respect to sex determination, *Hydra* appears to occupy an intermediate position between single-cell eukaryotes and higher metazoans. In higher metazoans, sexual phenotype appears to be controlled primarily by the somatic tissue; germ cells, even when chromosomally programed for male or female differentiation, differentiate according to the sex of the gonads. By comparison, in the primitive metazoan *Hydra*, sex is determined by the cell lineage, which gives rise to the gametes. At present there is no evidence for somatic influence on sex determination. In this sense *Hydra* appears to be more closely related to single-cell eukaryotes such as yeast, in which sex is controlled by the cell itself. Thus, it was particularly interesting to observe in *Hydra* switching of the sexual phenotype of stem cells analogous to the mating-type switching observed in yeast.

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