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Generation of Blood Cells from Human Embryonic Stem Cells and Their Possible Clinical Utilization

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1. Introduction

All cellular blood components in adults are derived from hematopoietic stem cells resided in bone marrow (BM). However, along with the events in ontogeny, the process of hematopoiesis is a long and complex progression over time and space. It is classically assumed that the first blood cells generated in blood islands of the extraembryonic yolk sac are large nucleated erythroblasts, representing a primitive wave of the initial hematopoiesis. A second wave of the blood cell generation, named definitive hematopoiesis, has its origin in the aorta/gonad/mesonephros (AGM) region. These definitive hematopoietic cells (HCs) are endowed with property of hematopoietic stem cells that can rescue lethally radiated animals and hold the potential to generate all blood cell progenies. Consequently, hematopoiesis is shifted to fetal liver in the midgestation and later to BM, where hematopoietic stem cells inhabit life long. Recent findings support a model that yolk sac also provides committed and mature blood cells with multipotential property, allowing survival until AGM-derived hematopoietic stem cells can emerge, and then seed the liver and differentiate into mature blood cells.

Transplantation of human hematopoietic stem cells (HSCs) in clinical therapies has been well applied to the patients suffering with malignant dysfunction of the hematopoietic system or after deadly radiation therapy for cure of leukemia. Although knowledge about the mechanisms underpinning the early development of hematopoiesis during embryonic and fetal stages have been largely expounded by various gene-targeting technologies, because of the restriction to use living human embryos, the early genesis of the human hematopoietic system, especially during embryonic / fetal stages, is largely unknown.

Recently, the establishment of human embryonic stem cells (hESCs) greatly expanded our view to elucidate the events in early human ontogeny. The ESCs derived from the inner cell mass of the human balstocyst are capable of growing indefinitely while maintaining pluripotency, namely to differentiate into all tissues of the body, including blood cells.

The characteristics of both stemness and multipotency provide two main expectations on hESCs in basic research and clinical applications. First, they provide models for studies of basic disease mechanisms, screens for drug discovery, and tissue engineering for new treatments for diseases such as diabetes, spinal cord injury, Parkinson's disease, myocardial infarction and cancers. On the other hand, the pluripotency and embryonic property of these stem cells provide a unique tool in exploring the basic mechanisms of early development and differentiation of human beings, which never can be mimicked in lower level species.

During the past decade, hESCs have been utilized to characterize molecular and cellular mechanisms controlling the differentiation of hematopoietic progenitors and mature, functional blood cells. Almost all types of blood cells derived from hESCs have recently been reported, including functionally mature erythrocytes and neutrophils, platelets, megakaryocytes, eosinophils, monocytes, dendritic cells (DCs), nature killer (NK) cells, mast cells (MCs), and B-, T-lineage lymphoid cells. The advances in research are leading to a clinical translation of hESC-derived HCs as novel therapies in near future. Based on recent success, the initial clinical application of blood cells derived from hESCs will possibly be in the field of transfusion therapies (erythrocytes and platelets) and immune therapies (NK cells and DCs). However, hESC-derived hematopoietic stem cells capable of long-term, multilineage engraftment are still under searching. On the other hand, ethical recognition must be appropriately addressed before clinical utilization of hESC-derived cellular therapies.

This review outlines the current progress, including data collected in our laboratory, in the research on hESC-derived hematopoiesis and the aspects of what needs to be tackled in future in this research fields. The possibility of hESC-derived cellular therapies in clinical application will also be discussed.

2. Methodology

ESCs are cells capable of being indefinitely growing with multipotency if provided appropriate culture conditions. At the same time, they undergo spontaneous and synchronous differentiation into all cell lineages when deprived from the optimal conditions. Because ESCs under the undifferentiated state form teratomas when transplanted into the living body, the efficient in vitro induction of ESCs to differentiate into a specific cell lineage are of importance.

The earliest report for mouse ESCs to generate hematopoietic and mature blood cells was published two and half decades ago (TC. Doetschman, et al. 1985). By a coculture system, Nakano et al. successfully induced undifferentiated mouse ESCs into almost all lineages of mature blood cells (T. Nakano, et al. 1994), providing evidence that ESCs may play a role as sources of blood cells, experimentally as well as clinically. After then, the mouse stromal cell line they used, OP9, became a widely and standard matrix to be used to induce hematopoiesis both in mouse and human ESCs.

Since the first study on hESC-derived hematopoiesis reported (D. Kaufman, et al. 2001), derivations of mature blood cells from hESCs have been confirmed by many groups including ours. The efficiency and stability of the in vitro blood cell inducing system have also been improved. However, by different culture systems, hESC-derived blood cells are more or less diverse in their maturities. The methods commonly used to develop blood cells

from hESCs are categorized as: (1) the formation of embryoid bodies (EBs, three-dimensional colonies of differentiating ESCs) and (2) the coculturing of ESCs with stromal layers.

When formed EBs in suspension cultures, hESCs develop into a sac-like structure mimicking the early development of the zygote. Within the EBs, hESC-derived cells interact with each other among a microenvironment semi-separated from the culture medium, providing a suitable condition for differentiation, mainly spontaneous. Because the EBs mimic the early yolk sac structure, when with the stimulation of hematopoiesis-directing factors, the early blood cells including primitive erythrocytes can be gained. However, because accessibility of the external factors into this complex structure is limited, EBs may be disadvantageous in regulating differentiation of hESCs toward definitive hematopoiesis to generate fully matured blood cells. (H. Sakamoto, et al. 2010).

While EBs serve a microenvironment for initiation of primitive hematopoiesis, the coculture of ESCs with stromal cells, most of them derived from fetal /newborn hematopoietic niches, provide a more subtle and efficient way to generate mature blood cells. There are a variety of cell lines employed in coculture systems with mouse and human ESCs, among them the OP9 being most widely used (T. Nakano,et al. 1994; Y. Mukouyama, et al. 1998). OP9 was established from an op/op mouse deficient in macrophage colony-stimulating factor (M-CSF), and has some deleterious effects on the early development of HCs. Results accumulated from murine experiments showed that Flk1-expressing cells, representing the development of mesoderm, in EBs are detected up to day 4 and their number declines thereafter (WJ. Zhang, et al. 2005). While cocultures of mouse ESCs and OP9 cells give rise to high-level expression of Flk1 up to day 6 in differentiation, suggesting a prolonged mesodermal development may provide a proper environment for the ESCs to differentiate to HCs when cocultured with OP9.

By coculture with OP9 cells, differentiation of the HCs from mouse and human ESCs into various blood cell lineages can be observed. In mouse, coculture of ESCs with OP9 to generate erythrocytes (T. Nakano, et al. 1994; N. Motoyama, et al. 1999), B-lymphocytes (T. Nakano, et al. 1994; SK Cho, et al. 1999), megakaryocytes, NK cells and DCs (T. Era, et al. 2000; N. Nakayama, et al. 1998; S. Senju, et al. 2003) have been reported. OP9 cells expressing Delta-like ligand 1 (OP9-DL1), a ligand of Notch, also induce the differentiation of hematopoietic progenitors into T lymphocytes (TM. Schmitt, et al. 2004). OP9 and OP9-DL1 cells now have been widely used to induce the differentiation of HCs from both nonhuman primate and human ESCs (II Slukvin, et al. 2006; K. Umeda, et al. 2004; M Gaur, et al. 2006; N Takayama, et al. 2008; F. Timmermans, et al. 2009).

We also have reported efficient methods to induce human and non-human primate ESCs to differentiate into HCs by coculture with mouse AGM region-derived and fetal liver-derived stromal cells (MJ. Xu, et al. 1998; F. Ma, et al. 2001; F. Ma, et al. 2007; F. Ma, et al. 2008a; F. Ma, et al. 2008b). With these mouse fetal hematopoiesis-centered tissue stromas, human and non-human primate ESCs generate functionally mature blood cells through a first primitive hematopoiesis wave, mimicking the early hematopoiesis during the yolk sac stage, and then definitive hematopoiesis pathway.

3. Hematopoietic progenitor cells derived from hESCs

Since the first establishment of hESC lines had been done 12 years ago (JA. Thomson, et al. 1998), knowledge about the early hematopoiesis during human embryonic stage has been

extensively refreshed by applying hESC-differentiating methods. Through a large variety of experiments, it has been identified that the first progenitor cells holding hematopoietic activity mostly share an endothelial cell (EC) potential, thus they are named as hemangioblasts (ET. Zambidis, et al. 2005; M. Kennedy, et al. 2007). The onset of the EC and HC bipotential progenitors derived from hESCs express FLK-1, CD31, VE-Cadherin, CD34, but lacking CD45 on their surface (CD45- PFV cells) (L. Wang, et al. 2004), giving rise to both ECs and HCs when properly induced. Furthermore, this fraction of CD45- PFV cells can reconstitute the hematopoietic system in immunocompromised mice when injected into the bone marrow (L. Wang, et al. 2005a, L. Wang, et al. 2005b), suggesting that the CD45- PFV population containing hematopoietic stem cells.

When continuously maintained on an environment favoring hematopoiesis development, such as coculture on OP9 or on fetal liver stromal cells, these hemangioblastic progenitors further differentiate into mature blood cells (ET. Zambidis, et al. 2005; F. Ma, et al, 2008a). The hESC-derived hematopoietic progenitors coexpress CD34, CD43 and CD45, and give rise to myeloid and lymphoid cells (MA. Vodyanik, et al. 2006). Through a coculture with OP9 cells, a hESC-derived common myeloid progenitor cell fraction that share a phenotype of lineage specific marker- CD34+CD45+ CD43+ are capable of generating functionally mature myelomonocytic cells with high efficiency, including neutrophils, eosinophils, macrophages, osteoclasts, dentritic cells and Langerhans cells (KD. Choi, et al. 2009). These techniques for generating hESC-derived hematopoietic progenitor cells, especially multipotential myeloid progenitors, may play roles in searching and expanding new clinical approaches by generating large number of patient-specific cells for in vitro study and drug screening.

4. Functionally mature blood cells derived from hESCs.

By applying lineage-specific stimulation methods such as addition of cytokines or culturing on normal or genetically manipulated stromal cells, hESCs can be further induced to functionally mature cells along to a specific lineage with high purity. This ensures a distinct scientific base to trace the early development of human hematopoiesis along with a specific blood cell lineage, especially when hESCs are used as models. In addition, hESCs may provide a novel source for regenerative medicine. To fulfill this aim, induction of hESC-derived blood cells with full maturation is critical. Recently, various mature blood cells with functional maturation have been produced in vitro, challeging to translate use of these cells to clinical application.

Notably, the hESC-derived erytrocytes and pletelets should be the most feasible products in near future clinical applications, because both RBCs and platelets do not have a nucleus and are with minimal genetic material thus role out the possibility for malignant transformation of these particular cell types.

4.1 Erythrocytes

The limitation of blood sources hampers the sufficient utilization of red blood cells (RBCs) in transfusion medicine. Sufficient blood supply is always in great demand from a therapeutic standpoint. Since the mature RBCs lack nuclei and are free of concerns for tumagenicity, they represent an attractive, maybe the first generation of, product from the stem cell derivations. Large-scale production of erythroid cells from hESCs may provide

us a novel and safer source of RBCs for transfusions. hESC-derived mature erythrocytes with a universal blood type such as blood group O and suppressed expression of HLA molecules will be an ideal source of erythrocytes in transfusions. Large-scale productions of mature erythroid cells from hESCs have recently been reported by several groups, including ours (F. Ma, et al. 2008a; SJ Lu, et al. 2008; EN. Olivier, et al. 2006). By co-culturing hESCs with murine fetal liver-derived stromal cells, we first produced multipotential hematopoietic progenitors that could give rise to huge pure erythroid colonies. After harvesting these pure erythroid colonies, we successfully obtained large quantity of mature erythroid cells. When we traced these hESC-derived erythrocytes at clone level, we found that hESC-derived progenitors were fated mostly to become definitive erythrocytes that finally undergo enucleation, switching to adult-type β-globin at almost 100% along times in culture. Furthermore, these hESC-derived mature erythrocytes functioned as oxygen carriers. As much as 1 $x10^4$ undifferentiated hESCs roughly generated 1 x 10^6 mature erythrocytes (F. Ma, et al. 2008a). Our study not only provide evidence that hESC-derived erythrocytes can be induced to a definitive stage with functional maturity, but also offer a method to scale up the production of erythrocytes that may be employed in future clinical use. Actually, by a multistage protocol involving EB formation, defined cytokines plus a recombinant tPTD-HOXB4 protein to produce hematopoietic differentiation, Lu SJ et al also achieved up to 10^{10} to 10^{11} RBCs from one 6-well plate of undifferentiated hESCs (SJ. Lu, et al. 2008). However, although a promising direction has been provided, substantial effort should still be paid to bring hESC-derived RBCs to a scale needed for future clinical applications. Since the transfusion therapy is routinely applied in daily surgeries and the insufficiency of fresh blood sources always remains an headeache worldwidely, research on hESC-derived erythrocytes should be predominantly pushed up and hESC-derived RBCs may serve the first product from the benifit of stem cells.

4.2 Megakaryocytes and platelets

For the same reason as hESC-derived RBCs, platelets derived from hESCs will also meet the potential need for future transfusion medicin.When cocultured with murine bone marrow stromal (S17) and yolk sac endothelial cell (C166) lines, Kaufman et al produced hematopoietic progenitors that could generate mature megakaryocyte-containing clononies in semisolid culture (D. Kaufman, et al, 2001). Gaur et al applied a coculture with OP9 stromal cells to generate megakaryocytes from hESCs with characteristic DNA polyploid nucleus, specific cytoskeletal and surface proteins, and ability to signal through integrin αIIbβ_3 (M. Gaur, et al, 2006). However, they did not confirm the production of pletelets from these hESC-derived magakaryocytes. Subsequently, using coculture with either OP9 or C3H10T1/2 cells in the presence of thrombopoietin for longer periods of time (over 3 weeks), Takayama et al made a comparitively large production of mature hESC-derived megakaryocytes (2–5 x 10^5 platelet-producing megakaryocytes per 10^5 undifferentiated hESCs) (N. Takayama, et al. 2008). These hESC-generated mature megakaryocytes produced platelets with morphology and function similar to those human pletelets isolated from fresh plasma. However, the lower yeilding of hESC-derived pletelets when compared to in vivo process indicate that further improvement should be paid to reach a possible clinical trial.

4.3 Neutrophils

Human neutrophils are the most primary constituent of the peripheral blood leukocytes and play a central role in host defense against the invasion of microorganisms. In some cases, congenital leukocyte function deficiencies and myelosuppressions caused by chemo- or radiotherapies need granulocyte transfusion therapy to protect the patient from lethal infections.

Saeki et al reported a two-step method to generate mature neutrophils from hESCs (K. Saeki, et al, 2009). They first made formation of hESC-derived spheres by adding cytokines favoring the development of hematopoietic progenitor cells. After replated to adherent culture for 2 to 3 weeks, these hESC-derived spheres form sac-like structures holding round mature myeloid cell, with an approximately 40-50% ratio of mature neutrophils. Although these hESC-derived neutrophils phenotypically and functionally mimicked human mature neutrophils, their production is comparatively low (1×10^6 undifferentiated hESCs generate 1×10^6 mature neutrophils). A more efficient method by first making EB formation and then coculturing with OP9 cells had been applied to generate hESC-derived mature neutrophils (Y. Yokoyama, et al, 2009). In this system, high purity of mature neutrophils could be induced within 2 week in culture. These hESC-derived mature neutrophils showed various functions such as superoxide production, phagocytosis, bactericidal activity and chemotaxis that were similar to those with peripheral blood counterparts. Although these studies provided good culture system to research on the development and functional maturation of hESC-derived neutrophils, they are still difficult to be used clinically as a transfusion therapy model.

4.4 Nature killer cells

NK cells stand at the center in immune defenses against pathogens and malignant tumors. Human NK cells provide critical cell-mediated antitumor activities. Furthermore, clinical trials have already confirmed the transplantable NK cells in recipient patients, suggesting the possible new therapy may be conducted by the NK cell transfusion to cure cancers (JS. Miller, et al. 2005; L. Ruggeri, et al, 2002). Thus, if properly induced to be mature NK cells, the unlimited potential of hESC may provide an ideal source of human NK cells that can be used in extensive antitumor therapies.

Actually, the first confirmation of hESC-derived functional lymphocytes was NK cells (PS. Woll, et al, 2005). By a 2-step culture method, $CD56^+CD45^+$ lymphocytes with a function like mature NK cells could be induced from hESCs. The hESC-derived NK cells express killer cell-specific markers such as Ig-like receptors, natural cytotoxicity receptors, and CD16. These hESC-derived NK cells were able to lyse human tumor cells by both direct cell-mediated cytotoxicity and antibody-dependent cellular cytotoxicity, showing their full function of antitumor activities. More recently, interesting result has been reported by the same research group, showing that hESC-derived mature NK cells are more efficiently to clear human tumor cells in vivo than human cord blood derived NK cells, suggesting a potential clinical use for hESC-derived NK cells in cancer therapy (PS. Woll, et al. 2009).

4.5 T- and B-lymphocytes

Some earlier reports suggested development of lymphocytes from hESCs based on surface staining of markers such as CD3 (T cells) or CD19 (B cells) and RT–PCR analysis (X. Zhan, et al, 2004; MA. Vodyanik, et al. 2005), but without functional assays. By first using coculture with OP9 stromal cells to differentiate GFP-expressing hESCs into $CD34^+$ and $CD133^+$ cells and then injected them into human thymic tissues engrafted immunodeficient mice (SCID-

hu mouse model), Galic et al successfully made engrafts of hESC-derived mature T-lymphcytes in vivo (Z. Galic, et al, 2006). These hESC-derived T cells expressed T-specific surface markers such as CD4, CD8, CD1a, and CD7. Sequentially, the same research group has applied EB-mediated differentiation to generate T-cell progenitor cells in the SCID-hu model (Z. Galic, et al. 2009). In addition, function of the hESC-derived T cells has been tested based on increased expression of CD25 after CD3/CD28-mediated activation. However, the engraftment of the hESC-derived T cells in the SCID-hu model is low (1% or less). Comparing to myeloid cells, it has proven difficult to induce hESC-derived hematopoietic progenitors to further develop into mature T- and B-lymphoid lineage cells.

To allow more access to developing cells and improving conditions that support or inhibit development of T cells, a Notch ligand–expressing OP9-DL1 stromal cells have been used to derive T cells from multiple progenitor cell populations expressing CD34 and CD45 such as human BM, umbilical cord blood, and mouse ESCs (RF. de Pooter , et al. 2003; CH. Martin, et al, 2008; TM. Schmitt, et al, 2004; RN. La Motte-Mohs, et al. 2005). However, the same hESC-derived $CD34^{+}CD45^{+}$ cells that effectively produce NK cells from hESCs were unable to produce T cells in this in vitro system (D. Kaufman, 2009), suggesting a different condition may be needed for hESC-derived T cell development.

Recently, Timmermans et al reported that a specific population of hESC-derived $CD34^{+}CD43^{low}$ cells that were present in hematopoietic zones morphologically similar to blood islands (F. Timmermans, et al. 2009). By first coculture with OP9 and then with OP9-DL1 cells, they demonstrated in vitro development of mature T cells from hESCs. In their system, hESC-derived T cells typically developed through a sequential pathway, initially committed to a CD34+CD7+ T/NK common potential stage, then to CD7+CD4+CD8- single positive and CD4+CD8+ double positive satges, and finally to CD3+CD1-CD27+ mature T cell stage. This promising study provided a new approach to use hESCs to generate T cells for novel immunotherapy.

4.6 Other mature blood cells

Derivation of dendritic cells (DCs) from hESCs have also been reported (X Zhan, et al, 2004; II Slukvin, et al, 2006; S Senju, et al, 2007; Z Su, et al, 2008). These hESC-derived DCs expressed high levels of HLA class II molecules and showed an ability to stimulate leukocyte reactions as an in vitro measure of immune activity. Function of antigen uptaking and processing, and stimulating allogeneic and antigen-specific T-cell responses have been demonstrated on these hESC-derived DCs.

By culturing clonal hematopoietic cells derived from hESC in semisolid culture, we demonstrated the derivation of mature mast cells (MC) that held tryptase, but few chymase (F. Ma. et al, 2007). Recently, functionally matured mast cells (MC) have been induced from hESCs (M. Kovarova, et al. 2010). These hESC-derived MCs respond to antigen by releasing MC specific mediators, providing a useful model to analyze human MC development and may be possibly useful in drug screening for allergic diseases.

5. Future prospect

The establishment of hESCs brought forth a totally new generation of regenerative medicine. The unlimited potential of hESCs ensures their ability to derive almost all the tissue types in our living bodies, thus constructing a base for the future clinical use. However, before the clinical application of using hESC-derived hematopoietic cells, there

are still several gaps should be overcome. Firstly, an efficient and animal source-deprived culture system is needed to ensure the safety from infectious diseases and species-crossing genetic transfections. Secondly, for applying transfusion therapy by using hESC-derived RBCs, more efficient in vitro culture system should be promoted to ensure a large-scale production of enucleated hESC-derived RBCs. Third, since the real hESC-derived hematopoietic stem cells that can fulfill reconstitution has still yet been defined, efforts should be paid to search for a way by employing novel method to characterize the properties of the possible hESC-derived stem cells. Finally, to guarantee efficient and safe clinical use, attention should also be paid to develop standardization and stability of the cell culture system. The clinical need for new and better therapies by using hESC-derived cellular products should remain greater than any barriers and unanswered questions.

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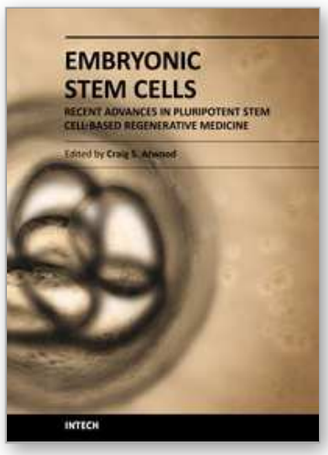

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Pluripotent stem cells have the potential to revolutionise medicine, providing treatment options for a wide range of diseases and conditions that currently lack therapies or cures. This book describes recent advances in the generation of tissue specific cell types for regenerative applications, as well as the obstacles that need to be overcome in order to recognize the potential of these cells.

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