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Modulation of activator diffusion by extracellular matrix in Turing system

By

Takashi Miura*, **

Abstract

It has been speculated that Turing pattern formation mechanism is working during chick feather bud formation, and candidates for activator and inhibitor molecules are specified. Although difference of diffusion coefficients of activator and inhibitor is crucial for pattern formation process, it has not been assayed in detail both from experimental and theoretical point of view. In the present study, we measured diffusion coefficient of activator and inhibitor in Matrigel, which mimics the extracellular matrix (ECM) environment of biological tissues by applying fluorescently-labelled proteins in the gel. We found transient high concentration region near the source of the activator molecule, which suggests the diffusion is not classic Fickian diffusion. We show that this diffusion pattern is reproduced when some part of the molecules are trapped by ECM. We also show that we can reproduce Turing instability with the 3-species model, but we need to rescale reaction term when morphogen trapped in ECM do not bind to its receptor.

§ 1. Introduction

Various spontaneous pattern formation phenomena take place during mammalian development. Examples include animal coat markings [1], feather bud [2], feather ridge formation [3], limb skeleton [4, 5, 6], lung branching morphogenesis [7], vasculogenesis [8, 9] etc. Spontaneous pattern formation has been studied mainly in chemistry and physics (convection, crystal formation, BZ reaction etc.[10]), but accumulating phenomenological and molecular data makes biological system promising area for future research.

The most well-studied biological example of pattern formation is Turing instability during development [11]. In 1952 Alan Turing formulated a hypothetical chemical

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*Department of anatomy and developmental biology, Kyoto university graduate school of Medicine. Yoshida Konoe-chou, Sakyo-Ku, 606-8501, Japan. e-mail: miura-takashi@umin.ac.jp.
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interaction which can generate a periodic pattern out of initial homogeneous state. The model assumes existence of two molecules, the activator and the inhibitor, and activator promotes its own production and that of inhibitor. The inhibitor suppresses activator production and diffuses faster than activator. In such a system certain range of wavenumbers becomes unstable, which lead to periodic pattern formation (an intuitive explanation can be found in [12, 13] and mathematical analysis is described in [14]).

In some biological systems, the candidates for activator and inhibitor have already been specified. For example, in limb bud cells, transforming growth factor beta (TGFβ) is assumed to work as an activator molecule [6]. In chick feather bud formation, fibroblast growth factor (FGF) works as an activator and bone morphogenetic protein (BMP) works as an inhibitor [2]. In chick skin ridge formation, FGF also works as an activator and BMP act as an inhibitor [3]. Interestingly, the key players in this mechanism fall into limited number of "toolkit" molecules - FGF, TGFβ superfamily including BMP, sonic hedgehog (Shh) and Wnt, which appear repeatedly in many developing organs [15].

Although difference in diffusion coefficient is necessary for the formation of Turing pattern, the difference has not been assayed directly in biological systems. Molecular weights of these toolkit molecules are around 10-30 kDa. Therefore, according to Einstein-Stokes equation, diffusion coefficient of these molecules should not be very different. [16] has assayed the diffusion coefficient of BMP4 in *Xenopus* and concluded the molecule diffuses more slowly than other morphogen molecules. Recently, morphogen gradient formation in *Drosophila* embryo has been studied extensively by visualizing distribution of extracellular protein [17] and several factors that affect diffusion of morphogen molecules are specified. However, these studies concentrate on formation of monotonic gradient and comparison of diffusion coefficient with spontaneous pattern formation has not been done.

In the present study, we measured the diffusion coefficient of two key morphogen molecules, BMP and FGF. We found that the effective diffusion coefficient of FGF is much slower than BMP in Matrigel, which mimics the extracellular matrix component of biological tissue. During diffusion process region of high morphogen concentration was observed, which suggests the diffusion cannot be explained by classic Fickian scheme. The diffusion pattern can be understood by including immobile fraction of morphogen molecule in the model. Numerical simulation and mathematical analysis show that by including immobile fraction of activator molecule we can construct a system which shows Turing instability even when the diffusion coefficients of activator and inhibitor are the same.

§ 2. Materials & Methods

§ 2.1. Preparation of Alexa Fluor-488-labelled protein

Morphogen molecules are purchased from Peprotech (FGF) and R&D systems (BMP), and labelled with Alexa Fluor-488 microscale labelling kit (Molecular Probes) according to the manufacturer's instructions. Since gel filtration method provided by the manufacturer results in considerable amount of unbound dye, we use polyacrylamide gel electrophoresis for isolating labelled protein. After electrophoresis, the gel was observed with UV transilluminator and labelled protein can be detected as a band with molecular weight around 20-30 kDa. The band was dissected out as the source of florescently-labelled morphogen protein.

§ 2.2. Numerical simulation

All the numerical simulations were done with *Mathematica* with the explicit finite difference scheme. All the simulations were done in one-dimensional domain with periodic boundary conditions. Simulation parameters are described in figure legends. In some cases, numerical simulation was implemented using **NDSolve** function. *Mathematica* source codes are available on request.

§ 3. Results

§ 3.1. Turing system in skin feather bud formation

In previous works [2] the molecular circuit for feather bud formation has been established, and here we deal with the most authentic ones - activator as FGF and inhibitor as BMP. We use simplest possible governing equation for Turing instability.

$$\begin{aligned} u' &= f_u u + f_v v + d_u \Delta u \\ v' &= g_u u + g_v v + d_v \Delta v \end{aligned} \tag{3.1}$$

u represents relative concentration of activator (FGF) and v represents relative concentration of inhibitor (BMP). However, molecular weights of these molecules are not very different - they are around 10-20 kDa, which means d_u and d_v are almost identical from chemical point of view. Therefore, we experimentally observe whether diffusion of activator and inhibitor are very different under biological settings.

§ 3.2. Diffusion pattern of morphogens in Matrigel

When we applied a small piece of polyacrylamide gel which contains fluorescently-labelled protein in thin layer of Matrigel, we could observe a gradual release of morphogen protein from polyacrylamide gel into Matrigel. With some proteins like BMP4, diffusion profile seems to obey Fick's law - protein diffuses outside PAG and changes diffusion coefficient outside the gel.

In morphogen molecules like FGF, we could observe very high concentration of morphogen at the interface - it was even higher than original FGF concentration inside the polyacrylamide gel (Fig. 1). Obviously, BMP diffuses faster than FGF in this case, so this is consistent with the hypothesis that FGF acts as activator and BMP acts as inhibitor. However, this distribution pattern cannot be reproduced by Fickian diffusion, which should not locally increase concentration of diffusible molecule.

§ 3.3. Biological background - FGF can bind HSPG in Matrigel

The proteins which show strange behaviour belong to heparin-binding proteins. Heparin is glycosaminoglycan which is widely used to stop blood coagulation process. A type of extracellular matrix protein - heparan sulfate proteoglycan (HSPG) consists of protein core and glycosaminoglycan side chains which consist of heparin. Therefore, heparin-binding proteins are known to bind to HSPG and to be immobilized [18].

§ 3.4. Modelling diffusion pattern by considering immobile fraction

The observed pattern can be understood by incorporating the above biological settings. We divide morphogen into mobile (u) and immobile (w) fraction, and suppose the immobile fraction is trapped by HSPG and does not move. We set association and dissociation rate constants as k_a and k_d, and ECM (HSPG) density as e. Usually, HSPG has numerous binding sites for morphogen molecules, so we neglect the effect of binding site saturation. Then the system is represented as follows:

$$\begin{aligned} u' &= d_u \Delta u + k_d w - k_a e u \\ w' &= -k_d w + k_a e u. \end{aligned} \tag{3.2}$$

In this situation e is dependent on space. In polyacrylamide region e is zero, while in Matrigel region they have some value.

Numerical simulation of this system can reproduce the observed pattern (Fig. 2). In this system, free FGF obey simple diffusion equation and immobile FGF bind to HSPG at Matrigel region. Therefore, distribution of total FGF is amplified in Matrigel region, which makes the high FGF concentration at the interface of PAG and Matrigel region.

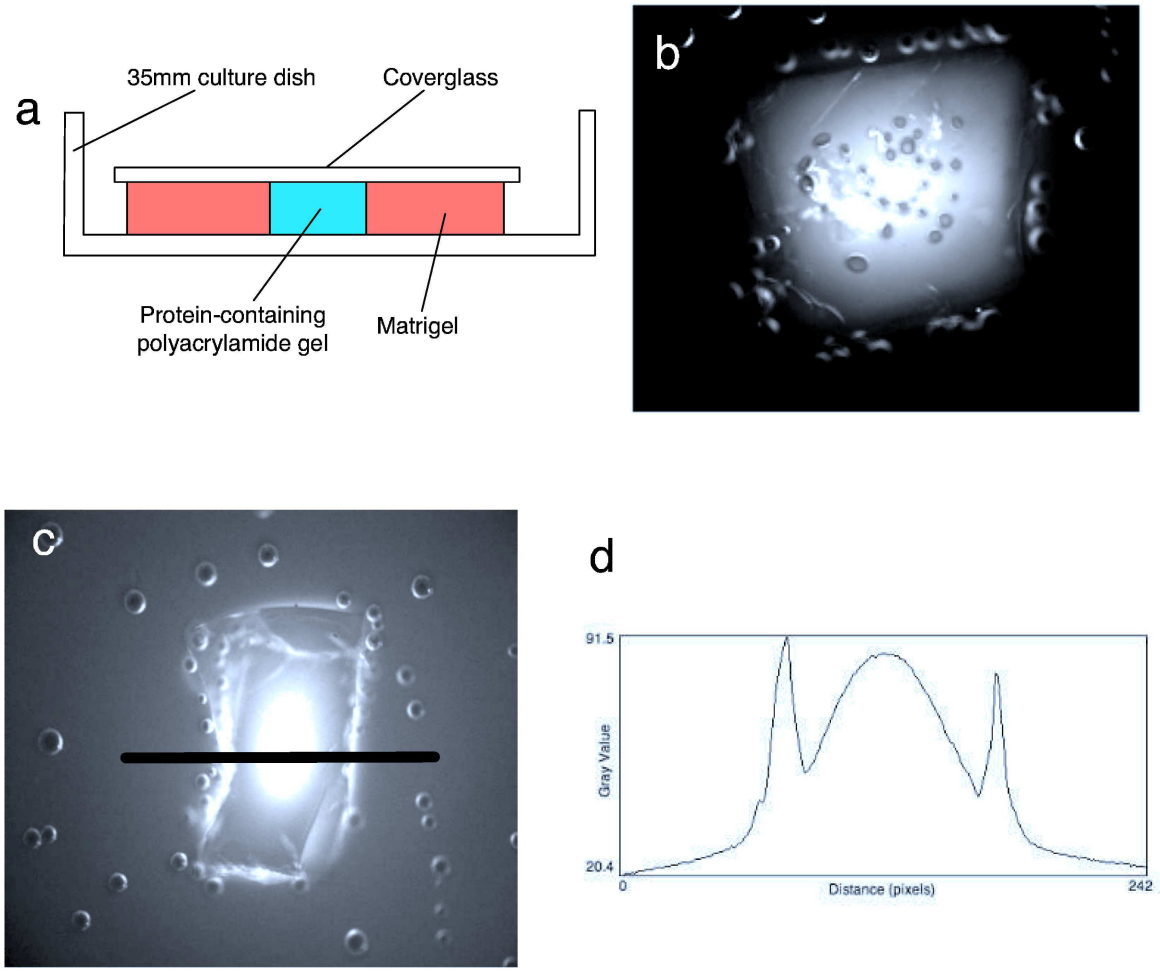

Figure 1. (a) Experimental setting. A thin layer of Matrigel was sandwiched by slide-glasses, and a piece of polyacrylamide gel was placed in Matrigel. (b) Distribution of fluorescently-labelled BMP4 molecule after 60 min. of incubation. (c) Distribution of fluorescently-labelled FGF10 molecule after 60 min. of incubation. (d) Concentration profile of (c). A region of high FGF10 can be observed outside the polyacrylamide gel.

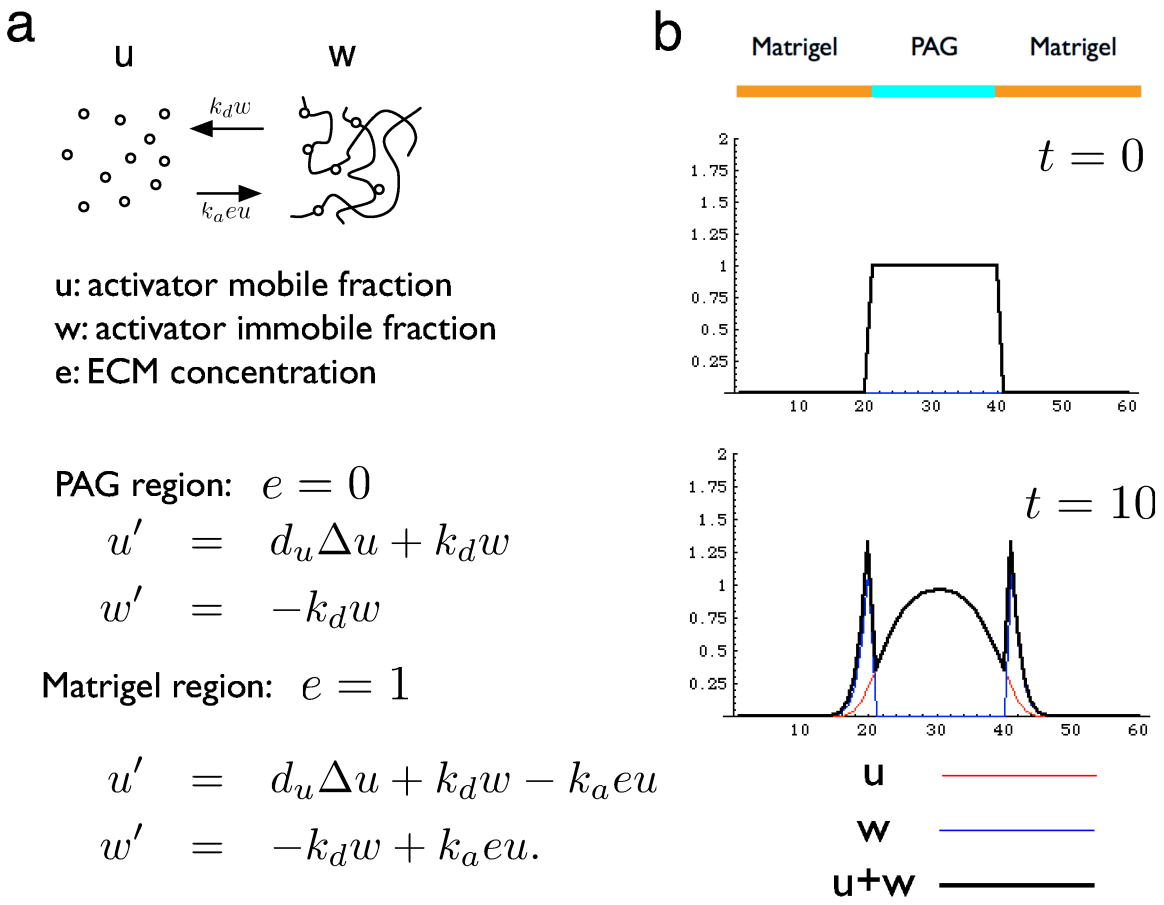

Figure 2. (a). Model description. Mobile FGF (u) binds to HSPG (e) by certain association/dissociation rate (k_a, k_d). (b). Result of numerical simulation at $t = 10$. Simulation parameters: domain size =60, mesh size=1, timestep=0.1, $D = 1, k_a = 5, k_d = 1$.

§ 3.5. Approximation of 3-species model by 2-species model

Now we go back to our original question and try to find whether the three-species model can generate Turing instability. We suppose the model as follows:

$$u' = f_u u + f_v v + d_u \Delta u + k_d w - k_a e u \tag{3.3}$$

$$v' = g_u u + g_v v + d_v \Delta v \tag{3.4}$$

$$w' = -k_d w + k_a e u. \tag{3.5}$$

In this case, we suppose $d_u = d_v$. However, the set of (f_u, f_v, g_u, g_v) which satisfies the diffusion-driven instability condition in 2-species model does not work, no matter how we increase k_a, which should reduce effective diffusion coefficient of activator (Fig. 3).

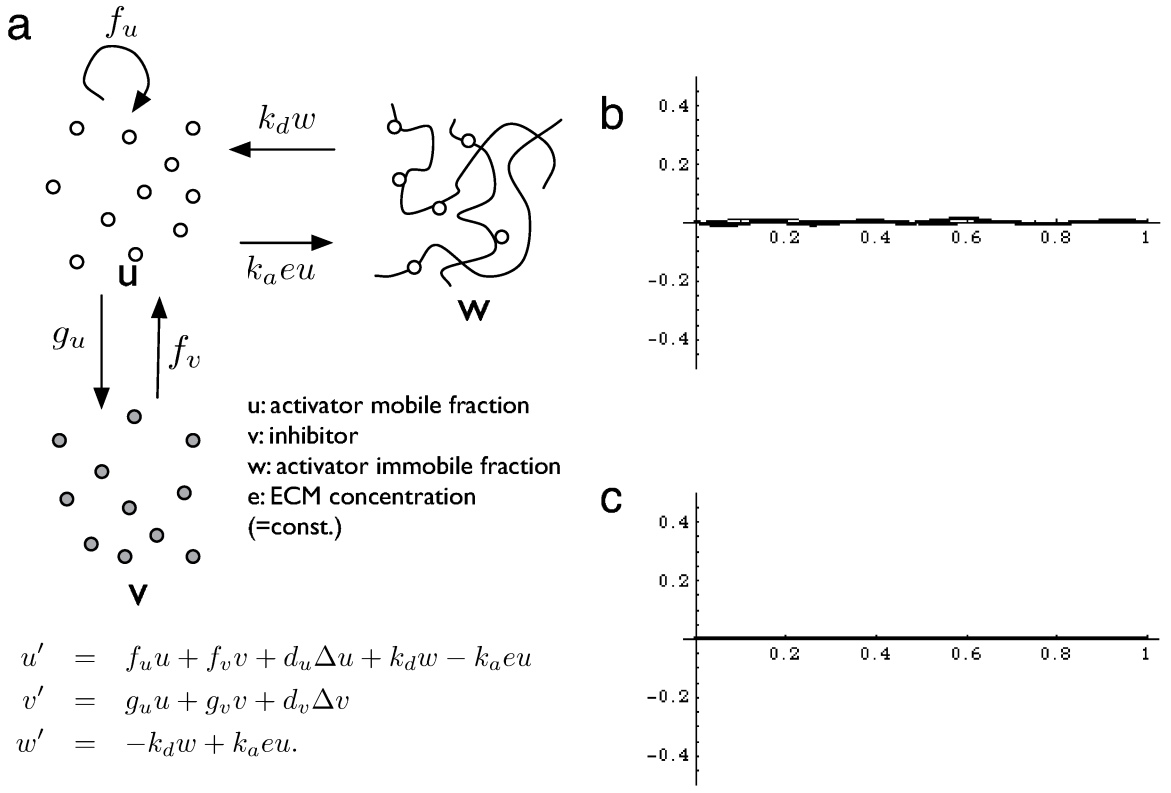

Figure 3. Mere addition of immobile activator fraction does not lead to Turing instability when $d_u = d_v$. (a) Model scheme. (b) $u + w$ and v distribution at $t = 0$. (c) $u + w$ distribution at $t = 100$. No pattern formation occurs. Simulation parameters: domain size=1, $(f_u, f_v, g_u, g_v) = (0.6, -1, 1.5, -2)$, $d_u = d_v = 0.0025$, $k_a = 100$, $k_d = 10$, e=1.

Estimating diffusion-driven instability condition using 3-species model is extremely cumbersome because characteristic polynomial is cubic. Therefore, we sought to reduce the system to 2-species using an approximation described in [19].

We start from simple diffusion equation (3.2). When k_a and k_d are large, i.e., association-dissociation reaction of morphogen-ECM is faster than activator-inhibitor interaction, equation (3.5) should quickly become equilibrium, that means

$$-k_d w + k_a e u = 0. \tag{3.6}$$

This is a biologically plausible assumption because we suppose activator-inhibitor interaction takes place via transcription regulation of cells which consist of the tissue, but association/dissociation reaction should be purely chemical. From this equation we obtain

$$w = \frac{k_a e}{k_d} u \tag{3.7}$$

$$u = \frac{k_d}{k_a e + k_d}(u + w). \tag{3.8}$$

Then, if we define total morphogen concentration $U = u + w$, the system can be reduced as follows.

$$U' = \Delta(\frac{k_d}{k_a e + k_d} d_u U). \tag{3.9}$$

Defining effective diffusion coefficient $d_e(x)$ as $\frac{k_d}{k_a e + k_d} d_u$, The equation becomes

$$U' = \Delta(d_e(x) U). \tag{3.10}$$

This is different from conventional Fickian diffusion equation ($U' = \nabla d \nabla U$). $d_e = d_u$ when $e = 0$, so this definition is also valid in PAG region. Numerical simulation of equation (3.10) can reproduce the observed diffusion pattern of morphogen molecule (Fig. 4).

§ 3.6. Turing instability by immobile fraction model

From above approximation the three-species model can be reduced as follows:

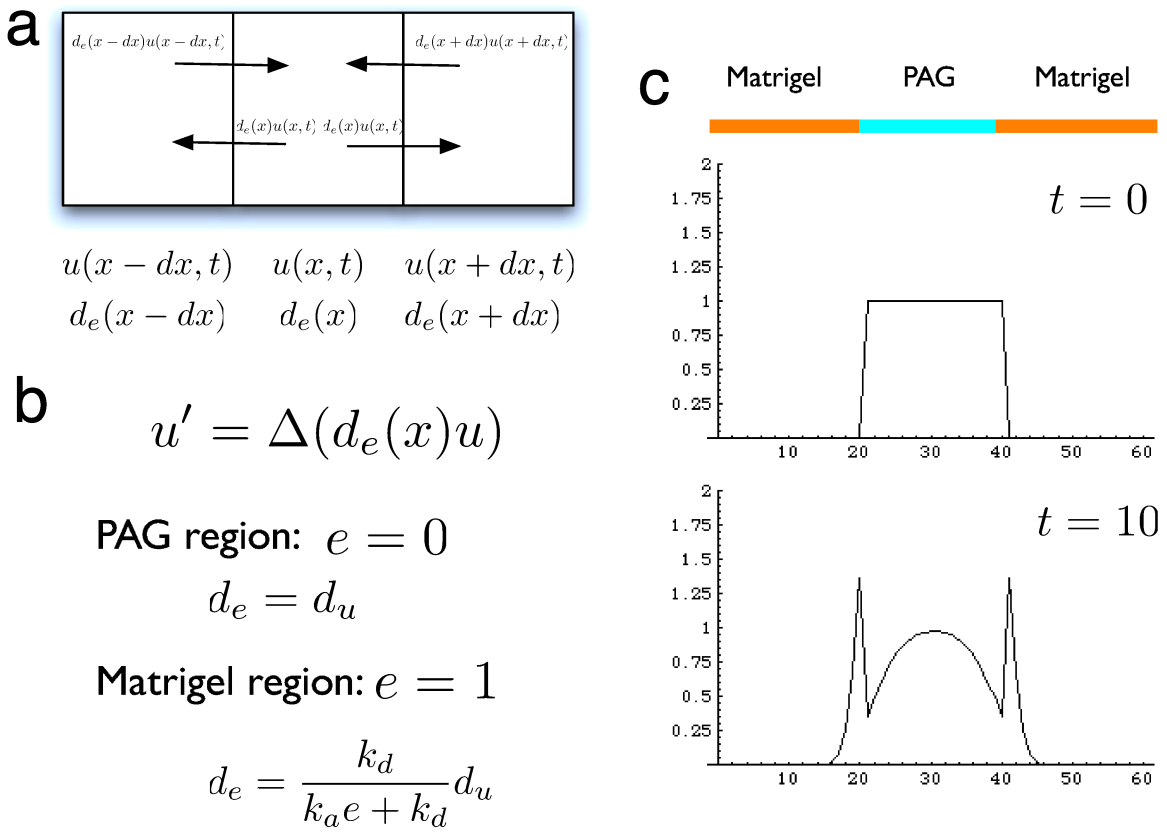

Figure 4. Non-Fickian diffusion term can reproduce the observed pattern. (a) Numerical scheme. We assign the effective diffusion coefficient d_e to each grid, and a certain fraction of morphogen in this grid is transferred to neighbouring grids. Since mobile fraction of morphogen is determined by d_e, amount of morphogen transferred to neighbouring grid is proportional to d_e. (b) If we take a limit $dx \to 0$, resulting governing equation is (3.10), which is different from Fickian diffusion. (c) Result of numerical simulation. We assume diffusion coefficient of morphogen is different between Matrigel region ($e = 1$) and PAG region ($e = 0$). Simulation parameters: domain size =60, mesh size=1, timestep=0.1, $k_a = 5, k_d = 1, d_u = 1$.

$$U' = f_u \frac{k_d}{k_a e + k_d} U + f_v v + d_u \Delta \frac{k_d}{k_a e + k_d} U \tag{3.11}$$

$$v' = g_u \frac{k_d}{k_a e + k_d} U + g_v v + d_v \Delta v \tag{3.12}$$

In this form, we can intuitively see why the above model (3.3-3.5) does not work - if diffusion is reduced by association to extracellular matrix, the immobile fraction should not reach to receptor and hence effect of morphogen molecule is decreased. To increase the effect of morphogen molecule on activator-inhibitor interaction, we rescaled f_u and f_v and we can reproduce Turing instability in 3-species model (Fig. 5).

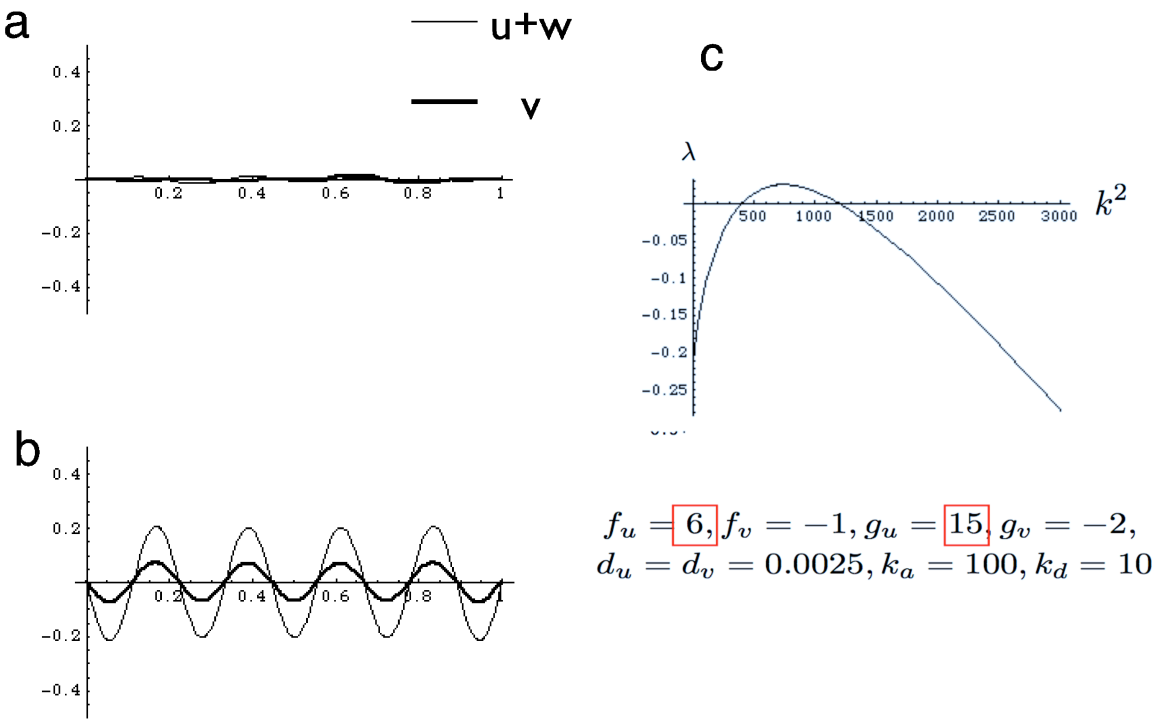

Figure 5. Turing instability in 3-species model. (a) $u + w$ and v distribution at the beginning of simulation. (b) $u+w$ and v distribution at $t = 100$. (c) Dispersion relation of the system. f_u and g_u have larger value than the rest of the parameters in this system (red box). Simulation parameters: domain size=1, $(f_u, f_v, g_u, g_v) = (6, -1, 15, -2)$, $d_u = d_v = 0.0025$, $k_a = 100$, $k_d = 10$, e=1.

§ 4. Discussion

Although some biological system BMP is a good candidate for inhibitor [2], which should have larger diffusion coefficient, [16] showed that BMP4 diffuses more slowly than other signalling molecules during early *Xenopus* development. There are several possible explanations for these seemingly contradictory data. First, BMP4 diffusion is slower than protein molecule of the same size like lysozyme which does not interact with the extracellular matrix, but FGF diffuses *much* more slowly than these molecules, Second, there are several extracellular modifiers which affect diffusion coefficient. For example, Noggin and Chordin are extracellular modulator of BMP function which blocks BMP-BMPR interaction [20], and they are shown to increase diffusion coefficient in *Drosophila* embryo [17]. Therefore, diffusion coefficient of morphogen can be highly context-dependent and should be assayed separately under different situations.

The feather bud formation [2] and ridge formation [3] utilize common molecular circuit (FGF-BMP) but resulting patterns have a different spatial scale - the feather ridge is mich smaller structure than the feather bud. This difference may come from the diffusion coefficient difference by HSPG. We can predict that amount of HSPG will increase at later stages of skin development, which can be experimentally tested.

Modulation of diffusion coefficient can be estimated using biochemical data. Heparin binding ability of various protein molecules have been studied in detail [21]. For example, ratio of association/dissociation constant $K_D = k_d/k_a$ of heparin binding was measured in many proteins. FGF2-heparin binding K_D is 20 nM [22] while BMP4-heparin binding K_D is 2 nM [23], which may reflect faster diffusion of BMP4.

The diffusion with absorption-dissociation reaction $\Delta(du)$ does not play a role in this case, but in some cases activator can act to promote expression of HSPG molecule (data not shown). In this case, activator diffusion is dependent on activator concentration, which may help generating instability or making higher mode structure. Activator-related domain growth has been done recently in modelling tooth development [24], and similar effect may occur in this system.

References

[1] S. Kondo and R. Asai. A reaction - diffusion wave on the skin of the marine angelfish pomacanthus. *Nature*, 376:765–768, 1995.

[2] T. X. Jiang, H. S. Jung, R. B. Widelitz, and C. M. Chuong. Self-organization of periodic patterns by dissociated feather mesenchymal cells and the regulation of size, number and spacing of primordia. *Development*, 126(22):4997–5009, 1999.

[3] M. P Harris, S. Williamson, J. F. Fallon, H. Meinhardt, and R. O. Prum. Molecular evidence for an activator-inhibitor mechanism in development of embryonic feather branching. *Proc Natl Acad Sci U S A*, 102(33):11734–11739, Aug 2005.

[4] S. A. Newman and H. L. Frisch. Dynamics of skeletal pattern formation in developing chicklimb. *Science*, 205:662–668, 1979.
[5] S. A. Newman. Sticky fingers: Hox genes and cell adhesion in vertebrate limb development. *Bioessays*, 18(3):171–4, Mar 1996.
[6] T. Miura and K. Shiota. Tgf beta 2 acts as an "activator" molecule in reaction-diffusion model and is involved in cell sorting phenomenon in mouse limb micromass culture. *Dev Dyn*, 217(3):241–9, 2000.
[7] T. Miura and K. Shiota. Depletion of fgf acts as a lateral inhibitory factor in lung branching morphogenesis in vitro. *Mech Dev*, 116(1-2):29–38, 2002.
[8] R. M. H. Merks, S. V. Brodsky, M. S. Goligorksy, S. A. Newman, and J. A. Glazier. Cell elongation is key to in silico replication of in vitro vasculogenesis and subsequent remodeling. *Dev Biol*, 289(1):44–54, Jan 2006.
[9] G. Serini, D. Ambrosi, E. Giraudo, A. Gamba, L. Preziosi, and F. Bussolino. Modeling the early stages of vascular network assembly. *EMBO J*, 22(8):1771–1779, Apr 2003.
[10] P. Ball. *The self-made tapestry.* Oxford university press, 1999.
[11] A. M. Turing. The chemical basis of morphogenesis. *Phil. Trans. R. Soc. B*, 237:37–72, 1952.
[12] S. Kondo. The reaction-diffusion system: a mechanism for autonomous pattern formation in the animal skin. *Genes Cells*, 7(6):535–41, 2002.
[13] T. Miura and P. K. Maini. Periodic pattern formation in reaction-diffusion systems: an introduction for numerical simulation. *Anat Sci Int*, 79(3):112–23, Sep 2004.
[14] J. D. Murray. *Mathematical biology.* Springer - Verlag, Berlin, third edition, 2003.
[15] S. F. Gilbert. *Developmental Biology.* Sinauer, Massachusettes, 2003.
[16] B. Ohkawara, S. Iemura, P. Dijke, and N. Ueno. Action range of BMP is defined by its N-terminal basic amino acid core. *Curr Biol*, 12(3):205–209, Feb 2002.
[17] A. Eldar, R. Dorfman, D. Weiss, H. Ashe, B. Z. Shilo, and N. Barkai. Robustness of the BMP morphogen gradient in Drosophila embryonic patterning. *Nature*, 419(6904):304–308, Sep 2002.
[18] U. Haecker, K. Nybakken, and N. Perrimon. Heparan sulphate proteoglycans: the sweet side of development. *Nat Rev Mol Cell Biol*, 6(7):530–541, Jul 2005.
[19] M. Iida, M. Mimura, and H. Ninomiya. Diffusion, cross-diffusion and competitive interaction. *Meiji Institute for Mathematical Science Report No.052005*, 2005.
[20] W. Balemans and W. V. Hul. Extracellular regulation of BMP signaling in vertebrates: a cocktail of modulators. *Dev Biol*, 250(2):231–250, Oct 2002.
[21] E. Conrad. *Heparin Binding Proteins.* Academic Press, 1998.
[22] D. Moscatelli. High and low affinity binding sites for basic fibroblast growth factor on cultured cells: absence of a role for low affinity binding in the stimulation of plasminogen activator production by bovine capillary endothelial cells. *J Cell Physiol*, 131(1):123–130, Apr 1987.
[23] R. Ruppert, E. Hoffmann, and W. Sebald. Human bone morphogenetic protein 2 contains a heparin-binding site which modifies its biological activity. *Eur J Biochem*, 237(1):295–302, Apr 1996.
[24] I. Salazar-Ciudad and J. Jernvall. A gene network model accounting for development and evolution of mammalian teeth. *Proc Natl Acad Sci U S A*, 99(12):8116–20, 2002.