Modeling Periodic Aspect of Limb Pattern Formation

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1. Introduction

Developmental biology deals with the development of an organ from single fertilized egg to very complex adult form. The process in which a simple shapeless fertilized egg becomes a very complex organismal structure without error attracts many biologists and the researchers in the related fields.

Attempts to explain this morphogenetic process in terms of mathematical model date back from Alan Turing's reaction-diffusion model (Turing, 1952) to recent complex systems study by Stuart Kauffman (Kauffman, 1996), hence there are considerable amount of theoretical studies accumulated now. However, these studies are more or less independent from the experimental studies. The present author and his collaborators made experimental studies by focusing on *in vitro* situations because genetic interaction *in vivo* is too complex. We are now trying to verify various theoretical models using these experimental systems. In this article we chose periodic pattern formation of limb mesenchyme cells as an example, and introduce the theoretical models and experimental verifications from our own study.

2. Limb Pattern Formation: Positional Information and Periodicity

Limb development is one of the most advanced area in the field of developmental biology. For example, the mouse limb appears first as outpocket of tissue called limb bud from side of the body (Fig. 1A). Next, the limb bud elongates, the distal region becomes flattened (Fig. 1C), and cartilages are formed in the limb bud (Fig. 1D) which later are substituted by limb skeleton. The cells between digits undergo cell death and removed.

In general, limb pattern formation was studied assuming there are three axis-anteroposterior (thumb-little finger axis), proximodistal (tip-shoulder axis) and dorsoventral (palm-back axis). Recent advance in molecular biology has identified extracellular signaling molecules involved in deciding the axis during development. For example, anteroposterior axis is determined by soluble signaling molecule called Sonic Hedgehog (SHH). Proximodistal axis is regulated by extracellular signaling molecule fibroblast growth factors (FGF). Dorsoventral axis is decided by protein called Wnt (Gilbert, 2000).

However, independent of these studies, we know some examples in which a mechanism to generate periodic structure in the limb bud is working during development. For example, we have pathological condition called polydactyly in humans with digits more than 6 (Fig. 1E). This phenomenon is thought to be caused by the fact that polydactylous embryos has larger limb bud. However, if the location of each digit is solely determined by some corresponding Hox code (genes that are thouoght to specify identity of each skeletal elements), the larger limb bud should result in thicker bones with the same number or simply excess soft tissue. From comparative anatomy point of view, the limb is homologous to the pectoral fin of the Ichthyosaurus. Their fin has many bony elements whose identity is not clear. It is difficult to assume that all these bone element is specified by the specific genes. From these observations, it has been suggested that there should be some mechanism to generate periodic structure during limb development.

To explore this mechanism, since the situation *in vivo* is too complex, we decided to use limb bud micromass culture system (Fig. 2A), which is considered to retain the periodic aspect of pattern formation. In this experiment, we isolate the mouse limb bud and dissociate it into single cell level using enzymatic treatment. Then, if the cells were cultivated again in high density, some of the cell becomes chondrocyte (future bone) while others remain fibroblasts (future soft tissue), which results in stripe-like pattern of cartilage (Fig. 2B). We utilize this culture system to elucidate the mechanism of periodic pattern formation during limb development.

3. Cell Sorting or Reaction Diffusion?

Two types of models has been proposed for the pattern formation. One is the cell sorting, and the other is the reaction-diffusion model.

The cell sorting model assumes that the cells are already differentiated at the beginning of the culture according to their positions in the limb bud, and that the cells from the same origin tend to adhere strongly to each other, resulting in formation of clusters of certain size. This model has experimental background; if we label the cells fluo-

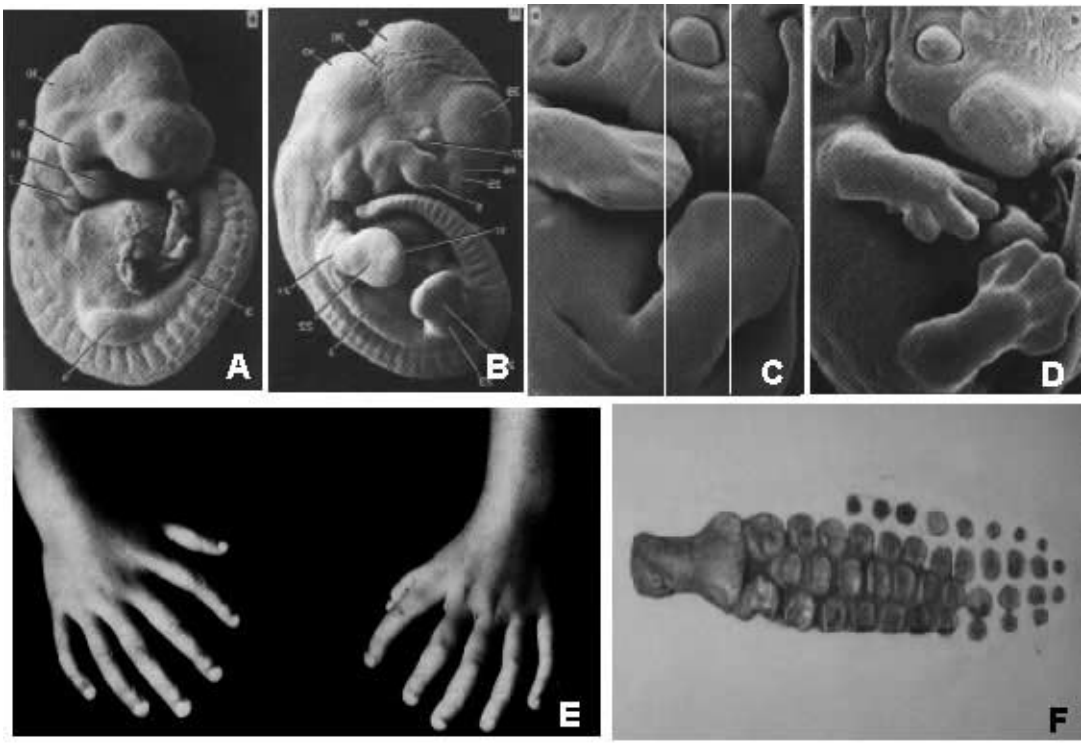

Fig. 1. Limb development. (A–D) Mouse limb bud development. A: E9.5, B: E11.5. C: E12.5. D: E13.5. (E) An example of human polydactyly (Langman's Medical Embryology). (F) An example of skeletal structure of pectoral fin of Ichthyosaurus.

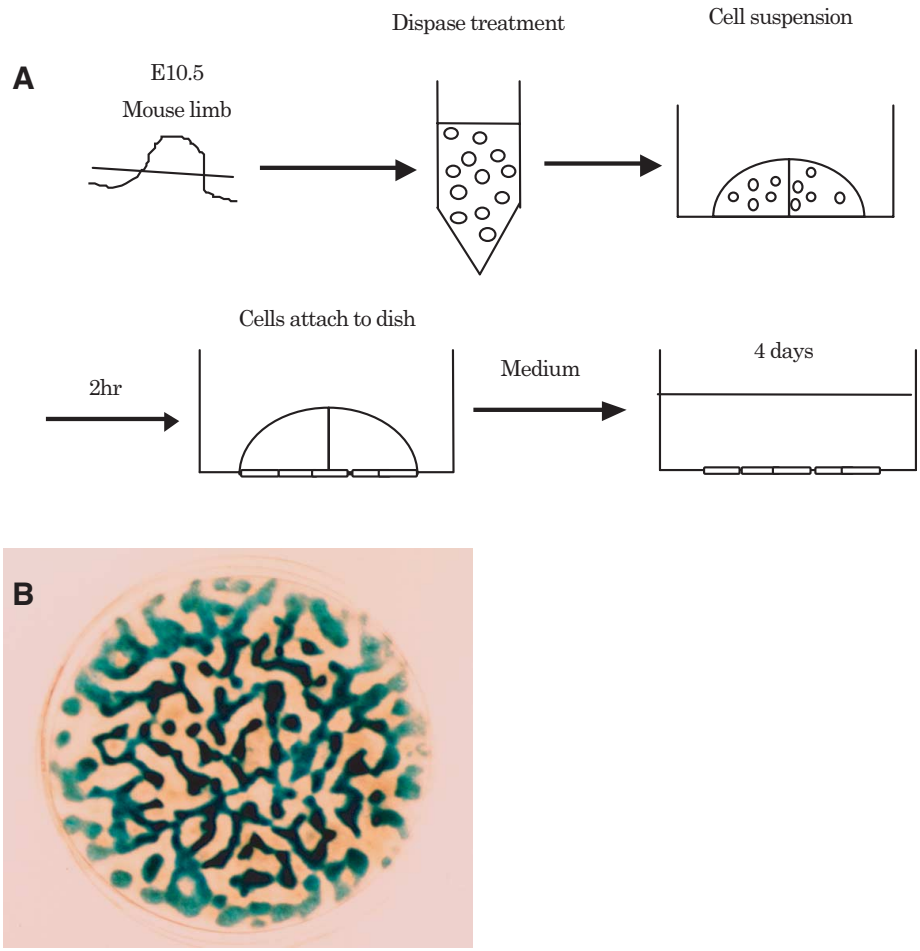

Fig. 2. Micromass culture of mouse limb. (A) Culture method. Limb bud tissue is dissociated into single cell level, and cultivated in high density on a culture dish. (B) Experimentally observed pattern. Blue part represents the cells differentiated to chondrocytes.

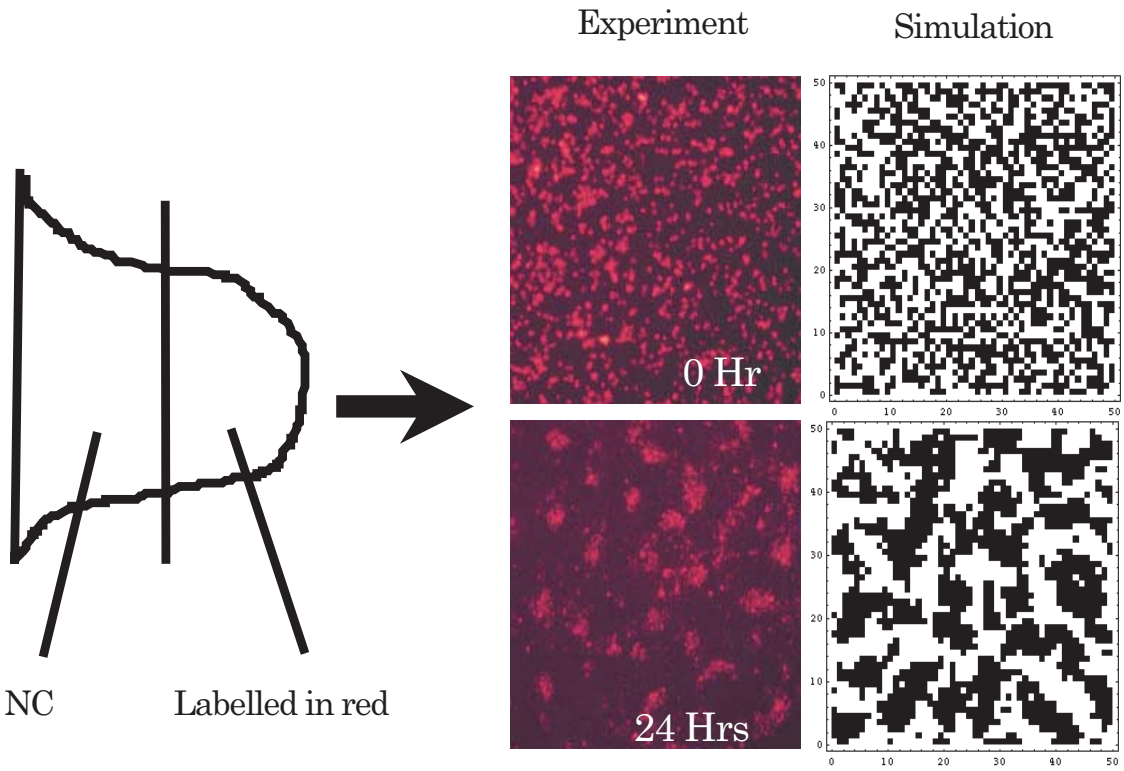

Fig. 3. An experiment supporting the cell sorting model. When the distal part of the limb bud cells were labeled with PKH-26 fluorescent dye and mixed with other cells (0 Hr), the labelled cells form clusters after 24 hours. The right two patterns are results of the model simulation by Mochizuki *et al.* (1998) where 24 Hrs distribution was obtained by introducing different adhesivity of cells and compared with experiment. They also tried to quantitate the difference of cell adhesivity.

rescently from the distal part of the limb bud, and mix it with other cells and culture for 24 hours, the randomly-distributed cells form clusters after 24 hours (Fig. 3). The region of cluster of the distal cells and the region of chondrogenesis (future bone formation) is identical, hence some researchers propose that the pattern in Fig. 2B is generated by cell sorting (Cottrill *et al.*, 1987). Moreover, Mochizuki *et al.* (1998) formulate a cell automaton model with quantitative difference of cell adhesivity.

On the other hand, reaction-diffusion model assumes the interaction of two hypothetical extracellular signalling molecules, the activator and the inhibitor. Then, under a certain condition, a perodic pattern is shown to form spontaneously (Turing, 1952). These molecules have the following characteristics (Fig. 4A):

(1) the activator promotes its own production,
(2) the activator upregulates the production of inhibitor,
(3) the inhibitor inhibits the production of activator,
(4) the inhibitor diffuses much faster than the activator.

Then, a periodic pattern emerges out of spatially homogeneous initial distribution. The intuitive explanation of this phenomenon is as follows; since the activator promotes its own production, initial small peaks of activator are amplified by this positive feedback mechanism. Then, since the activator promotes the production of inhibitor and inhibitor diffuses faster than the activator, shallow peaks of inhibitor are generated at the same location. Then, the action of inhibitor becomes dominant in the region slightly away from the peak and inhibits formation of new activator peak. As a result the pattern with relatively regular spacing is generated. (Fig. 4B) The pattern in the 2D space is also obtained (Fig. 4C), which is similar to the chondrogenic pattern of the culture system (Fig. 2B), which suggests that this model can explain the limb pattern formation (Downie *et al.*, 1995).

To compare these two models, we specify the most important factor that decide the pattern, and modify them experimentally to see whether the perturbation can induce the predicted pattern change. The most important factors that determine the pattern are the diffusion coefficient in the reaction-diffusion model, and the cell adhesion or random cell motion in cell sorting model. As a result, we could induce a change in characteristic length of the pattern relatively easily by changing the diffusion coefficient while changing cell adhesivity or cell locomotion did not induce the pattern change (Fig. 5). Thus, we conclude that reaction-diffusion model is more likely to predict the periodic aspect of the chondrogenic pattern during limb development (Miura and Shiota, 2000a).

4. Molecular Candidate of Activator or Inhibitor

The next question is the molecular nature of activator and inhibitor. It has been suggested that extracellular signaling molecule called TGF beta plays a role as an activator (Downie *et al.*, 1995). However, we assume that TGF beta 2 is the possible candidate for this pattern formation, and we showed experimentally that this molecule satisfies the folowing four necessary conditions for the activator:

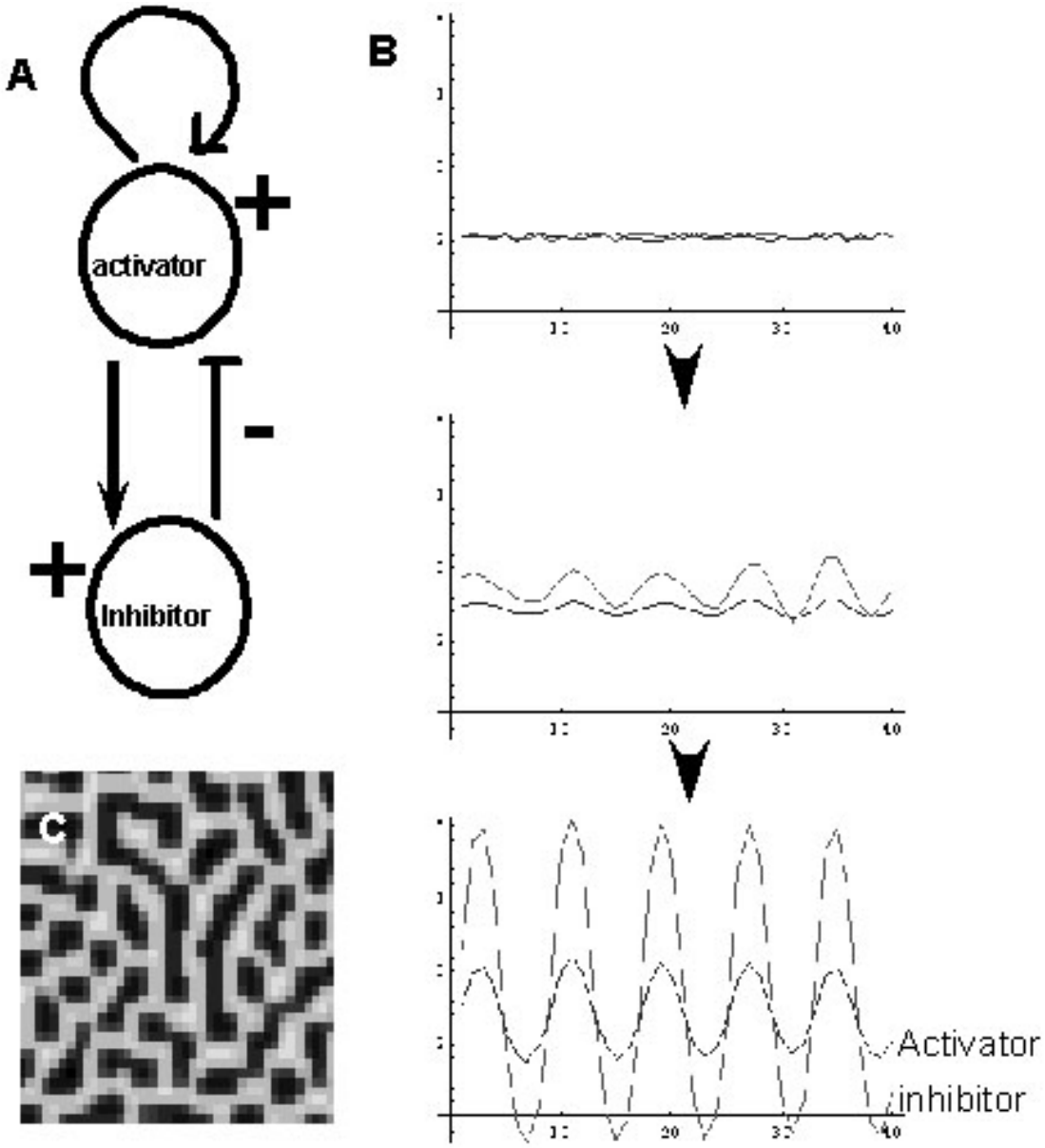

Fig. 4. Reaction-diffusion model. (A) The interaction of activator and inhibitor. (2) Numerical simulation of one-dimensional version of the model. (c) Numerical result of two-dimensional version of the model.

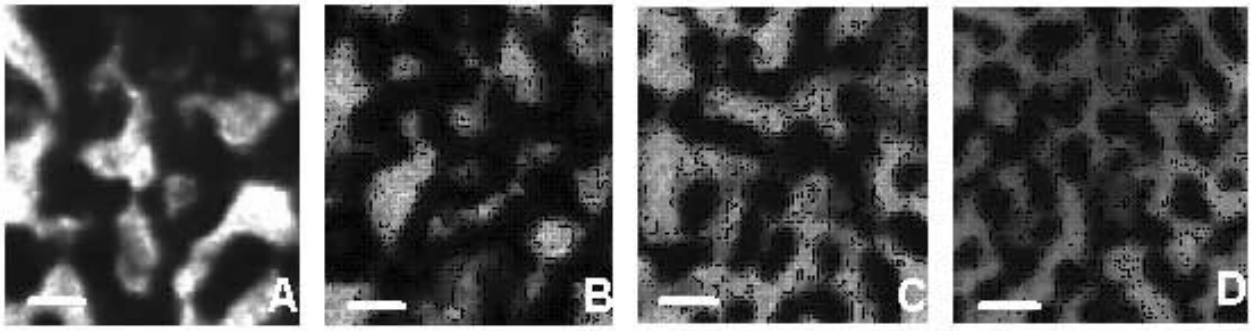

Fig. 5. Change of cartilage pattern by changing the diffusion coefficient. To change the diffusion coefficient, we embed the whole culture inside agarose gel and observed the pattern change. As a result, we could observe change in characteristic length of pattern. (A) Liquid medium. (B) 0.125% agarose gel. (C) 0.25% agarose gel. (D) 0.5% agarose gel.

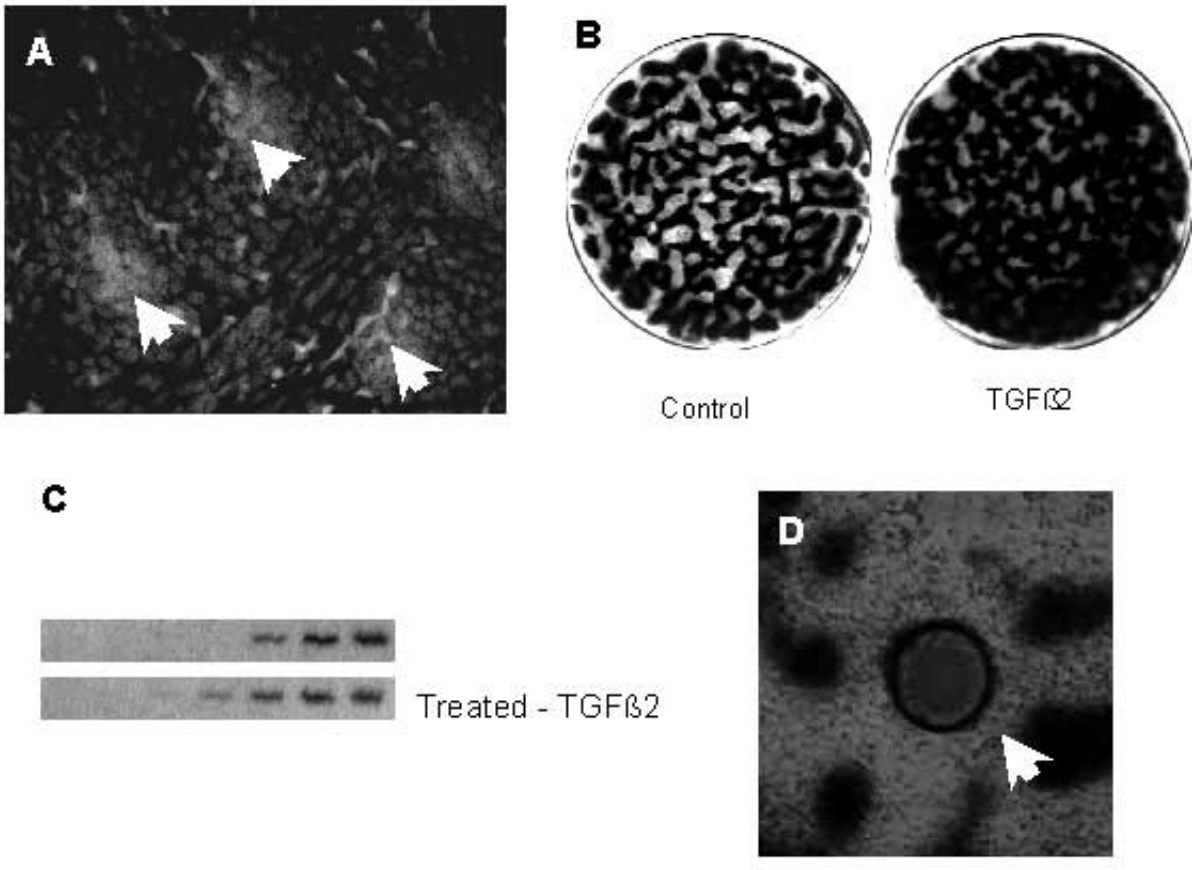

Fig. 6. Role of TGF beta 2 protein in limb bud micromass culture. (A) TGF beta 2 protein is localized at the region of chondrogenesis (arrowheads). (B) If we add TGF beta 2 to the culture medium, it promotes chondrogenesis. (C) Addition of TGF beta 2 promotes the production of tis own. (D) If we locally add TGF beta 2 (arrowhead), it laterally inhibits the formation of cartilage.

(1) localization at the chondrogenic region,
(2) promotion of chondrogenesis,
(3) promotion of its own production (Positive feedback),
(4) inhibit chondrogenesis in the neighbor when it is applied locally (lateral inhibition).

Therefore, we conclude that this molecule is a very good candidate for activator molecule in this system (Miura and Shiota, 2000b).

5. Future Prospects

We need to clarify whether the pattern formation *in vitro* can be correlated to *in vivo* situation. In the past, it has been argued that the *in vitro* pattern is independent of *in vivo* pattern because cell sorting was not observed *in vivo* (Cottrill *et al.*, 1987). However, we are skeptical to their opinion because they did not consider of the reaction-diffusion process,and because we showed that reaction-diffusion is more likely to take place *in vitro*. Currently we do not have any data on the relationship between *in vitro* and *in vivo* pattern formation. Direct proof is difficult, so we need to accumulate experimental evidences, for example, that the same teratogenic reagents or gene knockdown results in the same pattern change both *in vitro* and *in vivo*.

We also need to specify what the inhibitor is. There are two methods to determine inhibitor. One is picking up candidate for inhibitor molecule from literatures and examine the characteristics of each candidate. Now we have several good candidates for inhibitor (Wnt, PTHrP etc.), so by examining the localization and interaction with TGF beta 2 we can verify whether these factors act as an inhibitor. The other method is to screen the genes that has different expression pattern by application of TGF beta 2. Since the "inhibitor" molecule does not necessary consists of a single molecule, this approach becomes more important in the future.

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