Properties and applications of embryonic stem cells

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Abstract Mouse embryonic stem (ES) cells are pluripotent cells derived from the early embryo and can be propagated stably in undifferentiated state *in vitro*. They retain the ability to differentiate into all cell types found in the embryonic and adult body *in vivo*, and can be induced to differentiate into many cell types under appropriate culture conditions *in vitro*. Using these properties, people have set up various differentiated systems of many cell types and tissues *in vitro*. Through analysis of these systems, one can identify novel bioactive factors and reveal mechanisms of cell differentiation and organogenesis. ES cell-derived differentiated cells can also be applied to cell transplantation therapy. In addition, we summarized the features and potential applications of human ES cells.

Keywords: embryonic stem cell, differentiation *in vitro*, cell transplantation therapy, gene targeting.

Embryonic stem (ES) cells were first derived from the inner cell mass of mouse blastocyst in the early 1980s[1, 2]. More recently, primordial germ cells, cells of the early embryo that eventually differentiate into sperm and oocytes, were found to give rise to cells with characteristics of ES cells and were designated EG (embryonic germ) cells to denote their tissue origin[3]. The distinguished features of ES cells are their capacity of maintaining in an undifferentiated state in culture and the potential to develop into every kind of cells of the body. In addition, under appropriate culture conditions, ES cells differentiate into a broad spectrum of cell types and when injected into immunocompromised animals, they form teratomas composed of multiple lineages [4]. It is just this ability to develop into a wide range of cell types that has drawn so much attention to ES cells as a basic research tool and as a novel source of cell populations for new clinical therapies. ES cells have been used extensively in studies of embryogenesis, identification of gene function and development in the mammal.

1 Differentiation model systems *in vitro* based on mouse embryonic stem cells

ES cells are capable of differentiating autonomously *in vitro* into multiple cell types after alteration of the culture condition that maintains the undifferentiated state of ES cells. For example, after withdrawing the feeder cell layer, ES cells will differentiate into multicellular aggregates called embryoid bodies (EBs). EBs contain derivatives of the three primitive germ layers, i.e. ectoderm, mesoderm and endoderm. EBs can spontaneously differentiate into various cell types including skeletal myocytes, cardiac myocytes, neurons, erythrocytes, melanocytes, and others.

(i) Hematopoiesis and vasculogenesis. During embryogenesis, the murine embryonic hematopoietic system undergoes rapid and dynamic changes in both the lineages and the localization. The earliest stage of hematopoietic development, known as primitive hematopoiesis, occurs in the yolk sac blood islands and is restricted primarily to the generation of a unique population of erythoid cells, commonly referred to as embryonic or primitive erythrocytes. Three to four days after the initiation of yolk sac hematopoiesis, intraembryonic hematopoiesis is established, initially in the region destined to form the aorta-gonad-mesonephros and shortly thereafter in the fetal liver. The onset of fetal liver hematopoiesis marks the switch from the primitive to the definitive hematopoietic program, which is characterized by the production of a broad spectrum of lineages including adult type erythrocytes. With the establishment of the definitive hematopoietic program, yolk sac hematopoiesis declines and primitive erythrocytes are no longer produced.

Although these developmental changes within the hematopoietic system have been well characterized for many years, the relationship between the primitive and definitive lineages and the mechanisms of their regulation remain poorly defined. Recent gene targeting and naturally occurring mutations in mice have provided some insights into the molecular events involved in the establishment of primitive and definitive hematopoiesis and have documented clear differences in the transcriptional

regulation and growth control of these populations. Taken together, these studies suggest that primitive and definitive hematopoiesis represent separate developmental programs that are regulated by different molecular mechanisms and display their different growth requirements. Primitive and definitive erythropoiesis represent distinct hematopoietic programs that differ with respect to stage of development, transcriptional control and growth regulation. Although these differences have been recognized for some time, the relationship between these two erythroid lineages has not been well established yet. Further understanding of the relationship between primitive and definitive hematopoiesis, including the mechanisms involved in the establishment of the two systems, will require access to early precursor populations, from which they develop. Keller et al.[5] have used a model system based on the hematopoietic development of ES cells in culture to investigate the origins of the earliest hematopoietic populations. Used ES cells transduced with a retrovirus that overexpresses the *hoxl1* gene, immortalized hematopoietic cell lines can be generated from ES cell-derived EBs that overpress the *hoxl1* gene. The lines generated from these EBs are factor-dependent and display unique developmental potentials including the capacity of generating both primitive and definitive erythroid cells. These findings are the first demonstration of a clonal cell line with primitive and definitive hematopoietic potential and support the interpretation that these lineages may arise from a common precursor in early embryonic life.

Several methods of differentiation induction from ES cells to hematopoietic cells *in vitro* have been reported. However, these systems require formation of complex embryoid structures, or/and the addition of exogenous growth factors. Other limitations of these systems are the inability to analyze the development process from ES cells to blood cells and the lack of simultaneous induction of both myeloid and lymphoid lineage cells. To overcome these limitations, Toru Nakans et al.[6] developed an efficient differentiation induction system (OP9 system) by simple coculture of ES cells on a stromal cell line OP9 which does not produce functional M-CSF. Although differentiation induction by the OP9 system cannot give rise to self-renewing hematopoietic stem cells which produce spleen colonies, and achieve long-term reconstitution of hematopoiesis in lethally irradiated mice, the temporal pattern of hematopoietic cell development during the coculture of ES cells is very similar to that of mouse ontogeny. Herein, this system can offer an effective method to study formation and differentiation phases of hematopoiesis and genes involved in regulating in this period.

The hematopoietic stem cells derived from ES cells can reconstitute the lymphoid compartment of T- and B-lymphocyte-deficient *scid* mice and generate mature T and B lymphocytes in sublethally irradiated normal mice. Exogenous cytokines can dramatically alter the developmental fate of ES cells in culture. The *in vitro* system described here should facilitate the study of molecular events leading to cell-lineage commitment and to the formation of hemopoietic stem cells and their immediate lymphoid progeny.

During differentiation *in vitro*, ES cells generate progenitors for most hemato-lymphoid lineages. Can they differentiate lineage-committed functional cells? Eichmann et al.[7] studied the developmental potential of two ES cell subpopulations that share the lymphoid marker B220 (CD45R). Upon transplantation into lymphoid-deficient mice, the $B220^+$ population generated a single transient wave of IgM^+IgD^+ B cells but failed to generate T cells. In contrast, transplantation of the $B220^-$ fraction achieved long-term repopulation of both T and B lymphoid compartments and restored humoral and cell-mediated immune reactions in the recipients. The results suggest that multipotential and lineage-committed lymphoid precursors are generated during *in vitro* differentiation of ES cells and that both subsets can undergo complete final maturation *in vivo*.

The establishment of hematopoiesis in culture from ES cells provides not only the access to rare, early developing populations, but also a unique system for addressing questions relative to the effects of altered gene expression on development, growth and differentiation of hematopoietic pregenitors. The outstanding advantage of this approach is that genes can be introduced at a stage of the starting ES population and subsequently be expressed as the primitive hematopoietic precursor derived from prehematopoietic mesoderm. In addition to providing new insights into gene function, overexpression of genes, particularly those with transforming and/or immortalizing potential, provides a unique opportunity to establish cell lines that represent various stages of embryonic hematopoietic

development.

The gene-mutated ES cells can also be used to figure out the effects of the interaction of different tissues on the primitive erythropoiesis and vasculogenesis. Bielinska et al.[8] established an ES cell line deficient in *gata4*, a transcription factor expressed in yolk sac endoderm. When differentiated *in vitro*, those mutant cells do not develop an external visceral endoderm layer. During mouse embryogenesis, the first hematopoietic and endothelial cells form in blood islands located between layers of visceral endoderm and mesoderm in the yolk sac. Thus, using the *gata4*$^{-/-}$ ES cell line one can study the role of visceral endoderm in hematogenesis and vasculogenesis. *Gata4*$^{-/-}$ EBs are defective in primitive hematopoiesis and vasculogenesis as evidenced by lack of recognizable blood islands and vascular channels and by reduction in the expression of the primitive erythrocyte marker ε^{y}-globin. But the endothelial transcription factor was not affected in the mutant embryoid bodies. These results indicate that the visceral endoderm is not essential for the differentiation of primitive erythrocytes or endothelial cells, but this cell layer plays an important role in the formation and organization of yolk sac blood islands and vessels.

ES cells can spontaneously differentiate *in vitro* into the endothelial lineage, and ultimately form vascular structures in ES-derived EBs. This process is achieved through successive endothelial developmental stages that recapitulate the initial steps of *in vivo* vasculogenesis. ES derived from endothelial cell progenitors (*flk-1*$^{+}$ cell) can form primitive vascular plexus through coordinated regulation of differentiation, proliferation, migration and cell-cell adhesion, which is important during vasculogenesis[9, 10]. *Flk-1*$^{+}$ cells can differentiate spontaneously to mature endothelial cells independent of vascular endothelial growth factor (VEGF). VEGF regulates the proliferation of *flk-1*$^{+}$ cells in a dose-dependent manner. These results indicate that transduction of VEGF signals through flk-1 is strictly concentration-dependent during vasculogenesis of early embryos. In summary, VEGF has dual roles in regulating the cellular events of endothelial cells in vasculogenesis, supporting the proliferation of endothelial cells at low dose and enhancing cell motility at high dose. The full developmental potential towards the endothelial lineage *in vitro* seems to be a common and consistent feature of ES cell line. Thus, this *in vitro* model appears useful for studying the role of single genes in the early steps of endothelial differentiation.

(ii) Formation and differentiation of myocyte and myocardial cell. ES cells can differentiate into complex EBs which exhibit many characteristics of 4—10 d embryos, including areas of rhythmical contract. The expression of the four muscle isoactins has been identified in EBs by using transcript-specific probes for each of the muscle actin mRNAs and by selectively reactive MAbs to muscle actins. All of four muscle-specific actin genes are transcribed. The muscle actins present in at least two distinct cell populations within the EBs. Fibroblast-like cells exhibit significant levels of the two smooth muscle actins (vascular and enteric) localized to stress fibers. In addition, one or both of the striated muscle actins (cardiac and skeletal) are expressed in cardiomyocyte-like cells. Like the case in embryonic heart, α-smooth muscle actin and the striated muscle actin(s) are incorporated into well organized sarcomeres in these cardiomyocyte-like cells. Thus, differentiating EBs provides an *in vitro* system to study gene expression in both striated and smooth muscle cells[11].

Since Doetschman et al.[12] found that ES cell lines were capable of differentiating *in vitro* into multiple cell types, including spontaneously beating cardiac myocytes ten years ago, ES cell differentiation cultures have been used by many investigators as a model system to study cardiogenesis[13—15]. This system can be used to study aspects of the molecular basis of cardiac cell development, and the mechanical and electrical properties of cardiac myocytes as well, such as expression of different cardiac contractile proteins, ion channels or receptors. Differentiation of ES cells into cardiac myocytes appears to follow a number of programmed changes of gene expression just as that *in vivo*. Expression of several cardiac genes is activated during the *in vitro* differentiation of ES cells, and moreover, the pattern of gene expression recapitulates known changes in expression characteristic of developing cardiac myocytes *in vivo*.

The features of ES cells, such as easy manipulation of genetical modification and rapid differentiation into cardiac myocytes *in vitro*, make it useful for the study of cardiac gene expression and function. This cardiogenesis system *in vitro* may be particularly advantageous for pharmacological

studies, especially on discovery of cardioactive drugs and for identification of the functional alterations associated with ablated or mutated cardiac genes that result in a lethal phenotype *in vivo*. This system provides an effective tool to determine cardiac gene structure-function relationships *in vitro*. The use of ES cell-derived cardiac myocytes as a potential source of donor cells for cardiac repair might be realized in the near future.

(iii) Nervous system. Neural differentiation has been observed when mouse embryonic stem cells were treated with retinoic acid (RA). In the absence of serum and the presence of 2-mercaptoethanol, ES cells can directly differentiate to neurons, other than via EBs [16]. Using this system, one can reduce the time of differentiation significantly.

The gene expression pattern of neurons derived from ES cells is very similar to that *in vivo*. The morphological, immunological, molecular and physiological data argue that at least a subset of the genetic regulatory pathways in neuronal differentiation is activated in cultured ES cells. In the presence of RA, the expression of mRNAs encoding neuron-specific genes is activated significantly, but that of mRNAs encoding mesoderm-specific genes is inhibited. Therefore, RA may have a dual role in mouse ES cells *in vitro*[17]. It is important to point out that the neuronal phenotypes quite typical in these systems. In fact, some developmental biologists suggest that neurons may be in a "default" state for undifferentiated cells[18]. Perhaps because of that, it is relatively easy to turn ES cells into neurons. At the proper time, retinoic acid will make 90% of ES cells go down the neural lineage path, and differentiate spontaneously into neurons and glial cells.

One has learned some about the development of neuronal stem cells. Several proteins, such as neuregulin and bone morphogenic protein 2 (BMP2), can nudge neural stem cells into neurons, the neuronal supporting cells known as glial cells, or even smooth muscle. But it takes a lot of molecules to get stem cells to differentiate. For example, even with the best neuronal growth factors known, only about half of the neural stem cells become neurons. That may be because a developing brain cell relies on a specific combination of signals from its neighbors, which determine the specific type of cell it becomes.

2 Maintenance of undifferentiated state and proliferation of mouse embryonic stem cell

Although mouse ES cells are most noted for their ability to produce new strains of animals with specific genetic alternations, it is well-known for their capability to differentiate and generate multiple lineages in culture. After withdrawing the conditions that maintain them in an undifferentiated state, ES cells will spontaneously differentiate to form multicellular structures known as EBs that contain elements of all three embryonic germ layers, ectoderm, mesoderm and endoderm. As differentiation continues, a wide range of cell types, including hematopoietic, endothelial, muscle and neuronal develop within the EBs in a defined and reproducible temporal order. The evelopmental programs associated with lineage commitment in EBs show remarkable similarities to those found in the normal embryo. Thus, EBs provide plenty of normal developing cell populations with both embryonic and adult phenotypes. To date, various differentiation systems of many cell types which derived from mouse embryonic stem cells *in vitro* were well-established. ES cell-derived cardiomyocytes, neural precursors and hematopoietic precursors have been successfully transplanted into recipient animals, which displayed morphological and functional integration[19, 20]. The ability to generate animals with predetermined genetic alterations has made immense impact on our understanding of gene function within the animal, while the exploitation of ES cell differentiation *in vitro* has not only led to identification of cytokines and but also will provide a powerful model system for the analysis of gene function at celluar level.

Although many ES derived cell differentiation systems have been established, the molecular mechanisms for maintenance of the undifferentiated state of ES cells are still unknown. Recently, some people studied different mutant ES cell lines in order to find genes and factors involved in maintaining and regulating proliferation and undifferentiation of ES cells, and to get much information on molecule mechanisms of proliferation regulation of stem cell during embryogenesis.

The pluripotency of ES cells can be maintained by co-culturing with a feeder layer of fibroblastic cells, or by supplementing the media with purified leukemia inhibitory factor (LIF) or related cytokines.

This cytokine activates Janus tyrosine kinases and signal transducer and activator of transcription factor (Stat) via the signaling receptor component gp130. The level of tyrosine-phosphorylated Stat3 and the DNA-binding activity of Stat3 decrease rapidly during differentiation of ES cells induced by treatment either RA or by withdrawal of LIF. A correlation has been found between levels of Stat3 activity and maintenance of an undifferentiated phenotype. In contrast, variation in Stat3 activity did not affect cell proliferation[24]. These results demonstrate an essential role for a critical amount of Stat3 in the maintenance of an undifferentiated ES cell phenotype and of the pluripotential determinant of ES cells. The self-renewal represents a first step in the molecular characterization of pluripotency[23, 24]. However, Stat5 was absent from pluripotent proliferating ES cells, but appeared early after induction of differentiation. Expression of Stat5 mRNA was detectable as early as 12 h after treatment with RA, and its protein detectable 2 days after differentiation. These results indicated Stat5 as a novel marker of very early stages of differentiation of ES cells [25].

ES cells cultured *in vitro* would differentiate while the LIF is removed. To better define the mechanisms, it would be advantageous to have a mutated ES cell line that can maintain undifferentiated state *in vitro* without exogenous LIF. Anthony[26] used insertional mutagenesis and a robust screen for undifferentiated cells, and isolated one ES cell line, Poly27, that did not require exogenous LIF to remain undifferentiated phenotype, but could differentiate *in vitro* if cultured in the presence of chemical differentiating agents. In addition, in some ES cell lines that kept an undifferentiated pluripotent state in a way of independent of LIF, there was no detectable LIF protein in their medium, and no upregulation of LIF mRNA was found either[27]. These studies suggest that the signaling pathway involving diffusible LIF can be bypassed for maintaining the pluripotency in culture, and indicate a considerable variation in growth factor dependence and differentiation of different ES cells.

3 Construction of transgenic animal based on embryonic stem cells and gene targeting technique

In the last decade the transgenic technique has evolved from a technical triumph to a sophisticated assay system for studying diverse biological problems[28]. The next decade will undoubtedly see the widespread adoption of the sophisticated transgenic system based on embryonic stem cells and gene targeting[29–31]. These sophisticated techniques have already been used to generate a lot of new mouse lines, to study unknown gene functions and understand gene functions in the context of the whole animal. And the technology promises to provide enormous insight into mammalian developmental genetics, stimulate the application of gene targeting in embryonic stem cells, and unravel problems in complex regulatory pathways, specifically intermediary metabolism and physiology. Since the introduction of defined modifications at a genomic level by gene targeting, it has become a widely used technique. This new approach has already had major impact, especially in immunology, where the disruption of a particular gene not normally essential for the animal during development, can be studied *in vivo*. The development of transgenic technology, whereby genes (or mutations) can be stably introduced into the germline of experimental mammals, now allows investigators to create mice of virtually any genotype and to assess the consequences of these mutations in the context of a developing and intact mammal. In contrast to traditional "gain-of-function" mutations, typically created by microinjection of the gene of interest into a one-celled zygote, gene targeting via homologous recombination in pluripotential ES cells allows one to modify precisely the gene of interest. The central discoveries that have enabled these technologies are the isolation of *in vitro* permanent ES cell lines from pre-implantation embryos, and the demonstration that these cell lines are capable of recolonizing embryos contributing to the germ line after sustained *in vitro* growth. The most compelling advantage offered by ES cells as a transgenic system is the availability of large numbers of cells as an *in vitro* cell line. This presents an experimental opportunity to treat an entire population of cells. Thus it is possible to generate genetic modifications that might occur very infrequently, such as the consequences of gene targeting procedures[32–34]. Furthermore, rare clones of cells that have been generated with a desired genetic change could be isolated from the population and used to generate a mouse so that the modification could be established *in vitro*.

The most common genetic background of ES cell lines is 129Sv, a mouse strain that tends to

develop spontaneous teratocarcinomas and is widely used for studies of early embryonic development. However, the 129Sv strain is poorly defined and thus, to avoid extensive backcrossing of the targeted mouse strain, ES cells of genetic backgrounds other than 129Sv have been derived. Recently a gene knock-out technique using an ES cell line derived from (C57BL/6×CBA/JNCrj) F1 mice has been developed, two lines of C57BL/6 background have shown successful germ-line transmission and knock-out mice have been produced[35]. The C57BL/6 mouse strain has been extensively studied, in contrast to 129Sv, and many transgenic models have been established on this background .

Employing the gene targeting technology reviewed here, from the simple sequence replacement vector approach to the highly sophisticated Cre-loxP system[36–38], it is now possible to explore the functions of genes by assessing the impact of the absence of products for which they encode, this approach has proven to be of significant benefit in revealing the broader roles of molecules, which previously were not evident from *in vitro* studies. Spectacular examples include the inflammatory bowel diseases associated with the inactivation of the IL-2 and IL-10 genes knock-out mice[39, 40]. Applying gene knock-in to creation of gene function-gain mutation has been used extensively [33,34,41,42]. There could be little doubt that novel transgenic mice will continue to offer insights into the function and regulation of complex biological systems.

4 Human embryonic stem cells and potential application of ES cells

The benefits that ES cells have brought to the investigation of mammalian biology have been demonstrated by spectacular progress in mice. Although the mouse is the mainstay of experimental mammalian embryology, early structures including the placenta, extraembryonic membranes, and the egg cylinder all differ substantially from the corresponding structure of human embryo. For ethical and practical reasons, in many primate species including humans, the ability of ES cells to contribute to the germ line in chimeras is not a testable property.

A renewable tissue culture source of human cells capable of differentiating into a wide variety of cell types would have broad applications in basic research and transplantation therapies. A major step in realizing this goal has now been taken with the demonstration that human embryonic stem cells have been grown in culture[43, 44]. It now raises a whole new set of expectations. On the basis of the use and study of mouse ES cells, the research and clinical potentials for human ES cells are enormous. They will be important for *in vitro* studies of normal human embryogenesis, abnormal development through the generation of cell lines with targeted gene alterations and engineered chromosomes, human gene discovery, drug and teratogen testing and as a renewable source of cells for tissue transplantation, cell replacement and gene therapies. These latter applications could eventually preclude the direct use of fetal tissue in transplantation therapies. It could ultimately open the way to growing replacements for many types of tissue or even organs damaged by disease.

Over the past few years, one has already isolated stem cells from various tissues in animals and humans. No matter whichever kind of stem cells researchers work with, hoping to channel them into becoming certain kinds of tissue——say, dopamine-producing neurons for implantation into patients with Parkinson's disease, heart muscle cells to repair damaged hearts, or insulin-producting cells for diabetes patients——they will have to solve a great many problems first. To date, ES cell-derived cardiomyocytes, neural precursors and hematopoietic precursors have been transplanted into recipient animals. Although the analyses of the long-term outcome of such experiments are limited, the findings suggest that the transplanted cells are able to function in the host animal. However, before this goal can be reached, a number of obstacles need to be overcome, the most significant one of which is donor/recipient compatibility and graft rejection. Possible solution to this problem includes the banking of large numbers of ES/EG cell lines encompassing a significant fraction of the histocompatibility types in the population, and/or the genetic modification of the stem cells to make the graft more acceptable to the recipient.

Another application of ES/EG cells is for the study of development in both human and animal model systems. This approach includes the identification and isolation of novel precursor cells and of medically important genes. Such genes might encode proteins that have direct therapeutic applications, such as growth factors, or that are important targets for drug development. Human ES/EG cells will be

valuable as a test system for evaluating the toxicity and efficacy of new medicines or chemicals. It has the potential to reduce the need for animal testing, to increase the efficiency and to reduce the costs of developing safe and effective drugs and chemicals. The system is well suited to understanding the role of genes through 'loss-of-function' studies by developing ES cell lines that lack a specific gene. The ES cell differentiation system is also an ideal tool for 'gain-of-function' studies, as it is possible to analyze the consequences of overexpression of specific genes on the development of different cell lineages.

5 Conclusion

In summary, ES cells and cell differentiation model derived from ES cells could mimic and even recapitulate the complicated development and organogenesis processes *in vitro*. It is clear that ES cell technology has revolutionized modern biology and provides us with unique opportunities to understand the mechanisms that control basic biological processes. The development of human ES/EG cells is a major milestone towards applying the potential of this technology to the direct treatment of human diseases. Significant additional researches will be necessary to capitalize on the full therapeutic potential of these cells, but the resulting novel therapies should be more than to justify the effort.

We need a more thorough understanding of the efficiency and regulation of ES cell differentiation in culture. There are also basic technical issues relating to the growth and propagation of these cells in culture that need to be overcome. In addition, certain safety issues need to be addressed before this type of therapy can be moved to clinic. For instance, could genetic mutations be introduced during cell differentiation and expanded in EBs? What are the potential problems if the precursor cell population isolated for transplantation contains contaminating cell types? These problems would be overcome through more investigators making thorough investigations in ES cells with a will.

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