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14. ABSTRACT
This project was focused on production of megakaryocytes and platelets from human pluripotent stem cells -- originally human embryonic stem cells (hESC), but subsequently, as the technology became available, from human induced pluripotent stem cells (hiPSC). We developed a serum-free system, and also created our own cell-free extracellular matrix extracts instead of live feeder cells to support hESC and hiPSC growth and maintenance, followed by feeder cell-free cultures to create embryoid bodies, then mesenchymal cells, which then were induced to differentiate into functional megakaryocytes and platelets.

15. SUBJECT TERMS
human embryonic stem cells; human induced pluripotent stem cells; hematopoiesis; megakaryocytes; platelets; extracellular matrix; serum-free cultures

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ONR Grant # N00014-07-10951

Reparative Medicine: Production of Erythrocytes & Platelets from Human Embryonic Stem Cells
University of South Florida – PI: Kenneth S. Zuckerman, MD
2012 Final Progress Report – Technical Report

A. Scientific and Technical Objectives

The following were the primary scientific objectives of this project. The first objective was to establish optimal human embryonic stem cell (hESC) culture conditions and then to determine the optimal conditions to enhance differentiation of hESC to hematopoietic stem (HSC) and progenitor cells (HPC), with the eventual goal of terminal differentiation to erythrocytes and megakaryocytes and platelets, emphasizing megakaryocyte differentiation, based on recommendation of Dr. Givens. We progressively improved culture conditions for each step of differentiation from pluripotent hESC to embryoid bodies (EB) to HSC/HPC to megakaryocytes/platelets. Because of concerns over judicial injunctions against using hESC, we were concerned that there would be ongoing legal challenges to hESC use, which would continue to present an unacceptable level of uncertainty in our research. Therefore, we decided to gain ability to produce, maintain, and manipulate human induced pluripotent stem cells (hiPSC) derived from human fibroblasts and CD34+ hematopoietic cells, so that we could perform the proposed experiments with hiPSC that will not come under legal challenges. This need to establish hiPSC methodology in our laboratory set back our research timetable by about 2 years. Eventually, by late 2011 we were becoming successful in preparing hiPSC from human fibroblasts, and subsequently in 2012 with other funding we were able to continue this work to establish hiPSC also from human umbilical cord blood cells, and once from human peripheral blood CD34+ cells. The funding for this project terminated before we could analyze transcriptional regulatory factors involved in megakaryocyte production from hiPSC.

B. Approach

We went through several different approaches to establishing hiPSC creation in our laboratory – polycistronic lentiviral expression vectors with Yamanka or Thomson factor cDNAs; minicircle DNAs with the Yamanka factor (Oct4, Sox2, Klf4, and c-myc) or Thomson factor (Oct4, Sox2, Lim28, and Nanog) cDNAs or; microRNAs (miR302complex/miR367); the StemGent mRNA system; and finally Yamanaka factor cDNAs in episomal (non-integrating) vectors, which led to our consistent success in generating hiPSC from fibroblasts initially and more recently from umbilical cord blood mononuclear cells and human peripheral blood CD34+ cells. We utilized the serum-free and feeder cell-free system that we had developed for hESC, which we found also to work well for hiPSC. Our major approach to propagation of the undifferentiated/pluripotent cells and their differentiation into EBs, mesoderm, HSC/HPC, maturing megakaryocytes, and platelets, once the cell lines are established, is similar to the systems that we developed for hESC differentiation. After extensive manipulations and testing of culture conditions, we use ROCK inhibitor Y27632 to permit vigorous growth of hiPSC passaged as single cell suspensions and grow undifferentiated hiPSC in serum-free DMEM/F12-based medium with bFGF and 20% KO serum replacement. After extensive efforts to identify substitutes for feeder cells, we now use cell-free extracellular and cellular matrix derived by deoxycholate or Triton X100 treatment of human fibroblasts or E-cadherin-coated plates, which supports long-term growth of undifferentiated hiPSC. We have analyzed many combinations of growth factors to optimize culture conditions for differentiation to HSC/HPC and then megakaryocytes and platelets.

C. Concise Accomplishments

Our most obvious accomplishments to date are two-fold. First is in the area of improving culture conditions to expand undifferentiated hESC and accomplishment of establishing techniques in our laboratory for creating and propagating undifferentiated hiPSC (see above). (1) We passage undifferentiated hESC or hiPSC as single cells after Accutase or trypsin treatment of adherent undifferentiated hESC colonies, using the ROCK inhibitor Y27632. (2) We use defined serum-free medium with bFGF. (3) We use deoxycholate (DOC) or Triton X100 extracts from HFF fibroblasts as cellular and extracellular matrix (CECM) to support maintenance of undifferentiated hESC in culture. (4)

Using sequential combinations of growth factors to differentiate pluripotent stem cells to EBs, which subsequently differentiate into mesoderm, then HSC/HPC, and then megakaryocytes, which produce platelets in suspension cultures under appropriate culture conditions. The megakaryocytes produced in this manner demonstrated normal expression of CD41, CD61, von Willebrand Factor and platelet factor 4, had normal morphology by light and electron microscopy, and underwent proplatelet formation and produced apparently functionally normal platelets. Our second major accomplishment was adaptation of these methods to production of megakaryocytes and platelets from hiPSC.

D. Expanded Accomplishments

Our intention has been to establish optimal culture conditions, first for maintenance and expansion of undifferentiated hESC, then for differentiation of hESC along the desired hematopoietic lineages. The number of differentiated cells that can be produced is relatively fixed once the HSC stage is reached, barring new developments that permit much greater expansion of HSCs than is currently possible. Thus, the ability to continue to produce proliferating undifferentiated hESC is what theoretically permits large, potentially therapeutically relevant numbers of megakaryocytes and platelets to be produced in vitro. We developed methodology that has included growing hESC in low osmolality DMEM/F12 medium with 20% knockout serum replacement, and low to moderate-dose (12 ng/ml) basic fibroblast growth factor. A number of cell-free feeder layer systems have been described for undifferentiated hESC maintenance, which are more or less supportive of long-term self-renewal and maintenance of pluripotency of hESC in culture. We have used the very supportive HFF1 fibroblast feeder cells to produce cell-free extracts using deoxycholate (DOC) or Triton X100 digestion of the HFF1 cells. Residual cellular and extracellular matrix (CECM) adherent to culture dishes after DOC or Triton X100 treatment of HFF cells regularly supports long term maintenance and proliferation of undifferentiated hESC for >30 passages, and these cells, when transferred to ultra-low adherence culture dishes, yield high quality embryoid bodies (EB) capable of differentiating to multiple lineages. In the last year of support we also adapted this system to support propagation of hiPSC. We have found this self-created CECM to be far more supportive of maintaining the undifferentiated state of hESCs than is the commonly used commercial Matrigel. NP40 and water-treated extracts were less effective in supporting hESC growth. Commercial E-cadherin also has promise in supporting hESC and hiPSC maintenance and propagation. We tried to use serum-free, defined TeSR medium, but it is very expensive, and hESC grown in TeSR in our hands have a greater tendency to differentiate spontaneously than those grown in our standard medium. We have made significant progress in developing a multistage culture system for directing differentiation of hESC through EBs to mesoderm, hemangioblasts, hematopoietic stem and progenitor cells, to mature megakaryocytes that produce functional platelets. We use Y27632, a ROCK inhibitor, to generate our initial EBs from single or small clusters of hESC/hiPSC. It appears that VEGF + BMP4 for 2-4 days provides the best support for initial mesodermal differentiation. We have done considerable work with the optimal mixture of growth factors to support hematopoietic cell differentiation. The best combination that we have identified thus far seems to be VEGF + BMP4 + Stem Cell Factor + Flt3 Ligand (FL) + thrombopoietin (TPO) for the next 7-10 days, followed by Stem Cell Factor + Flt3 Ligand (FL) + thrombopoietin (TPO) + interleukin (IL)3 + IL6 + IL11 ± GM-CSF for the next several weeks, for generating megakaryocytes. Our work has generated 4 abstracts presented at national/international scientific meetings so far, 3 of which included summer research students, one presented at the 2009 American Society of Hematology, 2 presented at the 2010 International Society for Stem Cell Research meeting, and 1 presented at the 2011 American society of Hematology meeting, and we anticipate at least 1 and possibly 2 abstracts to be presented at the 2013 International Society for Stem Cell Research meeting. In addition, we have had a presentation by one of our summer students at the ASH Minority Medical Student Award Program annual dinner meeting, an award-winning presentation by another of our summer students at the USF annual research day. We are in the process of preparing several manuscripts – one on the CECM, one on the defined culture system for production of megakaryocytes from hESC, one on platelet production from hiPSC, and one on JAK/STAT signaling during hematopoietic cell production from hESC.

E. Work Plan

Not applicable, since this is a final report after conclusion of funding for this project. However, we are working on completing several manuscripts based on the work conducted for this project, which we hope

to submit for publication in peer-reviewed journals in the coming year. Our support from ONR will be acknowledged in those publications.

F. Major Problems/Issues (if any)

There were two main issues that slowed our progress over the course of this project. First, the judicial rulings and overall political climate that threatened hESC research caused us to re-think the stability of our research. This caused us to set aside most of our initial priorities in order to spend a considerable amount of effort trying to generate human iPSC in our lab, so that we would not be held hostage to the politics of stem cell research. It took nearly 2 years to establish the necessary technology in our lab for reproducible production of hiPSC. This was a serious setback that prevented us from having the sufficient time and resources to do the detailed study that we had planned on the regulation of transcriptional activation during differentiation from the pluripotent cell through definitive megakaryocytes. A second technical problem was that we had difficulty maintaining fully mature platelets in the unactivated state to allow us to define their activation characteristics, but we finally overcame that problem with newly instituted protocols for harvesting and handling of the platelets derived from these cultures.

G. Technology Transfer

None so far.

H. Foreign Collaborations and Supported Foreign Nationals

There have been no foreign collaborations with this project. One of our senior biological scientists in the laboratory, who is working on this project, Dr. Chun Fan, is a Chinese national who has US permanent resident status. He received partial salary and benefits support from this grant. We are still working on developing a collaboration with a small company in China and at the University of South Florida Connect incubator for creation of human pluripotent stem cell research-related reagents.

ONR Grant # N00014-07-10951

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University of South Florida – PI: Kenneth S. Zuckerman, MD
2012 Final Progress Report – Productivity Report

a. Refereed Journal Articles

None

b. Non-Refereed Significant Publications

None

c. Books or Chapters

None

d. Technical Reports

None

e. Workshops and Conferences

Fan C, Liu RY, Li K, Liu, QK, Zuckerman KS (2009) Megakaryocyte production from feeder cell-free cultures of human embryonic stem cells (hESC). Blood 116:995. (Presented at the American Society of Hematology Annual Meeting, New Orleans, LA, 12/2009).

Fan C, Liu RY, Li KX, Liu, QK, Garces A, Zuckerman KS (2010) Functional megakaryocytes and platelets derived from human embryonic stem cells (hESC). ISSCR Wednesday/Thursday Poster Session Abstracts, pp 42-43. (Presented at the International Society for Stem Cell Research Annual Meeting, San Francisco, 6/2010).

Liu RY, Li KX, Fan C, Jackson T, Zuckerman KS (2010) Cellular and extracellular matrix (CECM) extracted from human fibroblasts supports long-term feeder cell-free, serum-free maintenance of functional undifferentiated human embryonic stem cells (hESC). ISSCR Wednesday/Thursday Poster Session Abstracts, p 43. (Presented at the International Society for Stem Cell Research Annual Meeting, San Francisco, 6/2010).

Fan C, Liu RY, Li K, Zuckerman KS (2011) JAK/STAT signal transduction pathway activation during hematopoietic differentiation from human embryonic stem cells (hESC). Blood (ASH Annual Meeting Abstracts) 118:2346. (Presented at the American Society of Hematology Annual Meeting, San Diego, CA, 12/2011).

f. Patents: None

g. Awards/Honors: None