Perspective

Quantitative Analysis of Developing Tissues

Stanislav Y. Shvartsman
Dept of Chemical Engineering and Lewis-Sigler Institute for Integrative Genomics, Princeton University, Princeton, NJ 08544

DOI 10.1002/aic.10525
Published online April 5, 2005 in Wiley InterScience (www.interscience.wiley.com).

Introduction: Tissue Engineering and Embryonic Development

Tissue engineering as a field emerged over a decade ago, and its aim was defined as design and repair of tissues and organs.[1] Tissues are hierarchically structured, dynamic, and complex materials, composed of multiple types of cells and extracellular components. How can they be built in the lab? Currently, the predominant approach to building tissues is through assembly from parts, i.e., cells and natural or synthetic polymers that define the extracellular environment. Some impressive examples of this approach include "ink-jet" printing of stable three-dimensional cell aggregates with organ-like morphologies[2] and assembling heart tissue from isolated myocytes.[3] Increasingly complex materials with structural and functional attributes of living tissues are already being designed by combining "custom-made" cells and environments.[4]

Living tissues are the gold standard for any man-made materials. Living tissues, however, are not assembled from parts, but rather develop from simple components in a self-organizing process that involves active interactions between parts and irreversible modifications. Thus, tissue development and organogenesis are strikingly different from the assembly of a car or a computer: the end result does not equal the sum of the ingredients. Such an idea should come naturally to cooks and chemical engineers. To understand the nature of this qualitative change, one must look at embryonic development, where multiple cell fates and tissue architectures are established with remarkable speed and precision. Lessons derived from embryogenesis can lead to new strategies for tissue engineering.[5]

Both the number of cells and their diversity increase dramatically during the progression from a fertilized egg to an adult organism. The increase in the number of cells requires control of cell proliferation and death, while the increase in cell diversity requires control of cell differentiation. The elaborate architectures of living tissues depend on tight spatiotemporal regulation of these processes. From molecular studies of development,[6] tissue- and organogenesis emerge as control systems with levels of regulation that range from a few nucleotides in the sequence of a gene to hundreds of cells in a three-dimensional tissue. At this level of complexity, quantitative approaches are critical for interpreting the rapidly accumulating data and for design of new experiments. Today, quantitative analysis of embryonic development is becoming feasible due to the advances in developmental genetics that enable direct tests of quantitative models. As the confidence in these models increases, they can be used to guide the design of man-made tissues.

Epithelial Patterning: a "unit operation" of embryonic development

A common mechanism for cell fate diversification in embryonic development involves patterning of an epithelial layer, whereby a spatial pattern of gene expression is established within a layer of initially identical cells (Figure 1). During the subsequent stages of development, this pattern is used as a biochemical blueprint in building of tissues and organs. For example, cells at different locations within the patterned epithelial layer will move and divide in different ways, giving rise to complex three-dimensional organs.[6]

Cell communication plays a key role in the patterning of developing tissues. Many patterning mechanisms are based on the interaction between secreted protein ligands and their cell surface receptors.[7] Typically, a group of cells secretes a protein ligand that spreads through the tissue, binding to cell surface receptors. Ligand-receptor binding initiates a sequence of biochemical and biophysical intracellular processes, known as "signal transduction", which link extracellular signals to gene expression.

Ligand transport in tissues is usually accompanied by ligand degradation, either by cells themselves or by the enzymes in the extracellular medium.[8] The combination of localized ligand production, extracellular transport, and degradation can generate a gradient of ligand concentration in the patterned tissue. As a result, cells at different distances from the source of a diffusible ligand are exposed to different ligand concentrations. The combined activity of signal transduction and gene regulatory networks inside the cell can translate different amounts of extracellular ligand into different repertoires of gene expression and, as a result, into different cell fates. In principle, even a single diffusible signal can induce multiple cell fates in a naïve epithelial layer. This position-dependent control of gene expression by secreted molecules is called morphogenetic signaling.[9]

S. Y. Shvartsman's e-mail address is stas@princeton.edu.

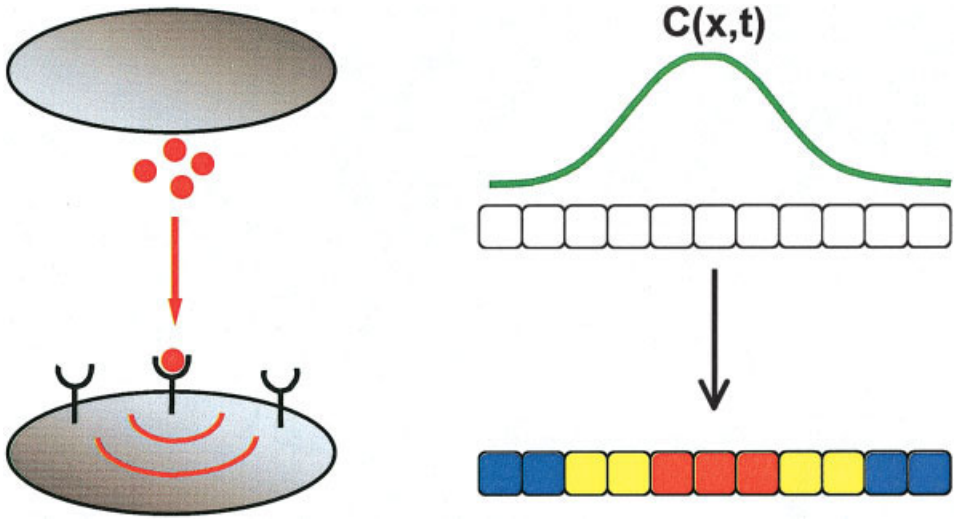

Figure 1. Pattern formation of developing tissues by diffusible chemical signals.

(A) Morphogens are diffusible chemicals that induce multiple cell fates in a field of identical cells; (B) the formation of morphogen gradients is frequently mediated by the interaction between secreted growth factors and their cell surface receptors.

While the notion of a morphogen gradient is almost 100 years old, the molecular nature of morphogens is being elucidated only now.[6] One striking conclusion of genetic studies is that the same morphogens and genetic networks are used recurrently in development, both across different organ systems within the same organism and across species. For example, molecules involved in the patterning of the fruit fly eye have human homologues that are repeatedly used in the development of human embryo. Another conclusion of genetic and genomic studies of embryogenesis is that the diversity of living tissues across species emerges from a coordinated action of a handful of distinct cell communication systems, categorized by the kinds of biochemical mechanisms of signal transduction events that link ligand-receptor binding to gene expression.

The initial response of a cell to a morphogen ligand can be followed by the activation of feedback mechanisms that establish increasingly complex patterns of gene expression in the field of responding cells.[10] For example, a cell receiving a high dose of a ligand can secrete an inhibitor that will sequester the ligand, thus interrupting the input to intracellular signaling circuitry.[11] Thus, morphogenetic signaling integrates ligand transport, signal transduction, and gene expression, are all linked by multiple regulatory mechanisms. At this level of complexity, it is highly nontrivial to determine whether a proposed mechanism is consistent with a given set of experiments or to predict the effects of new genetic and molecular manipulations. Given the increasing sophistication of genetic tools for perturbing the embryonic development, it is tempting to formulate and test the predictive models of epithelial patterning systems.

Systems with large numbers of nonlinearly interacting components are familiar to chemical engineers who are used to dealing with flames and polymerization reactors. Modern chemical reaction engineering relies on mathematical models that are formulated on the basis of the most current understanding of physics and chemistry of the system and analyzed using robust computational tools. Can something like this be attempted for pattern formation in developing tissues? The answer is "yes", and, recently, mechanistic models of embryonic pattern formation have been formulated on the basis of simple descriptions of ligand transport and signal transduction.[12-15] While none of these models can yet be used to accurately predict the outcomes genetic manipulations, the formulation of such models and their computational analysis is a critical step towards quantitative understanding of developing tissues. In the next section, we discuss the modeling and experimental issues associated with formulation, analysis, and validation of such models using the Epidermal Growth Factor Receptor (EGFR) system as an example.

Illustrative Example: Pattern Formation by Epidermal Growth Factor Receptor

The Epidermal Growth Factor (EGF) was discovered as a peptide that accelerates incisor eruption and eyelid opening in newborn mice (Cohen, 1962).[53] Shortly after its discovery, EGF and other peptide growth factors were identified in countless physiological and pathological contexts. EGF binds to a cell surface receptor (EGFR), inducing its dimerization and phosphorylation of several tyrosine residues within its cytoplasmic tail. The phosphorylated tyrosines provide binding sites for cytoplasmic proteins; this couples the activated receptor to the signal transduction cascades and, eventually, to gene expression and processes such as cell division, differentiation, or migration. Abnormal EGFR signaling leads to severe developmental defects and is also associated with many types of cancers. This much was understood about the EGF system when the 1986 Nobel Prize in Medicine was awarded to the Stanley Cohen and Rita Levi-Montalcini for their discovery of peptide growth factors.

Today, the EGFR is the subject of approximately 30,000 research papers (Figure 2). Many molecules mediating the EGFR-induced responses have been identified and are now drug targets in oncology and other areas of medicine. Tools of genomics and proteomics will soon make it possible to

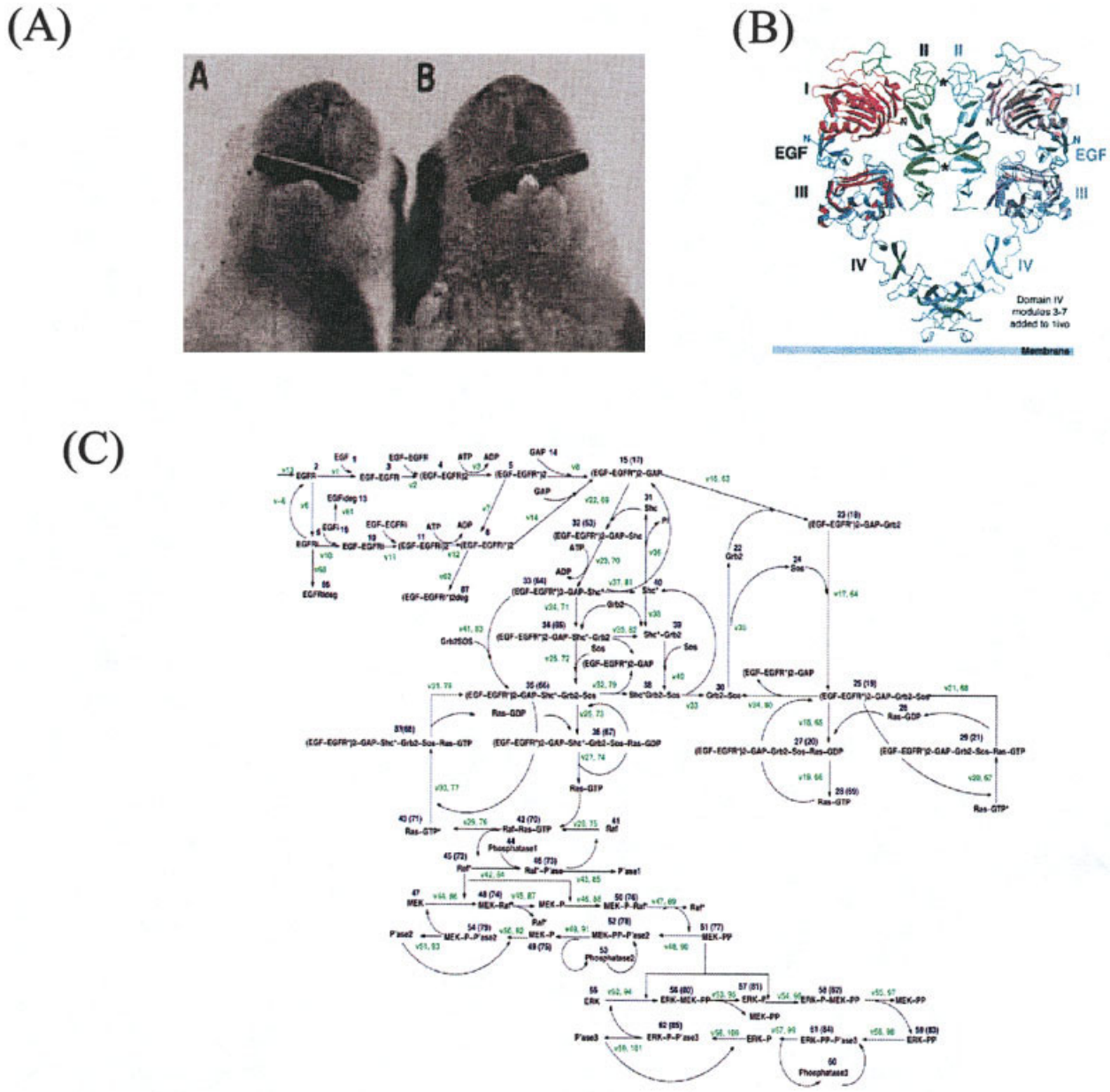

Figure 2. The Epidermal Growth Factor Receptor (EGFR) system.

EGF was discovered as a peptide growth factor that induced premature incisor eruption and eyelid opening in newborn mice and rats (A).[53] Today, EGFR is a subject of 30,000 research papers. We have high resolution crystal structures of EGFR in complex with its ligand (B)[19] and sophisticated mathematical models of EGFR signal transduction (C),[54] but the understanding of the mechanisms of EGFR signaling in tissues is far from complete. The same is true for all cell communication pathways in animal development. (A) Reprinted with permission from *Journal of Biological Chemistry*. (B) Reprinted with permission from *Molecular Cell*. (C) Reprinted with permission from *Nature Biotechnology*.

follow all genes and protein/protein interactions induced by EGFR activation.[16,17] The EGFR interaction with its ligands are becoming progressively understood at the atomistic level and can be followed in real-time with modern imaging tools.[18,19] However, the mechanisms of EGF-induced eyelid opening and incisor eruption are only beginning to be formulated.[20,21] To describe, predict, and manipulate such morphogenetic events, we need quantitative models of EGFR signaling in tissues.

EGFR network is the most extensively modeled signal transduction pathway in multicellular organisms.[22] However, most of the models have been formulated at the level of a single cell, and their "scale-up" to tissues is not straightforward.[23] First, while quantitative descriptions at the level of tissues must account for ligand transport, we know very little about the transport of EGF and related ligands in tissues. Second, cell-level models are "tuned" based on experiments with cells in culture, i.e., the cells in an environment that is rather different from a developing tissue. Third, tissue-level models are non-trivial to test experimentally. In mammals, for example, EGFR is activated by multiple EGF ligands, engages multiple intracellular signaling pathways, and interacts with other ligand-receptor systems. This complexity is difficult to control *in vivo* and next to impossible to reconstitute in a dish.

In addition to these issues of experimental validation, the

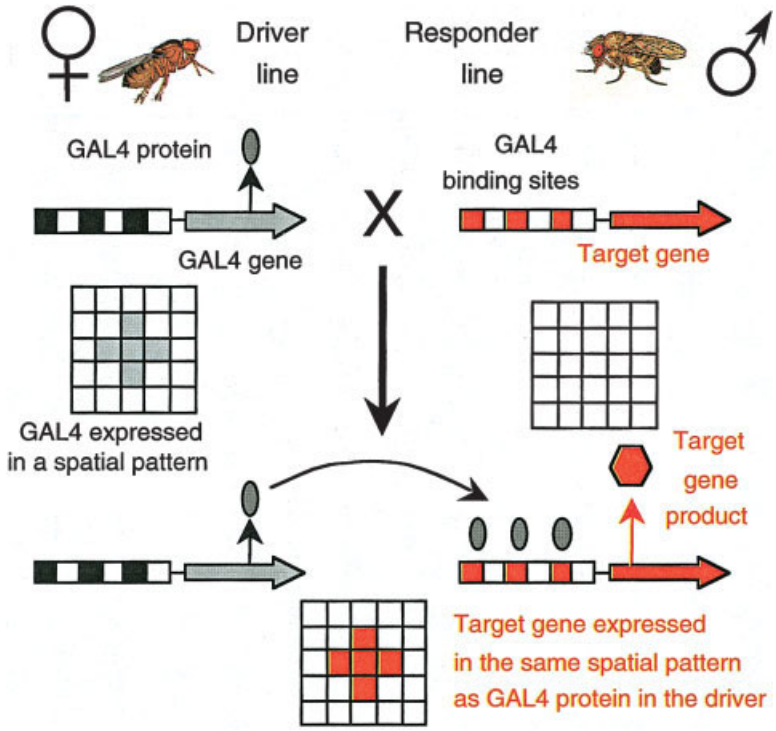

Figure 3. The experimental techniques of *Drosophila* genetics enable tissue-specific perturbations of cell communication systems across all stages of fruit fly development.

formulation of models themselves presents a number of challenges. These models must simultaneously account for ligand transport, receptor dynamics, signal transduction, and gene expression. Given the multitude of time and length scales associated with each of these processes and immense structural and parametric uncertainty associated with their description, the formulation and analysis of tissue-level models is a difficult task. Most current models are based on the assumption that the characteristic spatial length of gene expression patterns is much larger than the size of a single cell.[12,13,15] At the same time, genes controlled by morphogens can be expressed in domains that are one or two cells wide. Recently, we have started to develop discrete models of EGFR signaling epithelial layers, which explicitly account for "cellular" nature of patterned layers.[24,25] Analysis of these models suggests that their dynamics may be quite different from that of continuous reaction-diffusion systems. The main challenge, however, is to use these models to guide experimental analysis of developmental pattern formation.

Experimental Systems for Model Validation

Cell communication systems in general, and EGFR pathway in particular, are conserved across species, from worms to mammals.[6] Thus, model organisms of developmental biology can be used as experimental systems to formulate and validate quantitative descriptions of EGFR and other cell communication networks.[26] We are using the fruit fly *Drosophila melanogaster* as an experimental system for this purpose.[27] The fruit fly as a model organism is steadily gaining importance in biomedical research.[28,29] In addition to the sequenced genome and a wealth of cellular and molecular data available in *Drosophila*, there are specific reasons that make it an attractive system for the study of EGFR signaling in developing tissues.[30,31] The EGF receptor in *Drosophila* is homologous to its human counterpart. It is activated by four different ligands, all of which have mammalian homologues, and have been implicated in multiple diseases. The intracellular pathways mediating EGFR signaling are also conserved between humans and fruit flies. Alterations in *Drosophila* EGFR signaling produce distinct morphological phenotypes at all developmental stages, from the egg to the adult. Finally, versatile genetic tools enable highly specific manipulation of the *Drosophila* EGFR network.

In addition to a large number of publicly available mutants for specific genes in the *Drosophila* EGFR network, targeted gene expression systems allow one to express/repress a gene of interest in a given tissue.[32,33] The simplest version of the targeted gene expression system requires two lines of fruit flies, a driver and a responder (see Figure 3). In the responder line, the expression of the gene of interest is controlled by the presence of the UAS (upstream activating sequence), in this case a sequence of binding sites for the yeast GAL4 protein. The driver line expresses the GAL4 protein in a tissue-specific pattern. In the progeny of the cross between the driver and the responder lines, the gene of interest is expressed in the same pattern as the GAL4 protein in the driver line. At this time, responder lines exist for essentially every component of the EGFR system in *Drosophila*: from ligands and receptors to intracellular inhibitors and downstream transcription factors. In addition, numerous existing responder lines can be used to perturb the EGFR system in multiple stages of development, from egg to adult.

We are currently using these genetic tools in combination

with computational modeling to formulate quantitative models of EGFR in developing tissues. In *Drosophila* egg development, we are interested in determining all of the transcriptional targets of EGFR.[34] This is crucial for testing the proposed conceptual and computational models of EGFR signaling in this system.[15,35-38] In patterning of the *Drosophila* embryo, our analysis of the mechanistic model of EGFR signaling has revealed dimensionless groups that control the establishment of cell fates in the field patterned by the EGFR.[39] The data from genetic and biochemical experiments constrain the numerical values of these parameters.[11,40-42] Following that, computational analysis is used to predict the effects of genetic perturbations, e.g., increases in the levels of EGFR and its ligands. These predictions, in turn, are tested experimentally, using targeted gene expression systems.

Future Directions

The rapid progress of tissue engineering is fuelled by the advances in materials science and cell biology.[43,46] Developmental biology is a natural source of ideas for the design of man-made tissues. To take just one example, instead of assembling tissues from multiple cell types, one can pattern a layer of identical cells by applying a controlled gradient of a growth factor.[47] Experimental techniques to design such gradients already exist, and advances in stem cell biology makes the design of *in vitro* patterning systems a reality.

Tissue- and organogenesis rely heavily on cell communication by secreted protein ligands and their cell surface receptors. A small number of cell communication mechanisms is conserved across species and responsible for patterning developing tissues in worms, flies, mice, and humans. Defects in these cell communication systems lead to developmental abnormalities and severe pathologies, including a large number of human cancers. The concerted efforts of geneticists, biochemists, cell biologists, and medical scientists are resulting in systematic discoveries of crystal structures, maps of protein-protein interactions, and lists of transcriptional targets for each of these pathways. To understand how these patterning modules contribute to tissue and organogenesis, we must integrate this knowledge into quantitative models.

While we are still a long way from having a complete picture of any of these pathways, quantitative approaches and models are already becoming critical in dealing with a large number of components, feedback loops, and time and length scales. We are currently developing tissue-level models for the EGFR pathway, using the fruit fly as an experimental system for model validation. Similar efforts are currently underway for the EGFR signaling in worm development and for other developmental signaling pathways in *Drosophila*.[13,48,49]

Chemical engineers can play an important role in model formulation and analysis of pattern formation in development. A large number of important research problems is related to describing the concentration fields of secreted morphogens and mechanical forces accompanying morphogenetic events in tissue and organogenesis.[50,51] Some potential research problems include ligand transport in epithelial layers and inverse problems for morphogen gradients. The spatial range of secreted ligands is affected by its interaction with cell surfaces, components of extracellular matrix.[8] Furthermore, secreted ligand may be absorbed and then re-secreted by cells in the epithelial layer. Importantly each of these processes can be manipulated genetically. Chemical engineers have a long history of analysis of transport in complex media, such as porous solids and polymers. Some of the already existing tools may be applicable to problems epithelial transport, but most likely, new theories will have to be developed.[52]

The second problem is related to estimation of the concentration fields of morphogen ligands. At the present level of experimental techniques, morphogen gradients can be perceived only indirectly, through the spatial patterns of their intracellular targets. Models of morphogen gradients, in combination with their genetic perturbations, can be used to formulate parameter estimation and inverse problems for inferring the experimentally inaccessible concentration fields of morphogens in tissues.[39] Model-based analysis of spatial spread of pollutants is an example of productive fusion between reaction engineering and systems theory, and it motivates similar approaches to developmental pattern formation.

To work on these problems, one must learn genetics and developmental biology. These fields are now very receptive to the influx of engineers, mathematicians, and physicists interested in embryogenesis. Quantitative models of patterning events can be formulated in collaboration with biologists. Predictions of such models, e.g., changes in the spatial pattern of expression of a particular gene under the influence of a particular genetic perturbation, must then be tested experimentally. As a rule, in a biologist's lab, these experiments will not take priority over the work that focuses on the discovery of new network components, rather than systems-level properties of existing networks. After a while, engineers will be tempted to set up their own experiments. When this happens, worms, flies, and mice will become as common in the chemical and biological engineering departments as bacteria, yeast, and cultured mammalian cells.

Acknowledgments

SYS thanks Cyrill Muratov, Doug Lauffenburger, Trudi Schupbach, Mark Lemmon, Joe Duffy, Greg Reeves, Chris Bristow, Nir Yakoby, and Lea Goentoro for many valuable discussions. This work has been supported by funds from the NIGMS, NSF, Searle, Sloan, and Dreyfus foundations.

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