# Developmental Potential for Morphogenesis In Vivo and In Vitro

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*ABSTRACT*    Development is a complex process that involves differentiation into a variety of cell types. In spite of its complexity, the macroscopic pattern and cell types are robust to environmental and developmental perturbations. Even in vitro far from normal developmental conditions, ten normal tissues have been generated from *Xenopus* animal caps by successive treatment with activin and retinoic acid (RA). To describe both normal development and in vitro organogenesis, we introduce developmental potential following the pioneering study by Waddington. This potential value represents changeability of a cellular state, which decreases toward a local minimum through development. The attraction to a particular cell type through development is described as a process to decrease the potential value to its local minimum. By choosing an explicit potential form as a function of the concentrations of treated activin and RA, the concentration dependence of in vitro organogenesis is reproduced. The potential landscape is shown to have several local minima, each of which represents a stable cell type. This potential also explains why the induction of given tissues requires more treatment of activin at later stages. The consequences of the developmental potential hypothesis encompass the robustness of each tissue generation, the loss of competence through development, and the order of tissues in induction by tissues, which we have confirmed experimentally for in vitro organogenesis. The developmental potential hypothesis for a global description of early development is crucial to understanding the robustness of morphogenesis and explains the achievement of in vitro organogenesis using few molecules as well. *J. Exp. Zool. (Mol. Dev. Evol.) 310B:492– 503, 2008.*    © 2008 Wiley-Liss, Inc.

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Our understanding of development has expanded greatly with advances in knowledge of the gene networks that control this process (Davidson, 2001; Stathopoulos and Levine, 2005; Levine and Davidson, 2006). Numerous molecular processes have been elucidated. Surprisingly, the macroscopic developmental process is rather robust despite the inherent complexity: the spatial configuration of generated tissues, existing cell types, as well as their gene expression patterns are invariant in the face of the noise during developmental process that originates from stochastic

gene expression linked to intra-cellular reactions and environmental fluctuations (Waddington, '42, '57, '66; Nijhout, 2002; Forgacs and Newman, 2005; Kaneko, 2006). The reason why complex morphogenesis at the macroscopic level is so robust remains to be elucidated.

In discussing the differentiation and the robustness of different cell types, Waddington ('42, '57, '66) introduced the concept of *canalization*. A given cell state is the consequence of gene expression dynamics. The determination of a particular cell state reflects orientation toward a given gene expression pattern that corresponds to the cell type. Accordingly, the developmental process can be described schematically by the locus of a ball falling down an epigenetic landscape (Fig. 1), in which the axis represents deviation (in gene expression pattern) from the given cell type. According to the scheme of Waddington, the ball approaches a local minimum of the landscape, which describes differentiation to the cell type. Even if perturbed, the ball is attracted to the original minimum, which explains the robustness of the cellular state. The landscape shows canalization through development, such that novel minima appear successively, as shown in Figure 1. Several attracting states are generated in time, each of which corresponds to a differentiated cell type. Although this epigenetic landscape with canalization clearly explains both the differentiation and the robustness of each cell type, it is not yet clear how this epigenetic landscape can be explicitly determined in terms of controlled experiments. Furthermore, neither the meaning of the spatial axis in Figure 1 nor the number of

dimensions of the landscape required for the description of embryogenesis is known so far.

The choice of specific axes in development is an important topic in morphogenesis research. The body plan of the embryo consists of the dorso-ventral, the anterior–posterior, and the left-hand axes. Following the classic discovery of the organizer by Spemann and Mangold ('24), phenomenological models to describe morphogenesis along spatial axes have been proposed. Among these models, there is the pioneering study of Yamada ('39, '40, '58), who first introduced the idea of potential to describe cell plasticity, based on his transplantation experiments. From the ordering of the potential values along the spatial axes, he presumed the existence of two types of hypothetical chemical gradient in the early embryo, one for the dorso-ventral axis and the other for the anterior–posterior axis (Yamada, '58). He also proposed that each tissue of the early embryo is induced according to these two gradients. The theory put forward by Yamada was further developed by Toivonen and Saxen ('61), such that the gradients were explicitly assigned to the factors of Mesdermagenz and Neuralagenz. These gradients were later adopted by Wolpert ('69) in his positional information theory.

Yamada and Toivonen described the ordering of tissues along the axes as a result of chemical gradients. Considering the macroscopic robustness of development, the tissues organized along these axes should be attracting states, such as those in Waddington's valleys. However, neither a relationship between these spatial axes in development and that in Waddington's landscape nor a dynamical process that attracts to each tissue has been elucidated to date.

Considering the attraction of cells into the local minima of the potential, it becomes apparent that tissues can be generated in vitro under artificial conditions. If normal tissues are generated by a condition far from in vivo condition, it gives a strong support for robustness in development. In this study, we refer to experiments carried out by our group (Asashima et al., '89, '90; Nakano et al., '90; Ariizumi et al., '91, 2003; Moriya et al., '93, 2000; Okabayashi and Asashima, 2003). We used a mass of ectodermal cells of the amphibian blastula, termed as the "animal cap," which has pluripotency. Activin was identified as a potent inducer for all types of typical mesodermal tissues, as confirmed using the animal cap assay (Asashima et al., '89, '90); nodal is known to have a similar effect. Activin (or nodal) induces
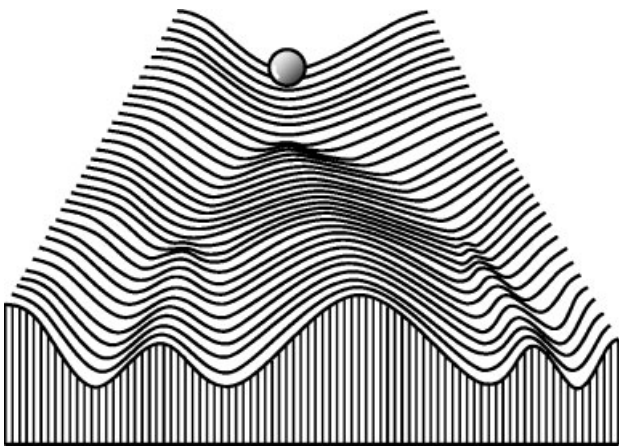


Fig. 1. Schematic diagram of Waddington's epigenetic landscape. In this scheme, canalization of the potential landscape leads to cell differentiation. Adapted from Waddington ('57).

mesodermal tissues in a concentration-dependent manner (Nakano et al., '90; Ariizumi et al., '91). Under in vitro culture conditions, intact animal caps are formed from atypical epidermis within a few days. Following treatment with 0.3–1.0 ng/mL activin, animal caps differentiate into ventral mesodermal cells and tissues, such as blood cells, coelomic epithelium, and mesenchymal cells. Treatment of animal caps with 5–10 ng/mL activin results in differentiation into muscle, whereas treatment with 50–100 ng/mL activin induces animal caps to differentiate into notochord (the most-dorsal mesodermal tissue) and anterior endodermal tissue (Asashima et al., '90; Ariizumi et al., '91; Okabayashi and Asashima, 2003).

This ordering of generated tissues is also observed by increasing the duration of activin treatment, rather than increasing the concentration of activin. In approximate terms, the effect of activin treatment is a function of concentration multiplied by duration time, over certain ranges of time and concentration. Importance of both the concentration and the duration of exposure of morphogen was theoretically discussed by Meinhardt ('78).

A series of studies has established various induction methods for the generation of organ-specific tissues from animal caps. For example, following 5 hr of treatment of dissociated *Xenopus* animal cap cells (approximately 1,000 cells in total) with 100 ng/mL activin, re-aggregated cells differentiate into autonomously beating cardiac tissues within a few days (Ariizumi et al., 2003). Kidney tissues, such as pronephric tubules and ducts, are formed in animal caps by simultaneous treatment with 10 ng/mL activin and $10^{-4}$ M retinoic acid (RA) (Ariizumi et al., 2003). Treatment with 100–400 ng/mL activin and culturing for 5 hr, followed by treatment with $10^{-4}$ M RA, result in the regeneration of pancreatic tissues (Moriya et al., '93). In the *Xenopus* embryo RA is distributed in a concentration gradient along the antero-posterior axis. This method of induction of pancreatic tissues (posterior endodermal tissue) demonstrates that the anterior endodermal tissue induced by high concentrations of activin is posteriorized by RA.

To sum up a series of experiments, more than a dozen tissues have been regenerated, simply by changing the concentrations of activin (or nodal) and RA used to treat pluripotent amphibian cells. These tissues have been generated in a manner that relies on the addition of different concentrations of activin and RA, as summarized in the

phase diagram displayed in Figure 2. The ordering of tissue generation in relation to increases in the concentration of imposed activin corresponds approximately to the animal–vegetal axis and is correlated with the dorso-ventral axis, although the correspondence of this "activin axis" with the dorso-ventral axis is not necessarily monotonic. On the other hand, increasing the RA concentration leads to changes in tissues along the antero-posterior axis, and thus the "RA axis" corresponds approximately to the antero-posterior axis. It appears that the activin and RA axes correspond to two-dimensional axes for the spatial configuration of tissues in normal development.

We note that the in vitro processes mediated by activin and RA do not reflect the in vivo situation. In vivo, such high concentrations of these molecules are not realized, and the in vivo developmental process is controlled by many other signaling molecules. These signaling molecules have synergetic effects on differentiation, so that the concentration of each molecule is lower than that adopted in our embryogenesis experiment. In spite of this difference, it is remarkable that the final tissue generated is normal and functions in the same way as that generated in vivo. Even though the transient developmental process required to reach the final pattern may be different from that in vivo, the final goal is identical. This strong robustness is concluded from the in vitro organogenesis experiment controlled by activin
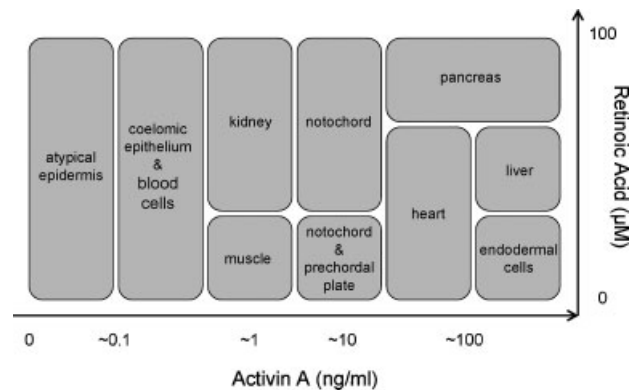


Fig. 2. In vitro embryogenesis. A summary of our experimental results (Asashima et al., '89, '90; Nakano et al., '90; Ariizumi et al., '91, 2003; Moriya et al., '93, 2000; Okabayashi and Asashima, 2003) is shown in the form of a phase diagram, in which different tissues are generated depending upon the activin and retinoic acid concentrations. *Xenopus* presumptive ectodermal explants (animal caps) were isolated, and various concentrations of activin A (abscissa) were applied, followed by retinoic acid (ordinate), resulting in differentiation into various types of cells, tissues, and organs.

and RA, which suggests the existence of an attracting state, as proposed by Waddington.

Now we come back to the problem of axes in the epigenetic landscape. From our controlled in vitro organogenesis, Waddington's landscape may be extracted in terms of measurable quantities. Our in vitro organogenesis of the early embryo is controlled by just two variables, the concentrations of activin and RA, which approximately correspond to the antero-posterior and dorso-ventral axes. Thus, it is suggested that the epigenetic landscape at the early stage of development is represented explicitly on a two-dimensional space by these axes. In this case, the molecules that represent the two axes need not necessarily be activin and RA. Other molecules, such as nodal, can have similar effects. We have used activin and RA, as they give prominent effects and have been studied extensively. It is important to note that a two-dimensional space is sufficient to describe the epigenetic landscape for early development.

The key novelty here, compared with Waddington's landscape that does not have explicit assignment of the axis, is the incorporation of physical space via the gradients of activin and RA. Furthermore, additional information is extracted from our experiment concerning the shape of Waddington's landscape. In this study, we will introduce a potential that corresponds to the landscape, which represents the plasticity of a cellular state. Using this assignment, the depth of each valley will be discussed in relation to the activin or RA concentration required to generate each tissue.

Understanding the formation of an appropriate epigenetic landscape through development is important. Information on the potential dynamics can be extracted from our organogenesis experiment. As the concentrations of activin required for organogenesis increase with the stage of development, we can examine how the potential is shaped over time. On the basis of Figure 2, it is possible to depict an abstract Waddington's landscape in a quantitative manner by using the activin and RA axes. In contrast to metaphorical landscape by Waddington, explicit meanings are assigned to the axes and peak heights, as discussed below.

In the next section, we introduce an explicit form of potential and its dynamics, so that normal (early) development and in vitro organogenesis by activin and RA can be explained consistently. Several consequences of the developmental potential theory are confirmed experimentally. In addition, direct measurement and theoretical derivation of developmental potential are provided and applications to other developmental processes are proposed.

## THE DEVELOPMENTAL POTENTIAL HYPOTHESIS

Considering Yamada's diagram, Waddington's epigenetic landscape, and the in vitro reconstruction of tissue organogenesis, we introduce the notion of "developmental potential $V$." $V$ is assigned to a cell within a given tissue, and ultimately should be represented in terms of the gene expression of a cell (see below). $V$ is not necessarily representative of a single isolated cell but is given for an ensemble of cells. As cells are not necessarily homogeneous even within a given tissue, we do not distinguish between the cells in a given tissue in assigning $V$ to a cell.

Considering the relaxation process to a cellular state ("attractor"), it is natural to introduce potential $V$ (Fig. 1) for each state of the cell. The important assumption here is the existence of a "global" potential $V$ for all the cell states, which is the function of a few variables, as shown below. As in a motion of a ball falling along a valley, each cell goes toward a state with lower $V$. Biologically, $V$ is assumed to represent the plasticity of a cell, i.e., the changeability of the cell state in a given tissue. Here, we adopt the term potential, by referring both to the concept in physics for motion and to that in biology for changeability or plasticity.

A cellular state with a higher value of $V$ is more easily changed to other states with smaller $V$ values as the result of an external stimulus, whereas a cellular state with a lower $V$ value is scarcely changed. Considering the experimental results discussed in the first section, we postulate the following for potential $V$:

(i)    The value $V$ represents changeability (plasticity) and is a function of cell state, which is represented by a few variables (see below).

(ii)   To each of the local minima of potential landscape $V$, there corresponds a stable cell state of a given tissue, following Waddington's epigenetic landscape. The configuration of the minima provides a fate map (see Fig. 3).

(iii)  Differentiation from one cell state to another is represented by a transition from one valley of potential to another across a barrier of potential $V$. If the potential barrier between two local minima is smal-

ler, transformation between the two corresponding cell states is more feasible. If the potential barrier is higher, the transition probability is lower.

(iv) Through the developmental process, the potential valley is deeper, i.e., the value of each local minimum in $V$ generally decreases with time, corresponding to a process from totipotency to multipotency, and thereafter to committed cell types. This decrease makes the barrier between two minima higher (see Fig. 4).

As mentioned above, the potential is assumed to be a function of only a few variables. We consider the morphogenesis of cells placed in a two-dimensional space $x$, $y$. Then, the state is given as a function of $x$, $y$, so that the potential $V(x, y)$ is a function of two variables, as depicted in Figure 3. Each valley of the final form of $V(x, y)$ corresponds to the cells in each tissue.

To represent the axes $x$ and $y$, we refer to the actions of activin and RA. We assume that the activity of activin works as a ''force'' to shift the cell state toward a higher value of $x$, whereas RA acts to shift the cell state toward a higher value of $y$, so as to overcome the potential barrier in each direction. Now, considering the phase diagram in Figure 2, we postulate that the potential has several minima (shown in Fig. 3), while each of the potential minima corresponds to the generated tissue in Figure 2, i.e., to a fate map at the early stage of development. In Figure 3, we further assume that the potential valley in $V(x, y)$ becomes

deeper with $x$ and $y$, as the organogenesis of tissues at larger $x$ or $y$ values requires higher concentrations of activin or RA. The potential shape in Figure 3 is introduced so that it is consistent with Figure 2. As the potential value represents changeability or plasticity of a cell, it is natural that the value for a differentiated cell decreases as the determination of a cell state progresses through development.

Next, consider the dynamics of the potential itself. As the cells are initially undifferentiated, the temporal change from an almost flat potential $V(x, y)$ to a structure with several valleys (Fig. 4) should occur. Considering that the variation in the potential increases with time, as postulated by (iv), the potential changes from an almost flat pattern to the final pattern (shown in Fig. 3) are displayed in Figure 4A–C (see also Supplemental Video at http://chaos.c.u-tokyo.ac.jp/study/JEZB-info.html). The depth of each potential valley increases with time. Indeed, this increase is consistent with the observation that more activin is required for organogenesis at a later stage of development. As the tissue is generated along the axis in time, the depth of the valley has to increase along the axes, so that the final pattern in Figure 3 and the temporal course in Figure 4 are the only possible choices.

It may be more natural to assign dorso-ventral and antero-posterior axes to the $x$- and $y$-axis for the potential. Instead, we used an ''activin axis'' for $x$ and an ''RA axis'' for $y$ as they are operationally defined in relation to the controlled experiment. However, these two assignments correspond to
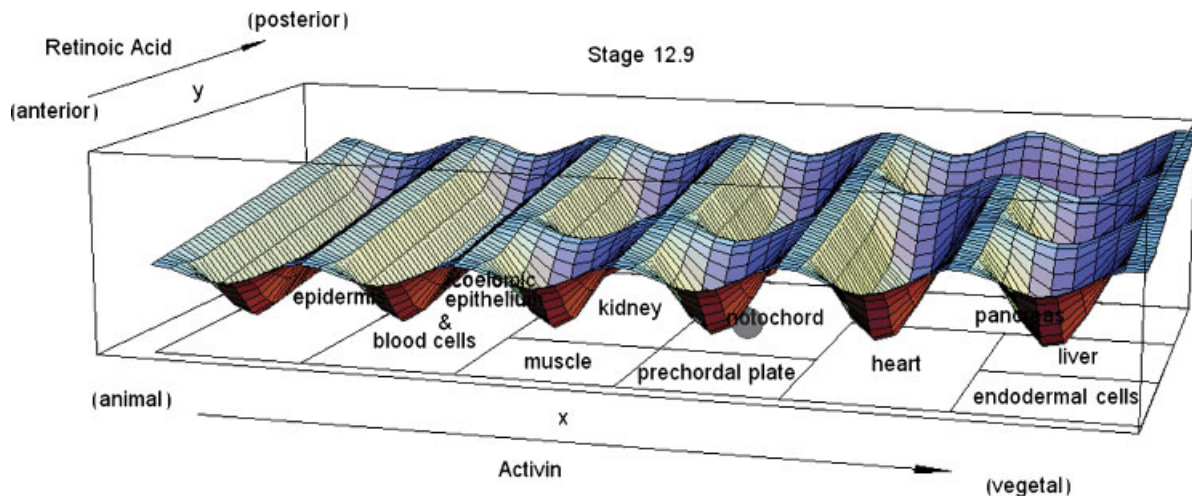


Fig. 3. Developmental potential in relation to normal development and in vitro organogenesis caused by activin and RA, with the corresponding fate map. The potential form at the end of the early developmental stage is plotted as a function of the two axes, which correspond to the concentrations of activin and RA. RA, retinoic acid.
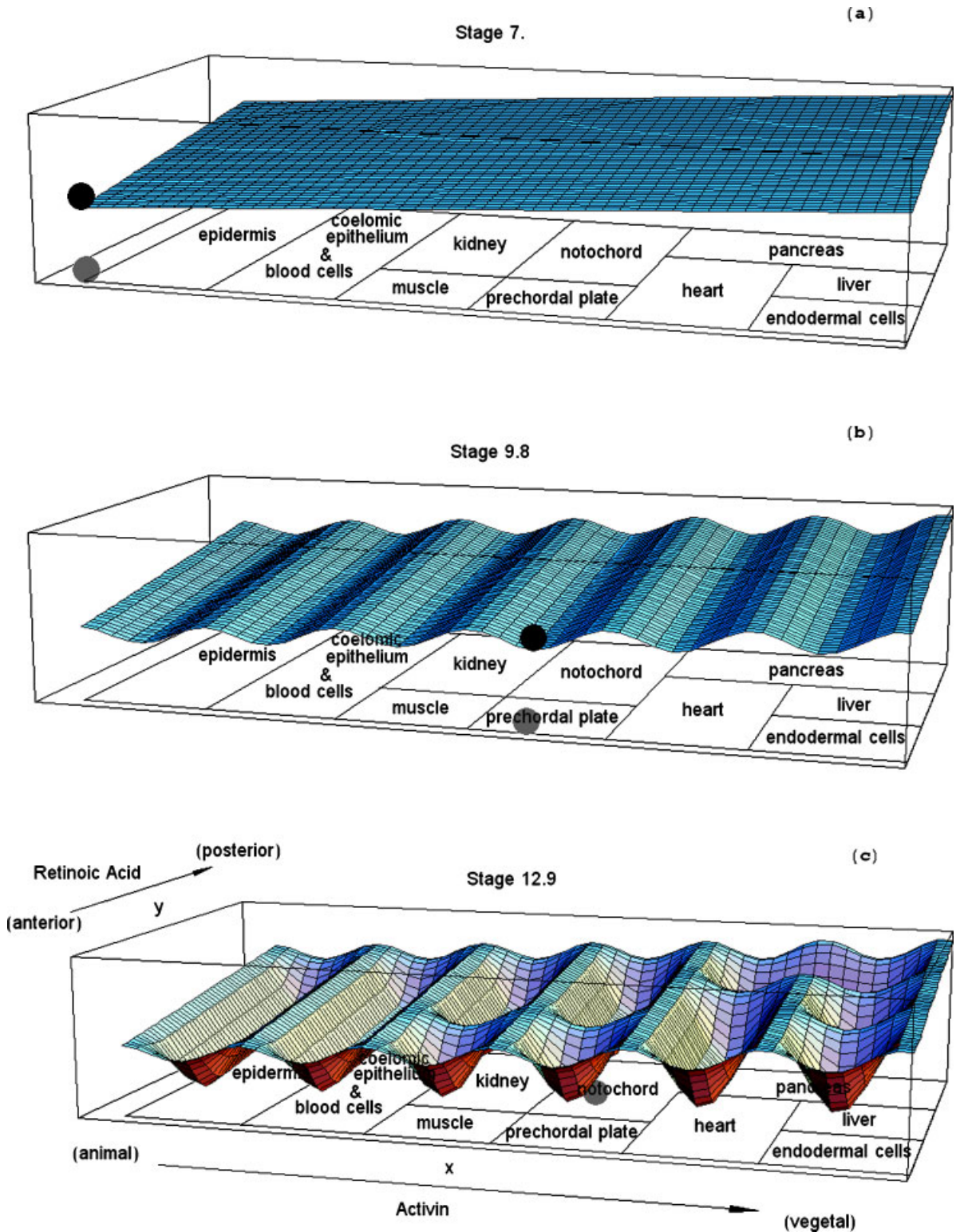
Fig. 4. Temporal evolution of developmental potential *V* through the early stage of development, in accordance with the experimental results obtained (Asashima et al., '89, '90; Nakano et al., '90; Ariizumi et al., '91, 2003; Moriya et al., '93, 2000; Okabayashi and Asashima, 2003). The potential shape changes in time through the developmental course: (**A**) stage 7, (**B**) stage 9.8, and (**C**) stage 12.9 of amphibian development.

each other. The activin axis is mapped onto the dorso-ventral axis by suitable transformation, whereas the RA axis approximately corresponds to the antero-posterior axis. We also note that a different set of chemical concentrations could be adopted for the axes, which is represented by the transformation of "coordinates" and does not lose information on morphogenesis.

The "motion" along the developmental potential represents attraction to a given cellular state following the gene expression dynamics. Our assumption implies the existence of a potential function that can be applied globally to all cell types. Indeed, the obtained potential landscape has a rather simple structure, with monotonic decreases in the potential values of local minima both in space and time. Temporal ordering supports the loss of plasticity or competence through development, which enhances the stability of each cellular state. Spatial ordering is observed as a monotonic decrease in the potential minima along the axes (Fig. 4). By simply changing the concentrations of activin and RA, generated tissues are simply shifted downwards along these potential values. The values of the local minima of the potential are ordered along each axis (Fig. 3).

For the moment, we do not specify what the potential value $V$ indicates in terms of directly measurable quantities; possible measurable candidates for the potential value will be discussed later. They include competence to signals and the degree of methylation in DNA or chromatin.

## CONSEQUENCES OF THE DEVELOPMENTAL POTENTIAL HYPOTHESIS

The basic hypothesis underlying our phenomenological description is the existence of a scalar potential $V$ that characterizes the plasticity of cells. Following the order of the magnitude of $V$, tissues are ordered with regard to the degree of this plasticity, i.e., the changeability of the cell state. Our hypothesis has several consequences that are either consistent with previous observations or are testable.

First, as a stable tissue exists only at each local minimum of the potential, cell types other than the normal tissues cannot exist, even under "artificial" in vitro developmental conditions. For example, when "intermediate" activin concentrations that lie between those used for the two neighboring tissues in Figure 2 are used, a mixture of the two tissues is generated (Ariizumi

et al., '91). No other stable states exist even for an intermediate concentration of activin. For example, the addition of activin at about 0.5 ng/ml leads to the formation of blood cells, whereas less than 0.1 ng/ml leads to atypical epiderm generation, and treatment with activin at less than 0.25 ng/mL gives a mixture of both generation, and treatment with activin at less than 0.25 ng/mL gives a mixture of notochord and muscle (for the fraction of each cell type obtained as a function of activin concentration, see Fig. 5). This attraction to a cell type corresponding to each tissue has been confirmed by direct macroscopic observations of cells, and is consistent with the data obtained for the gene expression patterns (Sedohara et al., 2006). Even though some genes are artificially activated, the expression pattern eventually settles to that of a cell type in each tissue. This supports our viewpoint that generated tissues are stable attracting states that are represented by local minima of the potential function.

The second consequence of our theory concerns the degree of external stimulus needed for transformation from one stable state to another through crossing a potential barrier (iv). The higher this barrier, the larger the amount of stimulus required. Thus, transformation from a tissue at a given valley to that at a more distant valley requires a larger amount of external influence than transformation to a tissue at a closer valley. This is simply the ordering of activin
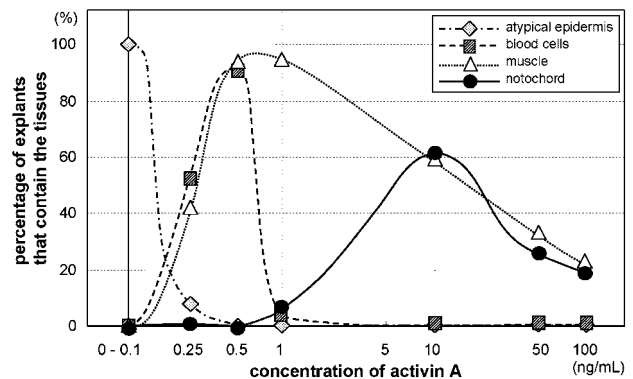


Fig. 5.  Dose-dependent induction of mesodermal tissues by activin. Graphical representation of the differentiation of tissues in explants treated with activin. Curves show the percentage of explants that differentiated into the tissues indicated above in total explants treated with each concentration of activin A for 1 day. Each peak of the curves indicates that 100% of the explants contained each respective tissue. As the concentration of activin A increases, the differentiation of the explant changes from ventral type (coelomic epithelium and blood cells) to dorsal type (notochord). The graph is based on the data reported by Ariizumi et al. ('91).

concentration required for each tissue (Fig. 2). Indeed, the potential is introduced so as to be consistent with our in vitro organogenesis model. Here, the stimulus needed to cross the barrier is represented by the product of the activin concentration and the duration of treatment. Its influence is integrated over the time span of treatment.

Third, we consider a consequence of temporal development of the potential. From the potential dynamics given in Figure 4, it is predicted that at a later stage of development, a higher concentration of activin is needed to generate the desired tissue. Indeed, previous reports have shown that competence for mesoderm induction by activin is stage-dependent. For example, treatment of animal cap cells with activin at the mid-blastula stage induces mesoderm formation, whereas treatment at the mid-gastrula stage does not induce mesoderm formation, which has been termed as "loss of competence" by Grainger and Gurdon ('89) and Grimm and Gurdon (2002). In particular, for animal caps dissected from stage 8 embryos, 2 hr of treatment with 5 ng activin caused elongation, whereas no superficial changes were observed following activin treatment of animal caps derived from stage 11 embryos (Grimm and Gurdon, 2002; Abe et al., 2005).

The extent of the external stimulus necessary for the generation of each tissue thus estimated is also relevant to the normal developmental process. During gastrulation, cells that are originally distant from each other are placed adjacent to each other and start to interact. This leads to induction toward new tissues. As the duration of the interaction is prolonged, the effect of this novel interaction is increased. Thus, the ordering of the generated tissues according to contact time must agree with the ordering of the depths of the potential valley of the tissue. In fact, the ordering of tissues according to contact time agrees with ordering according to activin concentration in the in vitro organogenesis. For example, cells of head organizer first involuted and emit inducible signals for a long period of time, whereas trunk–tail organizer, which is subsequently in contact with ectodermal cells, is under the influence of signals for a shorter period of time.

This ordering of tissues as to be liable to be induced is also observed in experiments with sandwiching cells treated with two different activin concentrations. When an animal cap treated with activin for 5 hr is placed between two normal caps, the sandwich explant differentiates into a head structure. On the other hand,
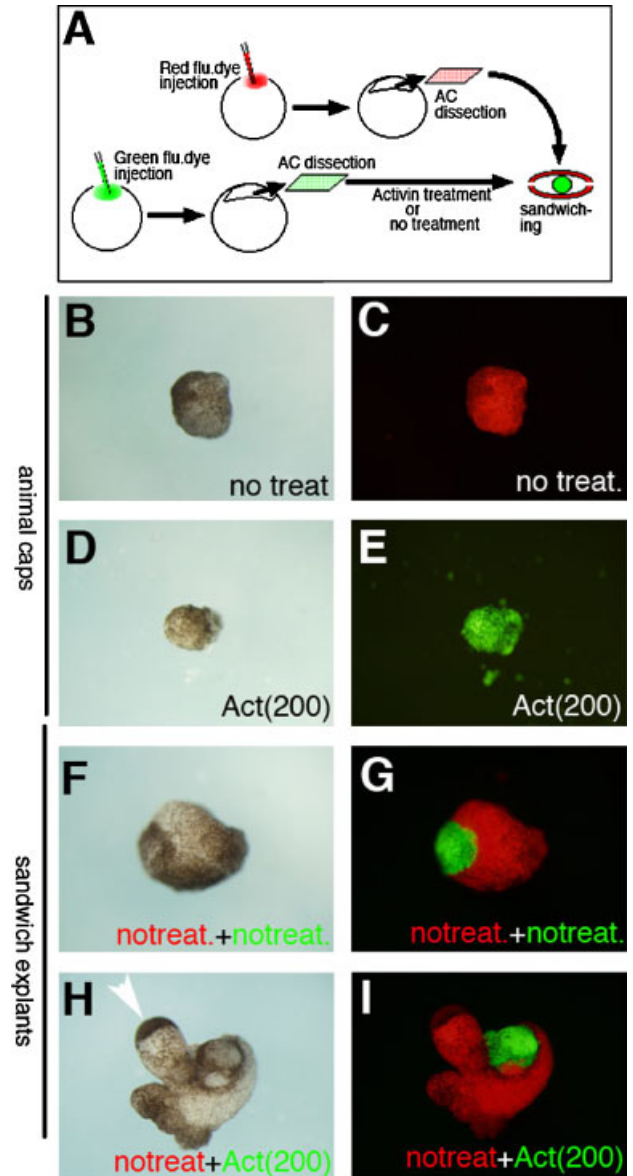


Fig. 6. The effect of activin treatment on animal caps. (**A**) Schematic diagram of a sandwich experiment. (**B–I**) Developed patterns of cells. (B, D, F, H) Bright-field images. (C, E, G, I) Fluorescent images that merge the red and green signals. (B–E) *Xenopus* animal cap cells without sandwiching. (B, C) Animal caps without treatment (red). (D, E) Animal caps treated with 200 ng/mL activin (green). (F, G, H, I) Sandwich explants. (F, G) A normal cap (no treat.; green label in G) sandwiched between two normal caps (notreat.; red label in G). (H, I) An activin-treated cap [Act(200); green label in I] sandwiched between two normal caps (notreat.; red label in I). Details of the procedure followed are provided in the Materials and Methods section.

when an animal cap treated with activin for only 2 hr is placed between two normal caps, the sandwich explant differentiates into a trunk–tail structure (Ariizumi et al., '91).

Fourth, the manner in which cells are liable to be induced is derived from the potential $V$, as the potential barrier gives a degree of external operation needed for differentiation to other cell types. Consider the operation of placing two different tissues adjacent to one another. According to the interpretation of $V$, it is expected that the tissue with the higher value of $V$ is more liable to be changed. Thus, cells with higher $V$ values are induced by those with lower $V$ values, but not vice versa.

Such ordering of liability to be transformed (Kaneko, 2006) can also explain the ordering of induction during the normal developmental process. If tissues $A$ and $B$ with $V(B) > V(A)$ are placed adjacent to one another, tissue $B$ is liable to be changed. For example, consider mesodermal cells [$V(A)$ in this case] that lie beneath ectodermal cells [$V(B)$] during gastrulation. In the developmental process, mesodermal cells secrete neuralizing factors, resulting in the induction of ectodermal cells to become neural tissues. In contrast, there has been no report to date that ectodermal cells cause mesodermal cells to induce any tissues, such as somites and notochord. This is consistent with our potential form satisfying $V(B) > V(A)$.

Ordering on the changeability according to the value of potential $V$ has been confirmed by sandwiching cells that are treated with activin (see Fig. 6A). Recall that animal cap cells normally differentiate into atypical epidermis without any treatment (Fig. 6B, C), whereas treatment of an animal cap with 200 ng/mL acitvin A induces endodermal tissue (Fig. 6D, E). When an untreated cap was placed between two untreated caps, no obvious change was observed (Fig. 6F, G). On the other hand, when an animal cap treated with 200 ng activin was sandwiched between two untreated caps, the cell types of the untreated caps (red cells in Fig. 6I) were dramatically changed, whereas those of the activin-treated caps were not superficially changed (green cells in Fig. 6I). Animal caps treated with activin (i.e., having a lower $V$ value) induced changes to nontreated animal cap cells having a higher $V$ value), whereas the converse was not true. This agrees with our prediction that changes to cells with larger $V$ values are induced by cells with lower $V$ values, but not vice versa.

## MATERIALS AND METHODS

### Sandwich analysis of Xenopus animal caps

At the two-cell stage, 5 ng of Alexa 488 (Invitrogen, Carlsbad, CA; gives green fluorescent signal) or Alexa 594 (red fluorescent signal) was injected into the animal pole, and the embryos were cultured. In this experiment, Alexa 594-injected embryos were dissected at stage 8 and the dissected animal caps were treated for 2 hr with $1 \times$ Steinberg's solution that contained 200 ng/mL of activin A. The Alexa 594-injected embryos (stage 8) were dissected for their outer caps at the completion of activin treatment. The inner caps were sandwiched between two outer caps and cultured to the appropriate stage. Explants were observed using fluorescence stereomicroscopy (Olympus Tokyo, Japan).

## DISCUSSION

By introducing the notion of developmental potential, we have succeeded in explaining coherently both in vitro and in vivo organogenesis. In contrast to the abstract epigenetic landscape proposed by Waddington or the potential put forward by Yamada, our potential is derived explicitly from experimentation. Considering that development is a highly complicated process involving a large number of genes, it is rather remarkable that so many aspects of the developmental process can be explained in terms of a single potential $V$. Indeed, the success of the organogenesis experiment itself (Ariizumi et al., '91, 2003; Moriya et al., '93, 2000; Okabayashi and Asashima, 2003; Sedohara et al., 2006) suggests the existence of such a potential.

This developmental potential $V$ explains both normal morphogenesis and organogenesis owing to physiological changes caused by external stimuli. In this sense, both the developmental and physiological changes are coherently explained as processes that decrease the value of $V$, which lends support to the correspondence between physiology and development that is often discussed (Waddington '53, Kirschner and Gerhart, 2005) following Waddington ('57).

We have assigned the axes in the potential landscape explicitly in terms of the concentrations of molecules required for in vitro organogenesis. These axes correspond to the dorso-ventral and antero-posterior axes in early development. It is remarkable that early development can be represented by a two-dimensional landscape. On the other hand, representation by only two axes is relevant to the robustness and controllability of the developmental process.

The obtained potential landscape has a rather simple structure. The value of local minima (which

corresponds to stable cell types) decreases both in space and time. This "funnel-like" landscape was first proposed for protein folding as a result of consistency between local attraction to a stable state and global relaxation dynamics (Abe and Go, '80; Onuchic et al., '95), where the potential value is simply the free energy. Although the free energy is not relevant in our case, the existence of global potential implies that there is a single quantity that characterizes a cell state, which we term the plasticity (changeability) of a cell.

In gene expression dynamics, a funnel-like landscape has been discussed recently (Li et al., 2004). Evolution to a funnel-like potential landscape has been confirmed in numerical evolution of gene expression dynamics under noise (Kaneko, 2007). The evolution of the dynamics shapes robustness to noise encountered during developmental process. Such robust dynamics is achieved by smooth and global attraction to a given target gene expression pattern (Siegal and Bergman, 2002; Wagner, 2005). Thus the funnel-like structure is shaped through the evolution. As a developmental process is an outcome of successive evolution over generations (Waddington, '42, '57, '66; Nijhout, 2002; Forgacs and Newman, 2005; Kaneko, 2006) and should be robust to noise, the existence of a funnel-like potential in developmental dynamics is a natural consequence.

In dynamical systems theory, the process of decreasing $V$ is expressed by the successive attraction of orbits into some domains (attractors) in the phase space (Waddington, '57; Kaneko, 2006). Correspondence of attractors with cell types was put forward by Kauffman ('93) (see also Huang et al. (2005)), whereas *isologous diversification* (Kaneko, 2006) theory explains the decrease in plasticity with the attraction of states in the phase space for gene expression dynamics, which is triggered by cell–cell interaction and increases in cell number. The process displayed in Figure 1 is represented as an attraction to each stable state by intra–inter-cellular dynamics, as schematically shown in Figure 7. Here, the decrease in plasticity is a result of cell–cell interaction, which is essential for the temporal change in potential function. In fact, the theory is demonstrated by a simple interacting cell model (Furusawa and Kaneko, 2001, 2006) for development, in which plasticity is characterized by variability of chemical concentrations (gene expression). This result from a simple model suggests the possibility of measuring potential $V$ in terms of the variability of total gene expression.
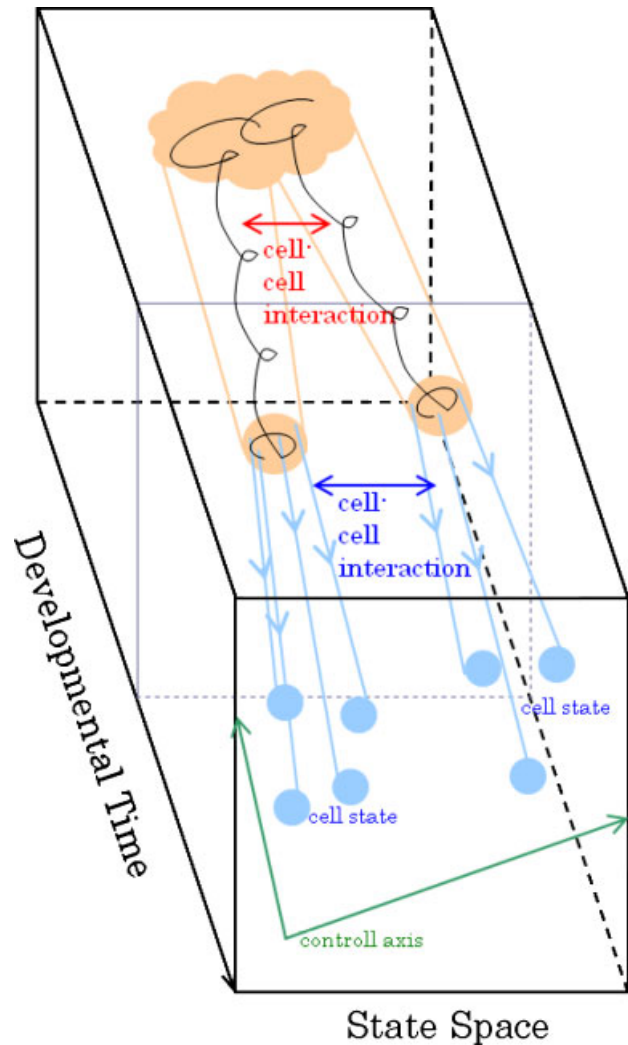


Fig. 7. Schematic representation of attraction to cell types. Through developmental process subject to cell–cell interaction, cell states are attracted into few types characterized by a set of state variables (e.g., concentrations of proteins). Selection of each cell types is controlled by a few sets of control variable. The process schematically shown here is demonstrated by numerical models with cell–cell interactions (Kaneko and Yomo, '97; Furusawa and Kaneko, 2001, 2006).

We need to consider a biological entity of potential $V$ in order to measure it experimentally. This potential represents plasticity, which is simply the degree of differentiation potency of the cell. Recently, changes in plasticity have been studied in terms of "epigenesis." At the genetic level, loss of plasticity during epigenesis is correlated with the methylation pattern of chromatin or DNA (Reik, 2007). Thus, the degree of methylation is a candidate for estimating $V$. The gene expression of methylated sites is fixed, so that the plasticity of a cell is lost successively with the

progress of methylation. Thus, the value of $V$ is expected to decrease with the methylation process.

At the cellular level, it is natural to correlate the plasticity $V$ with "competence" with respect to activin or RA (Grainger and Gurdon, '89; Grimm and Gurdon, 2002). Competence characterizes the degree to which external signals influence a cell state through signal transduction to the nucleus. Indeed, Toivonen and Saxen ('61) have proposed that competence is lost through the developmental process. Thus, a decrease in $V$, i.e., in competence, can be confirmed by measuring the stage-dependent changes in competence with respect to activin or RA.

Competence relates to changes in the receptors of a cell as well as its consequences for gene expression. The degree of plasticity is measured as the change in gene expression in response to an external stimulus. Here, the accumulation of variations of global gene expression may provide an estimate of plasticity, as it measures the changeability of the gene expression pattern.

Of course, plasticity is related to the capacity for regeneration. In our developmental potential hypothesis, regeneration is interpreted as the crossing of a potential barrier as the result of an increase in the plasticity value $V$. In fact, in a regeneration process, e.g., wound healing, some cells are dedifferentiated and novel differentiated cell types are produced from these cells. Thus, if the original value of $V$ is higher, the possibility for other cell types to regain differentiation potential in response to an external stimulus is increased. Indeed, regenerative ability is generally higher during the earlier stages of development, and is lost as development progresses, i.e., as the barrier to potential that increases, according to our theory.

In summary, we have proposed the potential dynamics to explain both natural early stage development and artificial embryogenesis, which involves treatment with activin and RA. The local minima of the potential describe cell types of each tissue in the fate map. Accordingly, Waddington's epigenetic landscape is represented by measured quantities derived from controlled experiments.

The comparison of our theory with experimental results is based on the early stage of amphibian development, and corresponds to in vitro embryogenesis using treatment with activin/nodal and RA, by extending the concept of Yamada's double-potential theory. Indeed applicability of the original Yamada's theory to blood cells in *Triturus pyrrhogaster* was in question (Nakamura and Matsuzawa, '67). However, formulation by poten-tial itself is not restricted to that by Yamada. The potential formulation can be straightforwardly extended by a suitable choice of axes, if there is temporal ordering in the differentiation process. For example, induction of the central nervous system by cell–cell interactions at a later stage can be similarly described by introducing an additional axis orthogonal to the activin or RA axis. In general, we expect that most developmental processes have developmental potential function with only a few variables. Plant cell differentiation, for example, is controlled by hormones, auxin and cytokinin (Aloni, '87; Fukuda, '96), and may well be described by a plasticity potential that is a function of the concentrations of these two hormones. Of course, generality of such a potential view, which we expect as a consequence of evolution of developmental robustness, needs to be examined in future.

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