Review Article

Development of *in vitro* differentiation systems using vertebrate stem cells

Koji Okabayashi[1)], Kiyoshi Ohnuma[1)], and Makoto Asashima[1,2,*)]

[1)]Laboratory of Cell Regulation and Organ Formation, Department of Life Sciences (Biology), Graduate School of Arts and Sciences, The University of Tokyo

[2)]Organ Development Research Laboratory, National Institute of Advanced Industrial Science and Technology (AIST)

Research on control of *in vitro* differentiation of stem cells into specific tissues and organs is critical not only for the realization of regenerative medicine, but also for creating experimental model systems for understanding the mechanism of organogenesis. We have been using undifferentiated cells of amphibians to develop *in vitro* differentiation systems for various tissues and organs by the treatments with activin and other inducers under simple culture conditions. We have been also searching for novel genes and proteins involved in cell differentiation and organ formation by these experimental systems. Recently, by culturing mouse and human embryonic stem cells (ES cells) in serum-free cultures, we have established highly-reproducible experimental systems to control cell differentiation. To search for novel factors that function in nuclear reprogramming of somatic cells, we are using proteomics to analyze protein factors specifically expressed in undifferentiated cells. In addition to developing techniques for controlling differentiation of stem cells, these research projects are useful for creating uniform criteria to evaluate various states of cellular differentiation and to classify various types of cells comprehensively.

Rec./Acc.11/26/2009, pp302-309

* Correspondence should be addressed to:
Makoto Asashima, Ph.D., Department of Life Sciences (Biology), Graduate School of Arts and Sciences, The University of Tokyo, 3-8-1 Komaba, Meguro-ku, Tokyo 153-8902, Japan. Phone: 81-3-5454-6632, Fax: 81-3-5454-4330, e-mail: asashi@bio.c.u-tokyo.ac.jp

Key words animal cap, embryonic stem cell, activin, retinoic acid, serum-free culture

Introduction

Studies on the embryonic development of vertebrate animals have been advancing at a torrid pace with the advent of molecular biology methods. In recent years, Yamanaka et al. have succeeded in generating induced pluripotent stem cells (iPS cells) from mice and human somatic cells[1,2)], and ushered in the reality of technologies for generating custom-made stem cells from a patient's own somatic cells without using human eggs or embryos. Applying basic knowledge of cell differentiation and organogenesis, the development of techniques to induce specific cell types from stem cells has further accelerated.

Research in stem cell differentiation with application toward regenerative medicine is predicated on the following: (1) cells used must not provoke immunological rejection in the patient

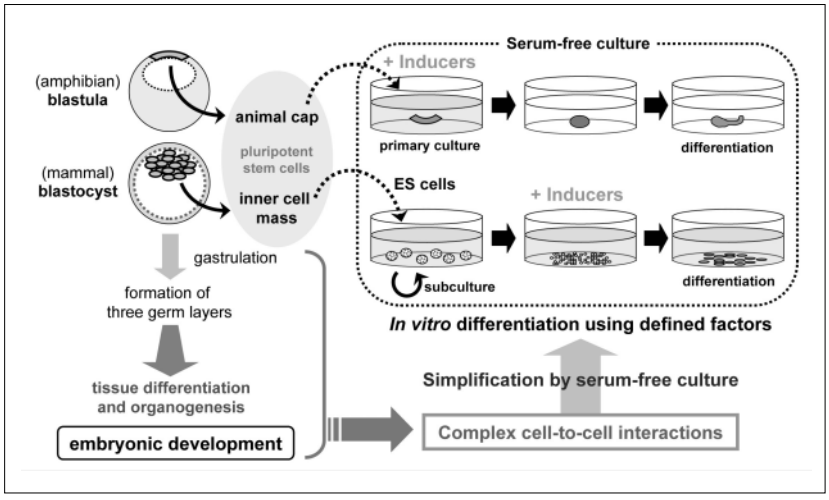

Fig.1 *In vitro* differentiation of pluripotent stem cells using serum-free culture systems

By using serum free culture systems, inducing effects of protein factors or chemical substances on the differentiations of pluripotent stem cells can be analyzed easily because various effects of unknown factors in the serum are negligible in these systems.

after transplantation, (2) it is necessary to prevent incorporation or infection of factors harmful to the patient, such as substances derived from other species and viruses, and (3) along with correctly controlling differentiation of specific cell and tissue types, techniques must be established that ensure safety after transplantation. Techniques to satisfy (1) include the generation of iPS cells from somatic cells and using somatic stem cells from a patient's own body. For (2), it is necessary to establish methods of culture and induction of differentiation in serum-free conditions by using xeno-free and chemically-defined culture media and inducers. For (3), it is necessary to develop culture systems that are simple and reproducible to the greatest extent possible. Also, if even a few undifferentiated cells remain in the differentiated cell populations derived from iPS cells, teratoma may form in the host body after the transplantation of these cells because of the high pluripotency of iPS cells. To prevent such unexpected cell differentiation, it is necessary to develop technologies to manage the "product quality" of cells. With such technologies, differentiated cells used in transplantation can be analyzed to determine if they are really normal tissue cells having identical quality, and whether the differentiation state of those cells are reliably controlled.

Because mouse ES cells are easier to culture than human ES cells and the results of differentiation induction are obtained quickly, research seeking to control cell differentiation *in vitro* has progressed rapidly. Cell differentiation *in vitro* does not necessarily reflect the same qualities as cells in the embryo during embryogenesis. However, an *in vitro* differentiation system using stem cells can reduce the complex cell-to-cell interactions during embryogenesis to a relatively simple system. This makes analysis easier (Fig.1). Also, because analyzing cell differentiation from a developmental biology point of view using different types of stem cells leads to the elucidation of fundamental mechanisms of cell differentiation, research using stem cells of laboratory animals such as mice is significant even as use of human iPS cells become possible. By using stem cells of vertebrate animals to identify novel factors involved in cell differentiation and analyzing their functions in developing embryos, mechanisms of vertebrate organogenesis can be clarified. We believe that such an approach, together with quality management as described above, will be beneficial for accumulating basic knowledge to ensure the safety of regenerative medicine.

We have been using the animal cap (undifferentiated cell mass) derived from amphibian blastula to develop *in vitro* differentiation systems that efficiently induce a variety of tissues and organs[3-5]. Using knowledge obtained from these experimental systems, we are further developing *in vitro* differentiation systems using mouse and human stem cells. In this paper we outline the *in vitro* differentiation systems we developed, and discuss future perspectives of research in stem cell differentiation.

In vitro differentiation of amphibian undifferentiated cells

The animal pole region of the amphibian embryo in the blastula stage has several layers of cells in a thin-sheet structure. This region is called the animal cap. If it is cut out and cultured in a simple saline solution, it differentiates into atypical epidermis. However, when inducing factors are added, animal cap differentiates into a variety of tissues. Therefore the animal cap is considered a region of undifferentiated cells. Previously, using the effects on the differentiation of the animal cap as indicators, we identified activin as a factor that can induce all types of meso-

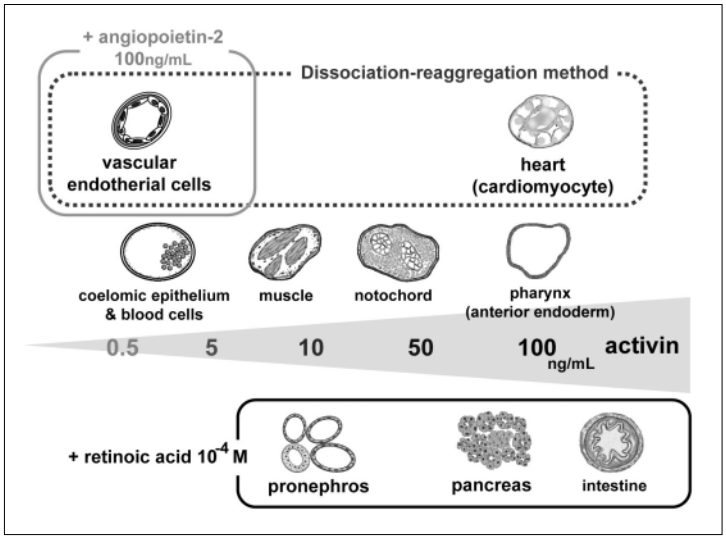

Fig.2 *In vitro* differentiation of *Xenopus* animal cap cells into various cells and tissues

Animal caps are differentiated into mesodermal and endodermal tissues by the treatment with activin in a concentration-dependent manner. Additional treatments with retinoic acid as a simultaneous treatment and a sequential treatment induce the differentiation of animal caps into pronephric tissue and pancreatic tissue, respectively. When animal cap cells are dissociated, treated with a high concentration of activin and then reaggregated, cardiac differentiation is induced.

dermal tissues, including the notochord, which is the most dorsal mesodermal tissue[6,7]. Furthermore, the mesodermal tissues induced from the animal caps are dorsalized by the treatments with higher concentrations of activin (Fig.2). Ventral mesodermal tissues including coelomic epithelium, mesenchymal cells and blood cells are induced by an activin treatment at the level of 0.5 ～ 1ng/mL; for muscle tissues, 5 ～ 10ng/mL; and for the notochord, 50 ～ 100ng/mL[6-8]. Activin at a high concentration of 100ng/mL or greater can differentiate the animal cap into anterior endodermal tissue. By the treatments with activin in combination with other inducing factors, we have succeeded in inducing the differentiation of the animal cap into a variety of tissues (Fig.2). As representative examples, we describe below the *in vitro* differentiation systems for pronephric (kidney) tissues and pancreatic tissues using retinoic acid, a derivative of vitamin A. We also describe the *in vitro* differentiation systems for cardiac muscle cells and vascular endothelial cells via dissociation and reaggregation culture of animal caps.

1) *In vitro* differentiation of animal cap cells into pronephros and pancreas

When the animal cap of *Xenopus laevis* was treated with 10ng/mL activin and 10^{-4} M retinoic acid at the same time, it differentiated into pronephric tissue[9] (Fig.2). Several marker genes of pronephric tubules and ducts were expressed in this tissue[10], and it showed histological features of normal pronephros. When an animal cap with such a treatment was transplanted into a late neurula embryo with the presumptive pronephros regions excised, about 20% of the embryos continued normal development, whereas embryos with the presumptive pronephros regions excised died[11]. These results show that the animal cap treated with activin and retinoic acid at the same time differentiates into normal pronephros, and it functions normally *in vivo* after the transplantation.

Furthermore, by treating the animal caps of *X. laevis* with 100 ～ 400ng/mL of activin for one hour and with 10^{-4} M of retinoic acid five hours later, we were able to specifically induce pancreatic tissues[12,13] (Fig.2). Pancreatic markers were expressed in these tissues. They showed histological features of normal pancreatic tissues, and both insulin- and glucagon- positive cells were found in the tissues. These results suggest that the differentiation of the anterior endoderm by a treatment of high concentration of activin is posteriorized by retinoic acid in this pancreatic tissue differentiation system. This system may reproduce the normal development of pancreas.

2) *In vitro* differentiation of animal cap cells into cardiac muscle cells and vascular endothelial cells by dissociation-reaggregation method

Animal caps can be easily dissociated into single cells by culturing in saline solution without Ca2+/Mg2+. These animal cap cells can be reaggregated in a solution that includes Ca2+/Mg2+. By using this “dissociation-reaggregation” method, we dissociated five animal caps of *X. laevis*, treated them with 100ng/mL of activin for 5 hours, reaggregated them as a cell mass and cultured for a few days. We found that more than 60% of the cell masses prepared by this method differentiated into beating cardiac tissue[15] (Fig.2). Cardiac differentiation markers were expressed in these tissues, and they showed histological features of normal cardiac tissues. When the presumptive heart region of

a host embryo was excised and the reaggregated animal cap cell mass prepared by this method was transplanted into the region, the host embryo developed normally with a normal heart. When the cell mass was transplanted into an ectopic region of normal host embryo, an embryo with two hearts was developed, and this embryo grew into an adult frog with two hearts after metamorphosis. These results suggest that this cardiac tissue differentiation system reproduces the cardiogenesis in normal embryonic development. By using several methods including this system, we have succeeded in identifying novel factors involved in cardiac development. In recent years, we identified XHAPLN3 and found that it is needed to maintain the hyaluronan matrix during cardiogenesis, and it is also necessary for cardiac development[16].

In addition, we developed an *in vitro* differentiation system for vascular endothelial cells by the improvement of above-mentioned method. When animal caps were dissociated, treated with 0.4 ng/mL of activin and with 100ng/mL of angiopoietin-2 simultaneously for one hour, reaggregated and cultured for three days, vascular endothelial cells were differentiated in these cell masses[17] (Fig.2). Using this system, we identified and analyzed factors related to the formation of blood vessels[18,19].

In vitro differentiation of mouse and human embryonic stem cells

In research on differentiation induction using stem cells, various unknown factors in the serum in culture medium may have effects on cellular differentiation. Because the composition of serums differs depending on the lot, such an uncertain element may lower the reproducibility of the experiment. Also, because experiments using amphibian animal cap use a simple saline solution as culture medium, when we apply the results of such experiments to mammalian ES cells, we believe it is critical to use a serum-free culturing system to develop highly reproducible differentiation systems (Fig.1). Therefore, we first identified conditions for inducing differentiation under conventional culture conditions. We then sought to apply these conditions to serum-free culturing systems by using commercially available serum-replacement products such as Knockout Serum Replacement (KSR, Invitrogen, CA, USA) or using chemically defined serum-free medium (ESF medium) developed by Furue et al.[20,21]. Here we describe the *in vitro* differentiation systems that we developed for cardiac muscle cells, ciliated cells, and pancreatic cells using mouse and human ES cells (Fig.3).

1) Induction of cardiac differentiation of mouse ES cells

We established a culture system for the differentiation of mouse ES cells into cardiac muscle cells by applying retinoic acid under a serum-free condition to embryoid body that has been cultured in serum-containing medium. In this system, we used retinoic acid derivatives that specifically activate (or antagonize) either one of two types of retinoic acid receptors (RAR and RXR).

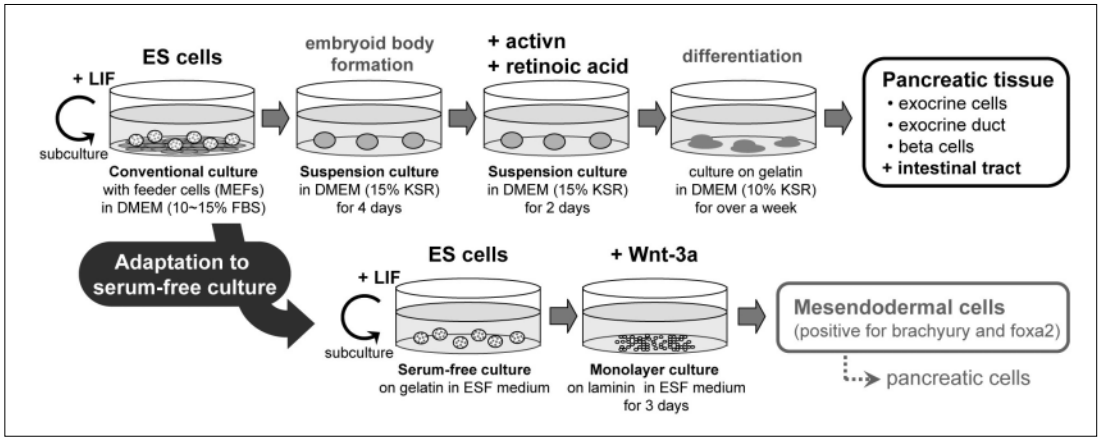

Fig.3 *In vitro* differentiation protocol of mouse embryonic stem cells into pancreatic tissue and mesendodermal cells in serum-free cultures

Pancreatic tissues as functional units in organ-like form are differentiated in the above method. Abbreviations: DMEM, Dulbecco's modified eagle medium; FBS, fetal bovine serum; MEF, mouse embryonic fibroblast; LIF, leukemia inhibitory factor; KSR, Knockout Serum Replacement. (ESF medium is a chemically defined serum-free medium developed by Furue et al.)

When an RXR-specific agonist (PA024) was added, differentiation of cardiac muscle cells was enhanced[22]. On the other hand, differentiation of cardiac muscle cells was inhibited by the addition of RXR-specific antagonist (PA452). These results suggest that RXR-mediated signaling have crucial role in the effects of retinoic acid signaling on the differentiation of cardiac muscle cells. Because we used serum-containing medium to prepare embryoid bodies from mouse ES cells in this cardiac differentiation method, we next sought to establish a differentiation system for cardiac muscle cells under a totally serum-free condition to completely remove the effects of serum. We were able to induce the differentiation of cardiac muscle cells at a higher rate than before by dissociating mouse ES cells, treating cells with BMP-4, and culturing reaggregated cell mass on culture dishes in Glasgow Minimum Essential Medium supplemented with 5% KSR for one week. Using this method to search for an appropriate surface marker, we found that N-cadherin is a more specific marker for cardiomyocyte progenitors than flk-1, which is a generally-used marker for cardiovascular progenitor cells. This shows that N-cadherin may potentially be a new cardiac muscle cell marker[23]. We are now searching for an appropriate condition to induce the differentiation of human ES cells into cardiac muscle cells at a higher rate by the improvement of these serum-free culture methods.

2) Induction of ciliated cell differentiation of mouse ES cells

By using attachment culture in a KSR-based medium, we found a condition that lead to efficient differentiation of mouse ES cells into ciliated cells[24]. The cilia of these ciliated cells had a 9+2 microtubule configuration, and these cells expressed differentiation markers of respiratory epithelial cells. Histological analysis showed that mucous cells were also differentiated in this method, and these ciliated cells and mucous cells formed a cell layer, which is similar to respiratory epithelium. We also found increased expression of gene markers for Clara cells by the addition of BMP-4 in this differentiation system. Clara cells are found in the respiratory epithelium on the side close to the alveoli. Because BMP signaling is known to regulate proximal-distal differentiation of respiratory organs in embryonic development, this differentiation system may simulate the development of respiratory organs.

3) Induction of pancreatic differentiation of mouse ES cells

We established a culture system for the differentiation of mouse ES cells into pancreatic tissue by applying activin and retinoic acid under a serum-free condition to embryoid body that has been cultured in serum-containing medium (Fig.3). By culturing on the gelatin-coated dishes in KSR-based medium for a week after the treatments with activin and retinoic acid for two days, embryoid bodies differentiated into intestine-like structures, which were surrounded by smooth muscles. These intestine-like structures showed spontaneous contraction that resembled smooth-muscle peristalsis. About 10-12 days after the treatments with activin and retinoic acid, over 20% of these structures formed a tissue that contained dark-colored spots. Histological analysis showed that these tissues contained pancreatic exocrine cells, pancreatic endocrine cells (insulin-producing cells) and pancreatic duct structures adjoining the intestine-like structure. Because pancreas is formed from the foregut during embryonic development, this differentiation system may reproduce the development of the pancreas. Also, we discovered that when 0.1 μM retinoic acid is used in this system, differentiation of exocrine cells is promoted with 10ng/mL of activin and differentiation of insulin-producing cells is promoted with 25 ng/mL of activin[25]. This pancreatic differentiation system has the advantage of being able to induce the differentiation of pancreatic tissues as functional units in organ-like form.

Next, we sought to increase the differentiation efficiency of endodermal cells because the pancreas is endodermal in origin. We tried to improve conditions for the differentiation using a serum-free monolayer culture[20]. It is known that in the development of mammals, definitive endoderm and mesoderm share a common precursor cell population, called the mesendoderm. Using the expression of mesendodermal markers, including brachyury, goosecoid and foxa2, as indicators, we searched for conditions that induce the differentiation of cells expressing these markers with high efficiency. We found that when mouse ES cells were cultured on laminin-coated dishes as a monolayer in ESF medium[20,21] supplemented with 50ng/mL of Wnt-3a for 3-6 days, the expression of these markers increased significantly (Fig.3). We also found that this result of mesendodermal cell differentiation by Wnt-3a is mediated through Wnt canonical pathway. The similar result was found using human ES cells[26]. Currently, we are searching for conditions that induce the differentiation of insulin-producing cells from human ES cells and iPS cells with high efficiency by improvement of this differentiation system using chemically defined serum-free medium.

Future perspectives of research in stem cell differentiation

Future research in stem cells can be roughly divided into the following two major directions: a) elucidating the mechanisms

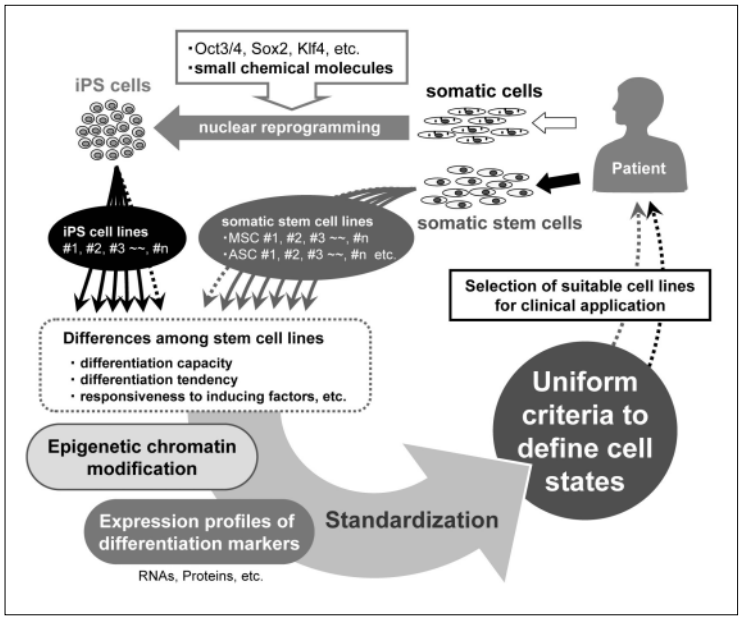

Fig.4 Selection of suitable stem cell lines derived from patients through the standardization of stem cells

Uniform criteria or standards to define cell states and to evaluate cell differentiation are needed for the selection of suitable stem cell lines. These criteria are also useful for controlling precisely the differentiation of stem cells.

of differentiation of stem cells into somatic cells and how to control them, and b) understanding the mechanisms of nuclear reprogramming of somatic cells and how to control them.

The goal of research described in this review paper was primarily a), but we also conducted research in the area of b). Using proteome analysis we attempted to analyze and identify novel protein factors that are specifically expressed in ES cells in their undifferentiated state[27]. Especially in recent years, we selectively purify proteins expressed on the surface of cell membranes by biotinylation, and analyze them using methods such as a newly developed highly efficient peptide separation method[28] and 2-dimensional fluorescence difference gel electrophoresis (2-D DIGE). We have identified 30 new membrane proteins that are specifically expressed in undifferentiated ES cells[29]. Additionally, from the analysis of extracted chromatin fractions of ES cells, we have identified dozens of chromatin binding proteins, and we are analyzing their function. We believe that by understanding the mechanism of nuclear reprogramming from the research described here, we can develop technologies that dramatically raise the efficiency of somatic cell reprogramming.

From 2008 to 2009, several new methods for iPS cell generation were reported. These include methods to temporarily express reprogramming factors (Yamanaka factors, etc.) without genomic integration using plasmid vectors[30] and adenovirus vectors[31] and methods to introduce reprogramming factor proteins directly into somatic cells using poly-arginine chain, a membrane-permeable peptide[32,33]. These are new techniques to overcome the disadvantage of iPS cells generated by using retrovirus vector or lentivirus vector. In addition, studies on the screening of small chemical molecules to identify novel molecule that can enhance somatic cell reprogramming have been flourishing. For example, Huangfu et al. have succeeded in the generation of iPS cells from human adult fibroblast by a combination of the treatment with valproic acid (VPA, a histone deacetylase inhibitor) and the introduction of only two factors, Oct3/4 and Sox2[34]. In the future, combining the search and analysis of novel reprogramming factors and improving the methods of iPS generation as described above may lead to techniques that make it possible to generate clinically safe human iPS cells simply by adding small chemical molecules to culture media. Such a goal can be said to be the ultimate goal of research b) described above.

Recent research trends with goal a) above also show great activity in approaches to induce cell differentiation by the treatments with small chemical molecules. For example, using human ES cells, Chen et al. carried out large-scale screening of small chemical molecules that promote the differentiation of pancreatic insulin-producing cells, and identified (-)-indolactam V[35,36]. It is anticipated that advancement of research that searches for such new chemical compounds will make it possible to develop techniques for inducing the differentiation of every cells and tissues of adult human body by using chemical compounds.

Regardless of whether the goal of research is a) or b), when using human ES cells and iPS cells, the differences such as differentiation capacity or tendency among various cell lines must be thoroughly considered[37]. For clinical applications, the most

suitable cell line must be selected from several iPS cell lines that are generated from somatic cells taken from the patient's own body. The differences in the quality among various stem cell lines make it difficult to determine the conditions for inducing differentiation of stem cells. It is also necessary to determine whether the reproducibility of an experiment depends on the cell line. We believe that from here on, when it comes to the use of a variety of stem cell types, it is necessary to create uniform criteria to evaluate various states of cellular differentiation and to classify various types of cells comprehensively. These criteria or standards will be established based on, for example, state of chromatin modifications, patterns of genomic methylation, expression of various differentiation markers and so on. In other words, a "standardization of stem cells" is required (Fig.4). With such standardization, we can obtain a powerful tool for comprehensively understanding the mechanism of embryonic development.

Comparing the results of research using amphibian and mammalian cells and studying shared signal pathways and shared activities of specific genes and proteins not only elucidate common mechanisms of organogenesis in vertebrates, but with the standardization of stem cells, it is an approach of upmost importance for building a knowledge base that supports advancement of regenerative medicine.

Acknowledgments

The authors acknowledge the financial assistance provided by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, Culture and Technology of Japan.

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