Mathematical modelling and experiments for the proliferation and differentiation of Drosophila intestinal stem cells II

Masataka Kuwamura*a, Kousuke Maeda and Takashi Adachi-Yamada

*Graduate School of Human Development and Environment, Kobe University, Kobe 657-8501, Japan; bGraduate School of Science, Kobe University, Kobe 657-8501, Japan; cGraduate School of Science, Gakushuin University, Tokyo 171-8588, Japan

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The Drosophila posterior midgut epithelium mainly consists of intestinal stem cells (ISCs); semi-differentiated cells, i.e. enteroblasts (EBs); and two types of fully differentiated cells, i.e. enteroendocrine cells (EEs) and enterocytes (ECs), which are controlled by signalling pathways. In [M. Kuwamura, K. Maeda, and T. Adachi-Yamada, Mathematical modeling and experiments for the proliferation and differentiation of Drosophila intestinal stem cells I, J. Biol. Dyn. 4 (2009), pp. 248–257], on the basis of the functions of the Wnt and Notch signalling pathways, we studied the regulatory mechanism for the proliferation and differentiation of ISCs under the assumption that the Wnt proteins are supplied from outside the cellular system of ISCs. In this paper, we experimentally show that the Wnt proteins are specifically expressed in ISCs, EBs, and EEs, and theoretically show that the cellular system of ISCs can be self-maintained under the assumption that the Wnt proteins are produced in the cellular system of ISCs. These results provide a useful basis for determining whether an environmental niche is required for maintaining the cellular system of tissue stem cells.

Keywords: stem cells; signalling pathways; self-maintenance

1. Introduction

Biological tissues that are continuously renewed, including blood, testes, and epithelia, are characterized by rapid and continuous cell turnover. Terminally differentiated cells have a short lifespan and are replaced via proliferation of a distinct subpopulation of cells known as stem cells. In each tissue, the processes of stem cell renewal and production of differentiated progeny is tightly coordinated by signalling pathways in order to meet the varying requirements of the body for differentiated cells.

In our previous paper [6], we focused on the proliferation and differentiation of stem cells in the adult Drosophila posterior midgut epithelium, which serves as a good model for studying the regulatory mechanism for the maintenance of the cellular system of tissue stem cells. According to Lin et al. [8], Micchelli and Perrimon [12], and Ohlstein and Spradling [13,14], the Drosophila...
posterior midgut epithelium mainly consists of intestinal stem cells (ISCs); semi-differentiated
cells, i.e. enteroblasts (EBs); and two types of fully differentiated cells, i.e. enteroendocrine cells
(EEs) and enterocytes (ECs), which are controlled by the Wnt and Notch signalling pathways. In
addition, Wnt was reported to be specifically expressed in the underlying circular muscles adjacent
to ISCs [8], which suggests that the microenvironment constitutes a niche in which stem cells are
present; Wnt thus plays a role in maintaining a balance between self-renewal and differentiation
of the cells.

On the other hand, Sato et al. [15] have recently demonstrated that ISCs in adult mammals
can be cultured in vitro: they showed that the intestinal crypt-villus units of adult mammals are
self-organizing structures, which can be formed from stem cells in the absence of a non-epithelial
cellular niche. Their result suggests that the cellular system of ISCs in the Drosophila posterior
midgut epithelium is self-maintained because Drosophila and mammalian ISCs have similarities
in their regulatory mechanisms.

The purpose of this paper is to investigate the conditions required for self-maintenance of the
cellular system of ISCs in the Drosophila posterior midgut epithelium. Drosophila has mostly
been used to study the functions of signalling pathways that control the development of various
organs such as compound eyes, wings, and legs. However, it is quite difficult to observe such
development in vivo by using the existing experimental techniques. This implies that purely
experimental approaches cannot provide quantitative data for time-based information such as
the mortality rate (i.e. average lifespan) of cells. In the Drosophila posterior midgut epithelium,
the ratio of the cell numbers of four cell types in the ISC lineage is the only quantitative data
available for our study. Information is also available on the cause-and-effect relationships between
the activity of signalling pathways and the proliferation and differentiation of cells in the ISC
lineage, which have been verified by experiments based on classical genetics or modern genetic
manipulation. It should be noted that these relationships cannot determine the time scale of cell
proliferation and differentiation.

In this paper, we experimentally show that Wnt proteins are specifically expressed in ISCs,
EBs, and EEs, and theoretically show that the cellular system of ISCs can be self-maintained
under the assumption that the Wnt proteins are produced in the cellular system of ISCs through a
simple ODE model. We determine the conditions required for maintaining the cellular system of
ISCs and investigate whether Wnt proteins are produced in the cellular system of ISCs or supplied
from outside the system. These results clarify the qualitative properties of the functions of Wnt
signalling pathways that control the proliferation and differentiation of cells in the ISC lineage.
In particular, they can help determine whether an environmental niche is required for maintaining
the cellular system of tissue stem cells.

2. Detection of Wnt proteins

In this section, we immunocytochemically detect Wnt proteins in the Drosophila posterior
midgut. The methods used for immunofluorescence analysis in the current study – except for
the method used for the primary antibody (anti-Wg in the current study) – have been described
in [6, Appendix]. One of the Wnt family ligands, Wingless (Wg), could be found in the EEs and
both cells in the ISC–EB pairs (Figure 1). No significant differences were observed in the levels
of Wg expression between these cells.

If this Wg protein is derived from outside these cells (e.g. underlying circular muscle cells),
then follows that it will be detected in the transmission pathway, at least at low levels. However,
we could not detect the Wg proteins in the circular muscle cells or in the basement membrane
between the circular muscle cells and the ISC–EB pairs. If the concentration of Wg protein is
lower than the detection limit, then it cannot be detected by microscopy. However, based on past knowledge using various other Drosophila tissues, the Wg protein in the transmission pathway is known to aggregate to generate a granule, the presence of which is considered to be sufficient for its detection. Thus, we may conclude that these three types of cells (EEs, ISCs, and EBs) are the major sources of the ligand Wg, although more detailed experiments will be required to precisely identify the source of the ligand Wg, as explained in Section 5.

3. Theoretical analysis

In this section, we propose an ODE model for the proliferation and differentiation of ISCs in the Drosophila posterior midgut epithelium under the assumption that the Wnt proteins are produced in ISCs, EBs, and EEs. Using this model, we show that the cellular system of ISCs can be self-maintained under certain conditions. Here, we present only an outline of our analysis, since it is analogous to that in [6]. It should be noted that the definitions of the cell types ISC and EB are given by Kuwamura et al. [6, Remark 1]. For more details, the reader can refer to [6].

The proliferation and differentiation of ISCs are summarized in a schematic form (Figure 2), which is based on the cause-and-effect relationships between the activity of signalling pathways and the proliferation and differentiation of cells in the ISC lineage; Wnt signalling promotes...
the proliferation of ISCs, and ISC daughter cells differentiate into either ECs or EEs depending on their Notch activity; EBs cannot sufficiently differentiate into ECs in the absence of Wnt signalling. These relationships have been verified by experiments based on classical genetics or modern genetic manipulation.

On the basis of the above scheme, we propose a simple ODE model for the proliferation and differentiation of ISCs, which are controlled by the Wnt and Notch signalling pathways; this model is similar to that described in [6]. Here, we suppose that the Wnt proteins are produced by ISCs, EBS, and EEs.

\[
\begin{align*}
\frac{dI}{dt} &= ((\alpha W - \varepsilon_1) - \rho NI - \sigma I)I - \beta I, \\
\frac{dB}{dt} &= f(N)\beta I - \mu WB - \varepsilon_2 B, \\
\frac{dC}{dt} &= \mu WB - d_1 C, \\
\frac{dE}{dt} &= (1 - f(N))\beta I - d_2 E, \\
\frac{dN}{dt} &= k_1 BI - m_1 N, \\
\frac{dW}{dt} &= k_2 I + k_3 B + k_4 E - m_2 W,
\end{align*}
\]

where

\[
f(N) = \frac{N}{r + N};
\]

\(I, B, C, \) and \(E\) denote the population densities of ISCs, EBS, ECs, and EEs, respectively; \(N\) and \(W\) denote the activities of Notch and Wnt signalling, respectively; and \(\alpha, \beta, \rho, \mu, \sigma, \varepsilon_1, \varepsilon_2, d_1, d_2, k_1, k_2, k_3, k_4, m_1, m_2, \) and \(r\) are positive constants, the meanings of which are given in Table 1. Equation (1) has the same form as Equation (1) in [6], except for the 1st, 2nd and 3rd terms of the right-hand side of the last row of Equation (1); these terms mean that the Wnt proteins are produced by ISCs, EBS, and EEs. The terms from Equation (1) that are

| Notation | Meaning |
|----------|---------|
| \(\alpha\) | Proliferation rate of ISCs controlled by Wnt proteins |
| \(\beta\) | Differentiation rate of ISCs into EBS |
| \(\rho\) | Inhibitory effect on proliferation of ISCs by Notch signalling |
| \(\mu\) | Differentiation rate of EBS into ECs controlled by Wnt proteins |
| \(\sigma\) | Density-dependent inhibition rate of proliferation of ISCs |
| \(r\) | Control parameter of differentiation ratio between EBS and EEs determined by Notch signalling |
| \(\varepsilon_1\) | Mortality rate of ISCs |
| \(\varepsilon_2\) | Mortality rate of EBS |
| \(d_1\) | Mortality rate of ECs |
| \(d_2\) | Mortality rate of EEs |
| \(k_1\) | Activation rate of Notch signalling by ISCs and EBS |
| \(k_2\) | Activation rate of Wnt proteins by ISCs |
| \(k_3\) | Activation rate of Wnt proteins by EBS |
| \(k_4\) | Activation rate of Wnt proteins by EEs |
| \(m_1\) | Decay rate of Notch signalling |
| \(m_2\) | Decay rate of Wnt proteins |
common with those from Equation (1) in [6] have been explained in detail in [6, Section 4]. We cannot specify the time scales of the variables and parameters in Equation (1), since it is derived from the scheme in Figure 2, which cannot determine the time scale of cell proliferation and differentiation in the ISC lineage.

Since EBs are juvenile cells and their mortality rate $\varepsilon_2$ is sufficiently small, we numerically investigate the behaviour of the solutions of Equation (1) for sufficiently small $\varepsilon_2$, starting from the initial values

$$I(0) = I_0, \quad B(0) = 0, \quad C(0) = 0, \quad E(0) = 0, \quad N(0) = N_0, \quad \text{and} \quad W(0) = 0, \quad (3)$$

because the entire cellular system of ISCs (clonal cell populations derived from ISCs) is generated from some ISCs with strong Delta activity, which is a trigger of Notch signalling. The solutions converge to either an equilibrium $(I^*, B^*, C^*, E^*, N^*, W^*)$ or the trivial equilibrium (Figure 3), where

$$I^* = 1, \quad B^* = 1, \quad C^* = \frac{18}{7}, \quad E^* = \frac{2}{7}, \quad N^* = \frac{k_1}{m_1}, \quad W^* = \frac{k}{m_2}, \quad (4)$$

and

$$k = k_2 + k_3 + \frac{2k_4}{7} \quad (5)$$

along with

$$\frac{\rho k_1 - \alpha k}{m_1} - \frac{\beta + \sigma + \varepsilon_1}{m_2} = 0,$$

$$\frac{\beta k_1 - \mu r k}{m_1} - \frac{\mu k_1 k}{(m_1 m_2)} = 0,$$

$$\frac{7\mu k}{m_2} - 18d_1 = 0, \quad \text{and} \quad \frac{2d_2 k_1}{m_1} - \frac{7\mu r k}{m_2} = 0, \quad (6)$$

Figure 3. Numerical solutions converge to either a stable equilibrium or the trivial equilibrium. We choose parameter values satisfying Equations (5) and (6): $k_1 = 0.6, k_2 = 0.3, k_3 = 0.3, k_4 = 0.7, m_1 = 1.0, m_2 = 1.0, \mu = 0.32, \alpha = 6.0, \beta = 0.26, d_1 = 0.1, d_2 = 0.01, r = 7.55, \sigma = 0.005, \tau = 0.0066, \varepsilon_1 = 0.005, \text{and} \varepsilon_2 = 0.0001. \quad \text{We set the initial values as follows:} \quad N_0 = 0.01 \quad \text{with} \quad I_0 = 0.05 \quad \text{and} \quad 0.03 \quad \text{for} \quad (a) \quad \text{and} \quad (b), \quad \text{respectively.} \quad \text{When} \quad N_0 = 0.01, \quad \text{solutions for} \quad I_0 \geq 0.039 \quad \text{converge to the stable equilibrium as seen in} \quad (a), \quad \text{whereas solutions for} \quad I_0 \leq 0.039 \quad \text{converge to the trivial equilibrium as seen in} \quad (b). \quad \text{The red, yellow, blue, and green curves represent the densities of ISCs, EBs, ECs, and EEAs, respectively.} \quad \text{The brown and black curves represent the activities of Wnt and Notch signalling, respectively.} \quad \text{We can obtain similar numerical results for other values of the parameters satisfying Equations (5) and (6).}
where we suppose $\varepsilon_2 = 0$. In this case, there exists a threshold value $I_c$ such that the cellular system of ISCs cannot be maintained for $I_0 < I_c$. This result suggests that the cellular system of ISCs can be generated from certain numbers of ISCs under the assumption that the Wnt proteins are produced in the cellular system of ISCs. This is different from the results of Equation (1) in [6], under the assumption that the Wnt proteins are supplied from the underlying muscles adjacent to ISCs.

Moreover, numerical simulations show that the dynamics of the solutions of Equation (1) depend on $k$, which is given by Equation (5), but do not strongly depend on the ratio of $k_2$, $k_3$, and $k_4$. Therefore, to understand the numerical results, we may consider

$$
\frac{dI}{dt} = ((\alpha W - \varepsilon_1) - \rho NI - \sigma I)I - \beta I,
\frac{dB}{dt} = f(N)\beta I - \mu WB - \varepsilon_2 B,
\frac{dC}{dt} = \mu WB - d_1 C,
\frac{dE}{dt} = (1 - f(N))\beta I - d_2 E,
\frac{dN}{dt} = k_1 BI - m_1 N,
\frac{dW}{dt} = kI - m_2 W,
$$

which allows us to perform an analysis in the same way as [6]. In fact, numerical simulations suggest that Equation (1) is well approximated by Equation (7) when the parameters and initial values satisfy Equations (3)–(6); this approximation may not be valid except for a neighbourhood of the trivial equilibrium and that of $(I^*, B^*, C^*, E^*, N^*, W^*)$, from a viewpoint of rigorous mathematical analysis.

By using a quasi-steady-state approximation (i.e. setting $m_1 N = k_1 BI$ and $m_2 W = kI$ in Equation (7)) under the assumption that the dynamics of Notch and Wnt signalling is faster than that of the cell densities, and applying a standard renormalization procedure to reduce the number of parameters, we reduce Equation (7) to the following two-dimensional ODE

$$
\frac{dI}{dt} = ((\alpha k - \sigma)I - (\beta + \varepsilon_1) - \rho k_1 B^2)I,
\frac{dB}{dt} = \left(\frac{\beta k_1 I^2}{r + k_1 BI} - \mu kI - \varepsilon_2\right)B,
$$

where we use the same letter $t$ for describing the slow dynamics. It is easily seen that the phase plane for Equation (8) can be classified into two generic types as presented in Figure 4.

Since the mortality rate of EBs is sufficiently low, by setting $\varepsilon_2 = 0$, we can easily obtain the following practical condition under which Equation (8) has a stable equilibrium $(I^*, B^*)$ for sufficiently small $\varepsilon_2$:

$$
\alpha k > \sigma \quad \text{and} \quad k\mu(\rho r + \alpha k - \sigma)^2 > 4\rho\beta k_1(\varepsilon_1 + \beta).
$$

In this case, almost every solution of Equation (8) converges to either the stable equilibrium $(I^*, B^*)$ or the trivial equilibrium. Moreover, the second inequality of Equation (9) is equivalent to

$$
\alpha > \frac{2}{k}\sqrt{\frac{\rho\beta k_1(\varepsilon_1 + \beta)}{k\mu}} + \frac{\sigma - \rho r}{k}.
$$
Recalling the numerical results, we can consider Equation (9) as a practical condition for maintaining the cellular system of ISCs defined by Equation (1). Thus, we see that the cellular system of ISCs can be self-maintained under the assumption that the Wnt proteins are produced in ISCs, EBs, and EEs.

4. Discussion

In this section, we compare the conditions for maintaining the cellular system of ISCs presented in Section 4 with those in [6, Section 5], and provide a basis for investigating whether the Wnt proteins are produced in the cellular system of ISCs or supplied from outside the system.

From Equation (10), we define

\[
\alpha_c := \frac{2}{k_i} \sqrt{\frac{\rho \beta k_1 (\varepsilon_1 + \beta)}{k_i \mu} + \frac{\sigma - \rho r}{k_i}},
\]

(11)

where \( k_i \) denotes \( k \) given by Equation (5), which controls the Wnt proteins produced in the cellular system of ISCs. Then, the condition (10) under which the cellular system of ISCs can be self-maintained is given by

\[
\alpha > \alpha_c,
\]

(12)

under the assumption that the Wnt proteins are produced in ISCs, EBs, and EEs.

Similarly, setting \( \varepsilon_2 = 0 \) on the right-hand side of the inequality (14) in [6], we define

\[
\alpha'_c := \frac{\sigma}{k_0} \sqrt{\frac{r \mu k_0}{\beta k_1} + \frac{\varepsilon_1 + \beta}{k_0}},
\]

(13)

where \( k_0 \) is a positive parameter that controls the Wnt proteins supplied from outside the cellular system of ISCs. Then, the condition (14) in [6] for the maintenance of the cellular system of ISCs is given by

\[
\alpha > \alpha'_c
\]

(14)

under the assumption that the Wnt proteins are supplied by the underlying muscles.

Comparing \( \alpha_c \) and \( \alpha'_c \), we find that the ratio of \( k_1 \) to \( \mu \) plays an opposite role in the conditions (12) and (14). In other words, the cellular system of ISCs cannot be maintained if the value of \( k_1/\mu \) is high, under the assumption that the Wnt proteins are produced in the cellular system. However, the system cannot be maintained if the value of \( k_1/\mu \) is low, under the assumption that the Wnt proteins are supplied from outside the system.
If the Wnt proteins are produced in the cellular system of ISCs, then the Notch signalling activity controlled by the parameter $k_1$ cannot have large values because Notch signalling inhibits the proliferation of ISCs. This implies that the differentiation of EBs into ECs controlled by the parameter $\mu$ must be enhanced because ISC daughter cells differentiate into EEs rather than ECs via EBs under weak Notch signalling. Therefore, the value of $k_1/\mu$ must be low for maintenance of the cellular system of ISCs.

In contrast, if the Wnt proteins are supplied by the underlying muscles, then Notch signalling activity can have large values (notice that $\alpha'$ defined by Equation (13) does not include the parameter $\rho$, which indicates that the activation of Notch signalling leads to a decrease in the proliferation rate of ISCs). In this case, the differentiation of EBs into ECs controlled by the parameter $\mu$ must be inhibited because ISC daughter cells differentiate into ECs via EBs rather than EEs under strong Notch signalling. Therefore, the value of $k_1/\mu$ must be high for maintenance of the cellular system of ISCs.

Thus, the ratio of $k_1$ to $\mu$ may be important for examining whether the Wnt proteins are produced in the cellular system of ISCs or supplied from outside the system.

5. Concluding remarks

In this paper, we experimentally show that Wnt proteins are specifically expressed in ISCs, EBs, and EEs, and using a simple ODE model, we theoretically show that the cellular system of ISCs can be self-maintained under the assumption that the Wnt proteins are produced in the cellular system of ISCs. Moreover, we determine the conditions required for maintaining the cellular system of ISCs and provide a basis for investigating whether Wnt proteins are produced in the cellular system of ISCs or supplied from outside the system. It should be emphasized that our theoretical results concern the qualitative properties of functions of signalling pathways that control the proliferation and differentiation of cells in the ISC lineage. In fact, we cannot determine the time scales of the variables and parameters in our models, we could not obtain precise quantitative data for time-based information because experimental techniques to continuously observe the *Drosophila* posterior midgut epithelium *in vivo* have not yet been established.

It is important to determine whether an environmental niche is required for the maintenance of the cellular system of tissue stem cells. In order to study this issue, detailed experiments are required to precisely identify the site of the Wg source because Wnt signalling plays a crucial role in maintaining the cellular system of stem cells. With regard to the cellular system of ISCs in the *Drosophila* posterior midgut epithelium, two different experimental results were obtained: In our experiments, the Wnt proteins were detected in EEs, EBs, and ISCs, whereas these proteins were specifically expressed in the underlying circular muscles adjacent to ISCs [8]. Therefore, we aim to clarify the source site of Wg by performing the following three experiments: (1) Detection of the Wg protein in clones without the $wg$ gene; (2) detection of the Wg proteins in the ISCs attached to the circular muscle without Wg protein; (3) detection of HA-tagged Wg expressed in circular muscles. Details of the study design for these experiments are described in the appendix.

Following pioneering works by [12–14], recent experimental studies have provided basic information about the maintenance of ISCs in the *Drosophila* posterior midgut epithelium [1–5,7–11]. From the viewpoint of mathematical modelling of the cellular system of ISCs under conditions of baseline homeostasis, we should pay attention to [9], who reported that Upd was another paracrine signal from the underlying circular muscles, which activates a canonical JAK/STAT signalling pathway in ISCs. In addition, Beebe et al. [1] and Lin et al. [9] reported that JAK/STAT signalling is a potent regulator of ISC proliferation and is necessary for the differentiation of EBs into ECs. These results suggest that JAK/STAT signalling functions in parallel and in cooperation with Wnt
signalling, and that these pathways have almost the same functions with regard to maintaining the cellular system of ISCs. Therefore, we should redefine the variable $W$ in our model as the activity of a cooperative function of Wnt and JAK/STAT signalling, or extend our model so as to accommodate a new variable for the JAK/STAT signalling pathway (Beebe et al. [1] and Lin et al. [9] reported different functions of JAK/STAT signalling in self-renewal of ISCs; precise mathematical modelling for this signalling should be considered in further studies).

Our model is a simple ODE model that has no spatial dependence. Even though it allows detailed analysis, our model can reflect neither the diffusive effect of Wnt proteins nor the characteristics of cell–cell interaction in Notch signalling. Although we may regard our model as dealing with a situation in which the diffusivity of Wnt proteins is sufficiently large, we have not yet been able to give a reasonable interpretation to the term concerning the activation of Notch signalling in our model. We therefore need to improve our model to reflect the characteristics of cell–cell interaction in Notch signalling. This is an important issue that needs to be investigated in further studies.

At present, we have no experimental evidence on the basis of which we can reject the possibility of self-maintenance of ISCs in the Drosophila posterior midgut epithelium. In fact, Sato et al. [15] recently revealed that in the case of the mammalian small intestine, Lgr5-positive ISCs have the ability to self-organize the crypt-like structure without any epithelial niches; they demonstrated this by performing in vitro culture of stem cells. For successful isolation and subsequent culture of ISCs, they manipulated the functions of Wnt, EGF, TGF-beta, Laminin, Rho, and Notch. On the basis of these experimental strategies, we think that it is possible to demonstrate the self-organization ability of the Drosophila midgut by culturing ISCs. The presence and functions of all of the above proteins are evolutionarily conserved between mammals and Drosophila; therefore, we can attempt to perform a similar in vitro culture experiment once we establish a method for isolating ISCs under sterilized conditions.

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Appendix. Experimental methods to identify the source site of Wg

1. Detection of the Wg protein in clones without the wg gene. Lin et al. [8] showed that the ISC clones mutant for wg did not show evident decrease in their growth rate as found in the clones mutant for Wg receptors or Wg signal transducers. However, they did not determine whether Wg proteins were present or absent in the Wg-mutant clones. Thus, we will try to detect Wg proteins in ISC clones that are homozygous deletion mutants for the wg gene. If Wg is produced in circular muscle cells, it follows that it will be detected in wg mutant clones. However, if it is produced in the ISC and functions in an autocrine manner, then the clones will not have Wg proteins.

2. Detection of Wg proteins in the ISC attached to the circular muscle without the Wg protein. Circular muscle cells have been reported to produce Wg, which is secreted from muscle cells and reach the closest ISC. Thus, we can test whether Wg proteins are detected in the ISC attached to the circular muscle cell in which Wg is knocked down by RNAi. Because several muscle cells have been known to fuse during differentiation, it is difficult to establish mutant clones by somatic recombination. Instead, we can use an inducible RNAi technique to remove the Wg protein from specific muscle cells, if it is present there.

3. Detection of HA-tagged Wg expressed in circular muscles. We can distinguish the endogenous protein from the artificially expressed protein tagged by HA (Haemagglutinin of Influenza). By doing this, we can trace the HA-tagged Wg artificially induced in the circular muscle cells to reach the ISCs by detection with anti-HA antibody.