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Transplantation analysis of developmental mechanisms in *Hydra*

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ABSTRACT Since the pioneering work of Ethel Browne (1909) who demonstrated for the first time the concept of organizer activity, i.e. the potency of an apical *Hydra* tissue to induce a secondary axis when transplanted onto a host, *Hydra* flourished as a fruitful model system for developmental studies. Over the next 60 years this efficient transplantation approach identified graded biological activities along the body column of *Hydra* named Head Activation and Head Inhibition. These properties inspired theoretical modelers including Lewis Wolpert, Alfred Gierer and Hans Meinhardt to propose models for morphogenesis, respectively the positional information (1969) and reaction-diffusion (1972) models. In 1973, Tsutomu Sugiyama and Toshitaka Fujisawa initiated in Mishima a unique project to analyze the properties of *Hydra* strains with distinct morphological and developmental characters. To this end, they collected in several areas of Japan multiple *Hydra* strains that they subsequently characterized and crossed. They also established a lateral transplantation strategy that was much more powerful than the previous ones, as it combined quantitative measurements with cellular analyses thanks to the chimera procedures developed by Campbell and colleagues. Indeed this approach provided a paradigm to quantify in any morphological phenotype the Head Activation and Head Inhibition levels along the body column. In this article, I review the various strains identified by Sugiyama and colleagues, the principles and the main results deduced from the quantitative lateral transplantation strategy. In addition, I briefly discuss the relevance of this approach in the era of molecular biology.

KEY WORDS: *Hydra, regeneration, transplantation, inhibition, positional information, gradient*

Introduction

Today genetic analyses are popular in many model organisms, however in the 1970s when Sugiyama and Fujisawa initiated a project to investigate the basic mechanism of pattern formation in *Hydra* at National Institute of Genetics at Mishima, genetic analyses were restricted to a limited number of organisms. *Hydra* is a freshwater cnidarian polyp with a simple anatomy that is shown and described in details in this issue in several places (e.g. Boettger and Hassel, 2012; Bossert and Galliot, 2012; Martinez and Bridge, 2012). As a specific trait, adult *Hydra* undergoes both sexual and asexual reproduction (budding). When well fed, it proliferates primarily by budding, as observed both in the lab and in the wild, whereas sexual reproduction is a relatively infrequent event (see in this issue the reviews by Boettger and Hassel, and by Nishimiya-Fujisawa). It is assumed that over a number of generations asexual reproduction could lead to the accumulation of mutations in the germ cells, which would yield phenotypes in the progeny resulting from sexual reproduction. If true, the hatching and survival rates of fertilized eggs might be relatively low due to a significant load of deleterious recessive mutations.

When Sugiyama and Fujisawa initiated genetic studies, several authors had already described the complete life cycle of *Hydra*, showing that it extends over several months as it takes weeks to months for fertilized eggs to hatch (McConnell, 1938; Moore and Campbell, 1973). In addition, Moore and Campbell (1973) found that the hatching rate and the survival rate of hatchlings were both very low. Altogether these data indicated that forward genetic screens following application of mutagens were not easily feasible in *Hydra*.

In place of forward genetic screening, Sugiyama and Fuji-

Abbreviations used in this paper: HA, head activation; HI, head inhibition; Hm, *Hydra magnipapillata*.

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TABLE 1

DIFFERENT STRAINS COLLECTED OR PRODUCED BY S. FUJISAWA (1979) WITH SOME MODIFICATIONS

***Hydra magnipapillata (Hm)* strains**	**Anatomy & Physiology**	**Developmental characters**	**Cellular characters**	**Origin**	**Reference**
Hm-104 (male)	normal			wild	(Sugiyama and Fujisawa, 1977a)
Hm-105 (male)				wild	(Sugiyama and Fujisawa, 1977a)
Hm-107 (female)				wild	(Sugiyama and Fujisawa, 1977a)
Hm-117 (female)				wild	(Sugiyama and Fujisawa, 1977a)
Hm L1				wild	(Sugiyama and Fujisawa, 1977a)
Hm L2 (male)				wild	
Hm L4 (female)	Large size	Low budding rate		wild	(Takano and Sugiyama, 1983)
Hm SSE (male)				wild	(Sugiyama and Sugimoto, 1985)
Hm SSC (female)				Wild	(Sugiyama and Sugimoto, 1985)
Hm mh-1	multiheaded			(F1)	(Sugiyama, 1982)
Hm reg-16		Head regeneration-deficient		(F2)	(Sugiyama and Fujisawa, 1977b; Achermann and Sugiyama, 1985)
Hm reg-19		Head regeneration-deficient		(F2)	(Sugiyama and Fujisawa, 1977a)
Hm nem-3			nematocyst (holotrichous isorhiza) deficient	(F1)	(Sugiyama and Fujisawa, 1977a)
Hm nem-4			nematocyst (stenotele) deficient	(F2)	(Sugiyama and Fujisawa, 1978b)
Hm maxi-1	Large size			(F1)	(Sugiyama and Fujisawa, 1977a)
Hm mini-4	Small size			(F1)	(Sugiyama and Fujisawa, 1977a)
Hm sf-1	Heat-shock sensitive		produces interstitial cell-deficient strain upon heat-shock (nf-1)	(F5)	(Sugiyama and Fujisawa, 1978a)
Hm sf-21			produces interstitial cell-deficient strain (nf-21)	(F1)	(Sugiyama and Fujisawa, 1979)
Hm nf-17	Food ingestion deficient			(F1)	(Sugiyama and Fujisawa, 1978a)

The main characteristics of each strain are indicated, for details refer to the original description. SexCr: sexual-cross.

sawa collected *Hydra* from various locations in Japan and let them undergo sexual reproduction in the laboratory to produce F1 and F2 progeny, with the expectation that the F2 generation would reveal recessive mutations present in the parental lines. Such offspring were termed "mutants" although the presence of a genetic mutation had not been rigorously demonstrated in any of them. From the strains obtained at the F1 to F6 generations, plus some chimeric strains constructed from some of these strains, they carried out systematic lateral transplantation analyses as initially described by Browne (1909) and later by Yao (1945), Webster and Wolpert (1966). In this article, I will mainly review the information that was obtained by Sugiyama and colleagues from these studies while referring to methodological contribution by Campbell and colleagues.

Strategy for analyzing head-forming mechanisms in Hydra

Production of a variety of Hydra *strains through inbreeding*

Once collected from the field the animals were maintained in the laboratory as described by Loomis and Lenhoff (1956), i.e. fed with brine shrimp nauplii and maintained at 18°C in fresh hydra medium modified according to Sugiyama and Fujisawa (1977b). Formation of eggs and testis was induced by altering the culture conditions, which depending on the strains might consist of aerating the culture medium to make it acidic, feeding animals poorly, shifting the temperature either to a higher or lower range, etc… *Hydra* strains are generally dioecious, i.e. either male or female, only occasionally hermaphrodites, the same polyp carrying both eggs and testis. To obtain fertilized eggs, male and female polyps of two different strains were kept in the same beaker for a week or two. As mentioned above, Sugiyama and Fujisawa conventionally called those strains obtained by cross-breeding "mutants", which, however, does not imply genetic mutation. In this review to avoid any confusion, we will simply call them here by name of the strains. A wild type strain of *Hydra magnipapillata (Hm)* that was collected in a pond in the neighborhood of National Institute of Genetics in Mishima, named strain 105 was used as the standard strain. The anatomical, developmental and cellular characters of these strains are listed in Table 1 as initially reported by Sugiyama and Fujisawa in 1979.

The lateral transplantation procedure

To take advantage of the different characters that were recorded on these strains, e.g. polyp size, polyp shape, head regenerative capacity etc., Sugiyama established experimental strategies aiming at measuring the morphogenetic properties of each of them. For this purpose they applied the lateral transplantation procedure that had led Lewis Wolpert several years earlier to propose the positional information theory (Wolpert, 1969; Wolpert *et al*., 1971; Wolpert *et al*., 1974). A critical issue in the regeneration phenomenon is how the cells at the regenerating site know that they are in charge of forming the missing organ. The positional information theory provided a very simple mechanism for it by setting up gradients of morphogenetic substances (morphogens) that are maintained by diffusion from the source at one end to the sink at the other end. According to this theory, cells obtain information about their position by the level of the morphogens to which they are exposed. As an example, the strain with a large polyp size, is expected to have a slower diffusion of morphogens and as a result a longer diffusion distance. This longer diffusion distance naturally stretches the distance between the source and the sink, which is reflected by the larger size of the animal.

Regeneration of any missing part occurs with high fidelity in *Hydra*. If one removes both head and foot from a polyp leaving cylindrical body column tissue, head regenerates at the apical end and foot regenerates at the basal end. This is also true for small pieces of tissue that are used in the lateral transplanta-

tion experiments (Shimizu *et al.*, 1993). When a piece of tissue excised from the donor polyp is immediately inserted into a slit made on the body of an intact host polyp, occasionally head-like structures consisting of one or more tentacles with or without hypostome is formed on the transplanted tissue within several days (Browne, 1909; Yao, 1945; Webster and Wolpert, 1966). Whether this ectopic head structure forms or not depends firstly on the original position of the donor tissue and secondly on the position where the donor tissue is inserted on the host polyp.

Sugiyama and colleagues carried out lateral transplantation experiments following the basic principles established by their predecessors (Fig. 1; see also Browne, 1909; Yao, 1945; Webster and Wolpert, 1966) but in a more sophisticated manner. First, they used animals cropped from a rigorously controlled mass culture as newly dropped polyps, fed daily for 3-4 days, and having the first bud protrusion when used (Takano and Sugiyama, 1983). Second, they labeled the donor tissue with Evans Blue, which made it easy to check whether the donor tissue stays at the site and the transplantation was successful. This was important because it occurred in some cases that the transplanted donor tissue was either sloughed off or swallowed into the gut through the slit resulting in the failure of head formation. Such failure may accidentally be recorded as no head formation although the transplantation was successful. Third, they strictly monitored the positional origin of the donor tissue and the position of tissue insertion on the host (Takano and Sugiyama, 1984).

Defining the head-forming properties in Hydra

Assessment of the head activation (HA) levels upon lateral transplantation

The concepts of head activation (HA) and head inhibition (HI) emerged from a series of transplantation experiments that were performed on hydrozoan polyps in multiple laboratories all around the world during the twentieth century (Mutz, 1930; Child, 1932; Barth, 1940; Yao, 1945; Burnett, 1961; Tardent, 1963; Webster and Wolpert, 1966; Wilby and Webster, 1970). To make the issue easier to understand and more reader friendly, a unique series of experiments is described in a simplified manner in this article. In the first series of experiments Takano and Sugiyama restricted the position of insertion to the lower body column region termed position-4, i.e. below the budding zone while the origin of the donor tissue varied from position-0 to position-4 (Fig. 1A).

The following results were observed: When the donor tissue was excised from position-4 and transplanted to the same position in the host, the tissue was absorbed into the host and neither apical (tentacles) nor basal (foot) structures formed at the transplantation site. When the donor tissue was dissected from a more apical region in the donor (position-3 to position-0), then ectopic head formation was observed with an occurrence rate that was maximal when tissue was taken from position-0 (100%) and progressively decreasing when tissues were taken from more basal positions (i.e. 91% from position-1, 47% from position-2, 29% from position-3, see Fig. 1A). These results demonstrated that the capacity of tissues from the body column to form ectopic heads after transplantation distributes as a gradient along the body axis with the highest value in the upper (apical) body column and the lowest, i.e. null value, in the basal region. This capacity of tissues to form ectopic heads was named head activation (Tanako and Sugiyama, 1983; MacWilliams, 1983b).

Assessment of the head inhibition (HI) levels upon lateral transplantation

With a slight modification of the transplantation procedure, the same authors could detect and quantify another tissue property. This time, they fixed the origin of the donor tissue to position-1 (a position where the donor tissue has a high head activation level) and they varied the position of transplantation onto the host from position-1 to position-4 (Fig. 1B). They recorded the following results: when the donor tissue was transplanted to the same position in the host, i.e. position-1 no head formation occurred. But when the site of transplantation was lower (i.e. more basal), then the occurrence rate of head formation was higher (Fig. 1B). This demonstrates that the capacity of head formation by the donor tissue can be affected by the contact with the host tissue, implying that the host tissue has the capacity to inhibit ectopic head formation induced by the transplanted donor tissue. This capacity of the host tissue to inhibit head formation was named

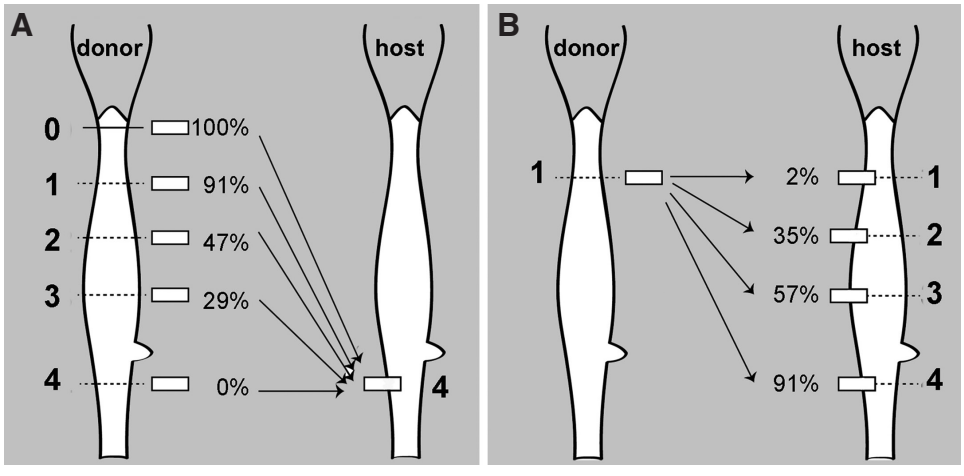

Fig. 1. Procedures to assess head activation (A) and head inhibition (B) levels upon lateral transplantation. *The body column between the lower end of the tentacle ring and the first bud protrusion was optically divided into four equal lengths and the three positions thus obtained are numbered from position-1 to position-3. The body column from the lower boundary of the bud protrusion to the basal disc was optically divided into three equal lengths and the upper (apical) third was numbered position-4.* **(A)** *The donor tissue was excised as a donut ring form from position-0 to position-3 of a donor polyp, cut into a half and one of the two pieces was inserted into a slit made at position-4 in the host polyp. The polyps were observed for 5-7 days to record tentacle formation. The percentages shown beside the donor tissues indicate the proportion of grafts that led to the formation of one or more ectopic tentacles (data from Takano and Sugiyama, 1983).* **(B)** *The donor tissue was excised from position-1 and inserted into the slit made at positions 1, 2, 3 or 4 in the host polyp. The percentages beside the donor tissues indicate the proportion of host animals where ectopic tentacle formation was observed (data taken from Takano and Sugiyama, 1983).*

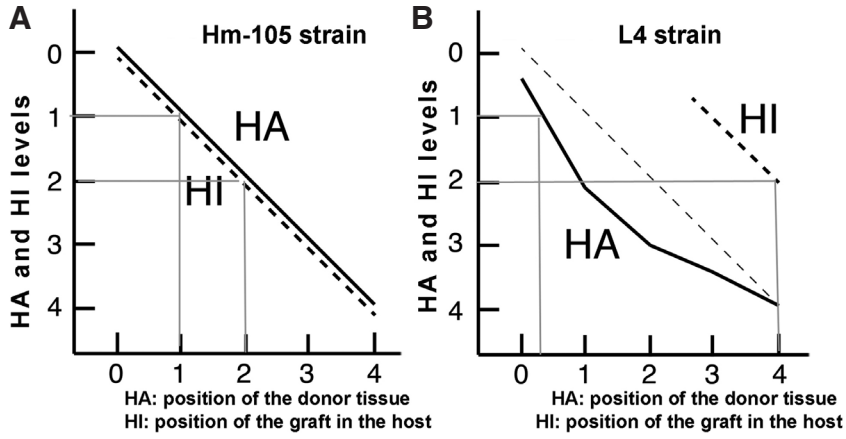

Fig. 2. Graded distribution of head activation (solid line) and head inhibition (dotted line) levels along the body column of the Hm-105 (A) and L4 (B) strains. *Head activation (HA) and head inhibition (HI) levels are deduced from the percentages of grafted animals forming ectopic heads at the site of grafting and thus correspond to inverted values: in Hm-105* **(A)***, the tissues of the upper body column exhibit the highest HA (high percentage of ectopic heads with donor tissue from position-0 or position-1), but also the highest HI with low percentage of ectopic heads when tissues are grafted at position-0 or position-1 (see Fig. 1). HA and HI are represented here on the y-axis by arbitrary units that can be used for comparisons between strains as indicated by the light grey lines: a HA level as high as 1 is obtained with donor tissues from a similar position in the Hm-105 and from an upper position in L4 strain* **(B)***. Meanwhile, a HI level of 2 is obtained when the graft is inserted at position 2 in Hm-105 or at position 4 in the L4 strain, showing that HI extends over a longer range along the body column of polyps of the L4 strain.*

head inhibition, and again its activity distributes as a gradient along the body axis, maximal in the upper (apical) body column and progressively getting lower towards the most basal region (Tanako and Sugiyama, 1983; MacWilliams, 1983a).

In summary, head formation induced by lateral transplantation is governed by two tissue capacities that form two parallel gradients extending from the head to the foot (Fig. 2A): The first one, named head activation (HA), is the capacity of the donor tissue to form head when transplanted onto the donor and the second one, named head inhibition (HI) is the capacity of the host tissue to inhibit head formation (Sugiyama, 1982; Takano and Sugiyama, 1983). It should be mentioned here that the concept of inhibition in cnidarian polyps was not discovered during 1960s-1970s, but had been repeatedly proposed over the past 30 years (Barth, 1940; Rose and Rose, 1941; Penzlin, 1957; Tardent, 1955; Tardent and Eymann, 1958; Trumdell, 1958; Tardent, 1963; Lenique and Lundblad, 1966; Rose, 1966, 1967; Rose and Powers, 1966). Interestingly several authors had already indicated that this inhibition activity distributes as a gradient along the body axis of the cnidarian polyps (Hyman, 1928; Goetsch, 1929; Burt, 1934; Child, 1941; Rose, 1966).

In complementary transplantation experiments the capacity of transplants to form ectopic foot was investigated, showing that foot formation is also governed by two tissue capacities, named foot activation and foot inhibition, both of them forming parallel gradients along the body axis of *Hydra* but now maximal at the basal end (foot) and progressively decreasing towards the apical end (head) (Achermann and Sugiyama, 1985). However, foot formation and foot regeneration were investigated far less extensively than head formation, therefore this review is focused on head formation.

Head activation (HA) and head inhibition (HI) exhibit different stabilities

Although graded in a similar manner along the axis of the body column, the two properties significantly differ by their stability. When the head of an animal is amputated at position-1 and the left part is allowed to regenerate, it takes about two days before the tentacle bumps become visible as a sign of head regeneration. The modulations in HA and HI activities during these two days were also examined in transplantation experiments (Fig. 3A). When the donor tissue was excised from the amputation site of the polyp 6 hours after bisection and transplanted to the host, the percentage of head formation was higher than when the transplantation takes place immediately after amputation. This result indicates that the HA level rose at the head-regenerating tip during the first 6 hours of regeneration (Fig. 3A), far before the morphological signs of head regeneration can be detected (Takano and Sugiyama, 1984). In contrast, the change of HI level after amputation was significantly quicker than the change of the HA level.

To estimate the HI level of the amputated host, the percentage of head formation induced by the transplant was measured on the amputated polyp rather than on the intact polyp (Fig. 3A). The percentage of head formation by the transplant taken from position-1 of an intact polyp and transferred to position-2 of an amputated polyp was 96%, a percentage significantly higher than the percentage of 30-35% recorded when the same transplant was grafted into an intact polyp. This observation strongly suggested that the HI level immediately dropped after amputation. Indeed complementary experiments showed that the HI level continues to decrease until 36hr and starts to increase from 48hr and later.

The drop of HI level occurs instantaneously, followed by the increase in HA level, suggesting that the two activities are mutually related. This possibility had already been supported experimentally by MacWilliams (1983a,b) who showed that when the increase in HA level is blocked by repeatedly removing the tissue at the regenerating tip, the recovery of HI level is partial suggesting that HA and HI levels regulate each other, their modulations reflecting a cross-talk between each of these two activities.

Analysis of the HA and HI levels in the L4 strain

To elucidate the mechanisms underlying the morphological properties observed in the different strains, Sugiyama and colleagues performed extensive lateral tissue transplantation experiments on the mh-1, L4, reg-16 strains as they exhbit interesting morphological characters (Sugiyama, 1982; Takano and Sugiyama, 1983; Achermann and Sugiyama, 1985). This procedure was possible because of the subtle tissue incompatibility between the strains collected in Japan. Although the strain mh-1 was studied first (Sugiyama, 1982), the most thorough and extensive analysis was carried out on the strain L4 (Takano and Sugiyama, 1984; Takano and Sugiyama, 1985). In this article, the results obtained in L4 are first described and some additional information is then referred.

Strain L4 was collected in the lake of Hachirogata (Akita pre-

fecture, northern Japan) as a wild-type strain. However, because of its larger polyp size and lower budding rate (when compared to strain 105) L4 was conventionally recognized as a "naturally occurring mutant". The question was then whether these morphological and developmental features were related to the HA and HI levels. A possible scenario was that a higher level of HI extending over a longer range might account for both characters, i.e. the larger polyp size and the lower budding rate. As to budding, it was known that the head indeed exerts an inhibitory effect on bud formation in its vicinity (Shostak, 1974; Cohen and MacWilliams, 1975). Therefore a high and long-range head inhibition would suffice to inhibit budding over a long distance, making the budding region of the animal more distant from the head, whereas even higher levels of head inhibition would definitely suppress the budding process.

The transplantation experiments showed that in L4 the HA level was slightly lower than in Hm-105 whereas the HI level was significantly higher, both values supporting the scenario predicted by the authors (Fig. 2B). Concerning the stability of the HA and HI levels after head amputation, the modulations were basically the same in Hm-105 and L4 with a rise in the HA level and a drop in the HI level, however a notable difference was the persistance of these changes over several days in L4 and not in Hm-105 (Fig. 3B).

A similar correlation between the HI level and the polyp size was obtained by Rubin and Bode (1982a) working on a population named the Aberrant. This population, which appeared in the mass culture of *H. vulgaris,* showed a significantly smaller polyp size than the standard culture. Lateral transplantation experiments performed indeed showed a lower HI level in the Aberrant than in the normal sized polyps, suggesting a causal link. Thus the results obtained in the L4 and Aberrant strains were consistent with the view that HI level is a key factor in the determination of the *Hydra* polyp size.

Strategies to map the cell lineages supporting HA and HI activities

Hydra consists of three cell lineages, ectodermal epithelial cell lineage, endodermal epithelial cell lineage, and interstitial cell (i-cell) lineage (see in this issue David, 2012; Hobmayer *et al.*, 2012). An interesting question to ask was which of these three cell lineages is responsible for the HA and HI levels. For this analysis, the techniques developed by Marcum and Campbell (1978) and Wanek and Campbell (1982) were of tremendous importance.

Construction of Ecto/Endo chimera

The epithelial cells of *Hydra* constantly proliferate in the body column, however the cell number is maintained steady in homeostatic conditions. In fact cells produced in excess are gradually sloughed off from both extremities (Campbell, 1967) and when polyps undergo budding, a significant amount of cells from the parental polyp are recruited into the growing buds. This loss of cells from the polyp is made possible by the displacement of the epithelial cells along the body column (Campbell, 1967). As a result, epithelial cells do not stay at the same position in the body column but change their position constantly. Shostak *et al.,* (1965) carried out grafting between two polyps that belong to different strains, one of them being vitally labeled in both epithelium in order to visualize the movements of the grafted tissues. Interestingly they noticed that the boundaries of the labeled ectoderm and the boundaries of the labeled endoderm did not stay adjacent to each other but with time became more distant from each other, demonstrating that the speed of the movement of the epithelial cells is not the same in the two layers.

Wanek and Campbell (1982) cleverly used this phenomenon to construct ecto/endo chimera strains. When the ectodermal and endodermal boundaries became significantly distant from each other, they excised a donut ring of tissue as such as the excised tissue consisted of the ectoderm from one strain while the endoderm was from the other strain. By allowing the tissue to regenerate, grow and proliferate, they had constructed an ecto/endo chimera strain formed of epithelial layers from different origins (Wanek and Campbell, 1982; Wanek, 1983).

Construction of nerve-free Hydra *and i-cell lineage chimera*

Epithelial cells and interstitial stem cells of *Hydra* have different cell cycle lengths, 40 to 85 hours for the epithelial cells versus 17 to 24 hours for the interstitial stem cells (David and Campbell, 1972; Campbell and David, 1974). Campbell (1976) used colchicine, a drug that inhibits microtubule polymerization to selectively induce cell death of the fast cycling cells (see in this issue Reiter *et al.*, 2012). Indeed a pulse treatment of several hours leads to the elimination of the interstitial stem cells while leaving the epithelial cells intact; after several days the progenies of the interstitial stem cells (nerve cells, nematocytes, gametes) are irreversibly eliminated. The polyps produced in this manner were termed "nerve-free *Hydra*" or "epithelial *Hydra*". By grafting

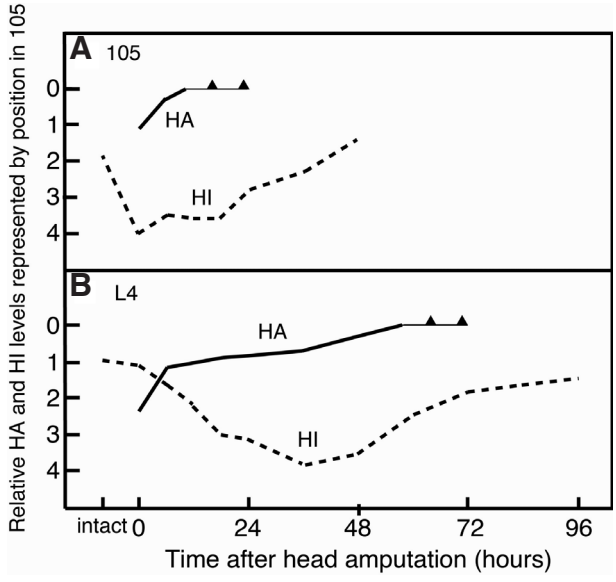

Fig. 3. Changes of head activation (solid line) and head inhibition (dotted line) levels after head removal at position-1 in the Hm-105 (A) and L4 (B) strains. *Head activation (HA) and head inhibition (HI) levels are represented with arbitrary units as specified in Fig. 2. Modified from Takano and Sugiyama (1984). Change of HA level was measured by excising donor tissues from the regenerating tip of the amputated polyps of Hm-105* **(A)** *and L4* **(B)** *strains and grafting them into position-2 of an intact Hm-105 polyp as the host. Change of HI level was measured by excising donor tissues from position-1 of intact polyps of Hm-105 and by grafting them into position-2 of the amputated polyps of Hm-105 and L4 strains as the host. Arrowheads in HA and HI levels show that the level at those time points is higher than the level at position-0 hence unable to evaluate.*

an intact polyp of other strains to the nerve-free polyp, Marcum and Campbell (1978) succeeded in introducing interstitial cells of a different strain into the nerve-free *Hydra* thereby substituting the original interstitial cell lineage and constructing i-cell lineage chimera. Finally by combining the two methods, it became possible to construct Ecto/Endo/Int chimera.

Chimeric analysis in the L4 strain

Takano and Sugiyama used the two methods mentioned above to construct Ecto/Endo/Int chimera of the Hm-105 and L4 strains. To examine the specific effect of each cell lineage on the tissue properties and more specifically on the HA and HI levels, seven chimeric strains were constructed, namely 105/105/L4, 105/L4/105, 105/L4/L4, L4/105/105, L4/105/L4, L4/L4/105, and 105/105/105 (used as control instead of intact Hm-105). To measure the HA levels of each type of chimera, tissue from the chimera were transplanted onto the Hm-105, whereas donor tissue from Hm-105 was grafted on chimeras to assess the HI levels (Fig. 4). In parallel the polyp size of each chimera strain was also measured to identify the cell lineage(s) responsible for the determination of this character. The results of these analyses indicated four types of conclusions: (1) in chimeras the HA level is close to that measured in the strain that provides the ectoderm, (2) in chimeras the HI level is close to that measured in the strain that provides the endoderm, (3) in chimeras containing the L4 i-cell lineage the HI level is slightly higher than in chimeras containing the Hm-105 i-cell lineage, (4) the polyp size of the chimera is close to the size of the strain that provides the endoderm.

These results suggested that (1) the ectodermal epithelial cell lineage is responsible for the HA level, (2) the endodermal epithelial lineage is responsible for the HI level and the polyp size, (3) the role of the i-cell lineage seems to be restricted to a moderate modulation of the HI level. Wanek and Campbell (1982) reached similar conclusions, however, they made another quite interesting observation. They constructed ecto/endo chimera

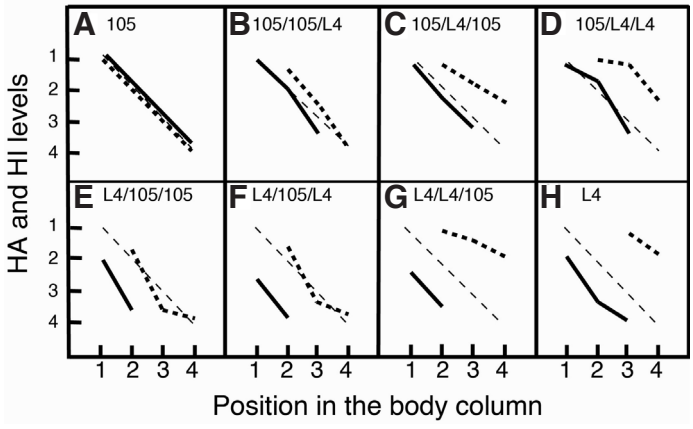

Fig. 4. Distribution of head activation (black line) and head inhibition (dotted line) levels in the chimeric strains constructed from strains Hm-105 and L4. *On the x-axis, positions in the body column are defined as in Fig. 1. They indicate the position of the donor tissue for head activation (HA) level and the position in the host for head inhibition (HI) level. On the y-axis HA and HI levels are represented with arbitrary units as specified in Figure 2. The code is as follows: 105/105/L4 means ectoderm from Hm-105 / endoderm from Hm-105 / and i-cells from L4. Modified from Takano and Sugiyama (1985).*

from two strains of *H. vulgaris* that exhibit a significantly different morphology of their body column. In one strain the body column was thin and tall whereas in the other strain the column was short but fat. The shape of the chimera resembled the shape of the strain that provides the ectoderm supporting the view that the ectoderm has the dominant role in shaping the body column.

However if the conclusion that the ectoderm plays the dominant role in shaping the body column was well accepted, the mechanism involved in this regulation remained unknown. In 1995 Shimizu *et al.,* examined the orientation of the epithelial cells of the two layers at the time they divide and found that in the upper body column cells were rather dividing following a longitudinal orientation, whereas in the lower body column, which is fat, most cells were dividing following a circumferential rather than longitudinal orientation. Hence these observations showed that the pattern of epithelial cell division is region-dependent, fitting with the development of polyp shape, and suggesting that the orientation of cell division plays a role in maintaining the body column shape.

However, the question remained as to what mechanism regulates the pattern of cell division. Takaku *et al.,* (2011) recently provided a clue to solve this problem. They found that in the elongated region of the body column where epithelial cells more likely divide along a longitudinal orientation, the microtubules in the ectodermal epithelial cells are aligned at the base of the cells following a longitudinal orientation. In contrast, this alignment was not observed in the endodermal layer. If this organization of the microtubules in the ectoderm influences the orientation of cell division by for instance, elongating the cell shape, both patterns, i.e. cell division and development of body shape, would be explained by the dynamics of the microtubules.

Another pending question was how the ectoderm and the endoderm can interact with each other while being separated by mesoglea, the extracellular matrix of *Hydra*. It was found that the ectodermal epithelial cells and the endodermal epithelial cells have direct contact with each other thanks to numerous protrusions that penetrate the mesoglea (Murate *et al*., 1997; Shimizu *et al*., 2008) thus enabling direct interactions between the two epithelial layers.

Transplantation analyses in the head regeneration deficient reg-16 strain

The reg-16 strain was originally obtained as a F2 progeny by crossing F1 polyps constructed by crossing wild type *Hydra magnipapillata* strains collected in Ugowada (Akita prefecture) that exhibit slightly lower head regenerative capacity (Sugiyama and Fujisawa, 1977b). The reg-16 strain displays a budding rate that is normal (i.e. close to that observed in Hm-105) but a significantly lower head regenerative capacity. Achermann and Sugiyama (1985) examined the HA and HI levels in reg-16 and found as striking features a lower HA level together with a higher HI level when compared to those measured in Hm-105. This HA/HI pattern in homeostatic conditions is similar to that observed in L4. However during regeneration they recorded in the reg-16 strain immediate modulations of HA and HI that are quite different from those recorded in the Hm-105 and the L4 strains: Firstly the HI level did not drop after amputation, but rather remained high during 12 hours after amputation to subsequently progressively decrease; second, the rise in HA level was not prominent. These results suggested a causal link between the abnormal HA and

HI properties and the reduced head regenerative capacity of the reg-16 polyps.

To examine the relationships between the cell lineages and the HA and HI levels in the reg-16 strain, Ecto/Endo/Int chimeric strains were constructed and their properties were examined by lateral tissue transplantation. Previous analyses performed on Hm-105/L4 chimeras had suggested that the ectoderm controls the HA level whereas the endoderm and the i-cell lineages would control the HI level (Takano and Sugiyama, 1983, 1984). The results obtained in the 105/reg-16 chimeras actually differed significantly from this scenario: the HA level was altered not only by the replacement of the ectoderm but also by the replacement of the endoderm, suggesting that both the ectoderm and the endoderm regulate the HA level in this strain. As to the HI level, the results suggested a predominant role for the endoderm in its regulation. In addition, unlike the 105/L4 chimeras, the replacement of the i-cell lineage from Hm-105 to reg-16 origin elevated the HI level suggesting that the i-cell lineage is also involved in regulating HI level. Interestingly Rubin and Bode (1982b) made similar observations on *Hydra vulgaris* strains.

The relationship between the cell lineage and the low head regenerative capacity of this strain was also examined. Replacement of the ectodermal epithelial cell lineage with Hm-105 (105/reg-16/reg-16) elevated the percentage to a similar level in 105 (Wanek *et al*., 1986) whereas replacement of the endodermal epithelial cell lineage with Hm-105 (reg-16/105/reg-16) did not elevate the percentage of regeneration. This demonstrates that the Hm-105 ectodermal epithelial cell lineage suffices to rescue the efficiency of the regeneration process. However the number of tentacles formed was larger in the reg-16/105/reg-16 chimera than in the reg-16 strain (Nishimiya *et al*., 1986). These observations suggested that beside the regulation of the efficiency of the regeneration process by the ectoderm, the endoderm affects the patterning of the new head, i.e. the number of tentacles to be formed on the regenerated structure.

Involvement of injury in the regeneration of reg-16 and Hm-105

One of the drawbacks of the lateral transplantation procedure is the injury effect, i.e. the fact that lateral incisions per se activate head formation in the tissue (MacWilliams, 1982; 1983a,b). MacWilliams who found that the HA level of the transplant is elevated by the incision, proposed that this “injury effect” also works during head regeneration. Indeed this hypothesis is supported by several experimental evidences. In the reg-16 strain Kobatake and Sugiyama (1989) found that reopening the wound once healed, i.e. 24-36 hours after head amputation, elevates both the HA level and the percentage of head regeneration. This time window actually coincides with the time required for the HI level to drop in this strain. It is therefore possible to postulate that wound opening at the time the HI level drops, induces a rise in HA level. In Hm-105 independent reports relying on a different experimental approach showed the positive effect of wound opening on head formation and regeneration (Shimizu *et al*., 1987; Shimizu *et al*., 1993).

Hence as initially proposed by MacWilliams (1982, 1983a,b) the wound appears to elevate the HA level whatever the *Hydra* strain, and two types of mechanisms can be postulated. The first one would be direct, i.e. a direct increase of the HA level upon injury but the molecular mechanism remains unclear. The second possibility is that the leakage of the inhibitory substance(s) from the wound would trigger a rise in the HA level. To discriminate between these possibilities, head regeneration was performed in two distinct contexts. In one case normal head regeneration was performed in amputated polyps whereas in the other case regeneration was performed in mirror-image grafts, as such as the wound opening of two amputated polyps are immediately covered by grafting two halves to each other thanks to a nylon fishline to thread the tissues together. During the course of regeneration, the HI levels in the two types of regenerates did not differ significantly, whereas the HA level was higher in normal regenerates with open wound than in mirror-image grafts. This results shows that the level of HA can vary independently of the level of HI, suggesting that injury and open wounds do not play a major role in the decrease of the HI level but are directly involved in the increase in the HA level.

Additional questions addressed by transplantation studies

Role of nerve cells in* Hydra *morphogenesis

Amphibians efficiently regenerate their appendages, either as tadpoles when frogs or as adult organisms when salamanders (Byrnes, 1904). When the nervous system of the limb is eliminated, either surgically or pharmacologically, the remaining tissue loses its regenerative capacity showing the essential role played by the nervous system in blastema proliferation (Stocum, 2004). Recent characterization of signaling molecules released by the neurons in the vicinity of the wound provides a molecular mechanism for this nerve-dependence of limb regeneration in vertebrates (Kumar *et al*., 2007).

In *Hydra* it had also been thought that head regeneration and budding require the involvement of i-cells and/or neurons (Lenz and Barnnett, 1963; Bode *et al*., 1973; Moore and Campbell, 1973b). Indeed both theories that describe pattern formation in *Hydra*, the positional information theory (Wolpert, 1969) and the theory of biological pattern formation (Gierer and Meinhardt, 1972; Meinhardt and Gierer, 1974) are based on diffusion-induced instability (Turing, 1952). Therefore nerve cells were seen as ideal candidates to regulate positional information along the body axis and set up at the site of head regeneration an environment to provoke neuron differentiation. Indeed cellular studies have proven that nerve cells accumulate in the *Hydra* head, providing a much higher density in the apical region than in the body column (Bode *et al*., 1973). The role of the nerve cells in patterning and regeneration could be tested as the i-cell lineage can be completely eliminated either by treating *Hydra* with drugs as Colchicine, Nitrogen mustard, Hydroxyurea (Campbell. 1976; David, 1983; Sacks and Davis, 1979) or by exposing the sf-1 strain to transient heat shock (Marcum *et al*., 1980). As explained above, both types of strategies produce “nerve-free” animals, i.e. *Hydra* that have lost their nervous system. Surprisingly the analysis of such nerve-free *Hydra* provided results that were entirely different from those previously predicted as Campbell showed that the nerve-free polyps retain the capacity to regenerate head and to form buds (Campbell, 1976). Moreover, Sugiyama and Wanek (1993) reported that in the head-regeneration deficient reg-16 strain, head regeneration can be rescued by the elimination of the interstitial cells (stem cells, nerve cells, nematocytes). From this

result they discuss the possibility that in homeostatic conditions, nerve cells rather play a suppressive role on the morphogenetic potential of the epithelial cells, this negative function would be enhanced in the reg-16 mutant. These observations clearly demonstrate that head regeneration can occur independently of nerve cells in *Hydra*.

What is the substance that is responsible for gradients along the body column?

The morphological gradients along the body column of hydra made researchers to expect that related morphogenetic substances (morphogens) would be identified rather soon. Unfortunately, however, the substances are still yet to be discovered.

Head activator (Schaller, 1973) was proposed as a neuropeptide that is closely related to HA level and extensive analysis was performed. However, the relevance of head activator is currently questionable in that the coding sequence for it is not found in the genomic sequence of hydra (Steele, 2012). Future analysis is supposed to provide definitive information.

Currently the most probable candidate for morphogenetic substance is wnt protein. Wnt-3a is expressed at the hypostomal end of a polyp and induces secondary axis formation when ectopic expression is induced (Hobmayer *et al*., 2000). The wnt protein is a secreted protein which is released from the hypostome to the extracellular space. Frizzled, the receptor of wnt protein, is localized in the basal surface of epithelial cells that faces the extracellular matrix. Therefore, a possible scenario is that secreted wnt protein diffuses (see Footnote below) in the extracellular matrix from the head to the foot region forming concentration gradient and binds to Frizzled receptor thereby affecting gene expression in a position dependent manner (Meinhardt, 2012). Such a graded distribution, however, has not been found yet.

In sum, it still remains to be elucidated what kind of molecules are involved in forming and maintaining gradients of HA and HI levels.

Self-organizing property of the Hydra *tissues from the body column*

Here we have reported about a body of coherent information resulting from transplantation of tissue from the body column. However it is important to stress that head formation in this context does not rely on head induction but rather results from the self-organizing property of the grafted tissue. Indeed labeling experiments have shown that after grafting tissues of any region of the body column, the tissue in the ectopic head contains exclusively cells from the donor and not from the host (Broun and Bode, 2002; see also in this issue (Bode, 2012)). This is in sharp contrast to transplantation experiments as initially performed by Ethel Browne, who used tissue from the hypostome, which acts as an organizer as the grafted cells recruit cells from the host to form a secondary body axis (Browne, 1909; Mutz, 1930). In fact, the tip of the hypostome can be compared to the Spemann's organizer that induces axis formation in the *Xenopus* embryo and both organizers actually express a *wnt-3a* orthologue (Liu *et al*., 1999; Hobmayer *et al*., 2000). Therefore, the head inductive mechanism in *Hydra* has common properties to notochord inductive mechanism in frog embryos. Unfortunately, the mechanism of construction and maintenance of the graded self-organizing property along the body column of *Hydra* is still very poorly understood at the molecular level, although the *wnt* proteins are candidates as powerful morphogens.

Footnote Fick's law of diffusion is not powerful enough to maintain the gradient of the wnt protein with MW of more than 30kD over the distance of 5-10 mm (Crick, 1970). Nevertheless, possibility exists that "anomalous diffusion" (Bouchaud, 1990; Havlin and Ben-Avraham, 2002) could enable formation of gradient over a long distance for such large molecules.

Current status of Hydra strains at the National Institute of Genetics

After Sugiyama and Fujisawa performed their initial field collection, extensive *Hydra* collecting in the wild was not carried out for many years. In the late 1990s Campbell and Martinez started to collect *Hydra* in various parts of the world to perform phylogenetic analyses (Martinez *et al*., 2010). A similar phylogenetic analysis was also performed by the Japanese school (Kawaida *et al*., 2010) and both studies provided a similar picture of the distribution of the *Hydra* strains in four major species. Many of the strains collected by Sugiyama and Fujisawa are currently maintained both in California and in Japan, with approximately 220 strains available at the National Institute of Genetics, Mishima. These include strains collected in Japan, in Europe and in the US in the 1960s, 1970s, 1980s, but also strains collected by Campbell and Martinez in 21st century, chimeric strains, and transgenic *Hydra* strains constructed recently in several laboratories worldwide. These strains are distributed on request to scientists for research purposes, to universities and schools for educational purposes (http://www.nig.ac.jp/labs/OntoGen/home.html).

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