

06SCA02**CT Stem Cell Research Proposal**

Title of Project: **Function of the fragile X mental retardation protein in early human neural development**

Amount requested: \$200,000; Amount funded: \$200,000

Principal Investigator: **Yingqun Joan Huang**

Institution: **Yale University**

This Project's purpose is to understand the molecular mechanism of and to find a cure for the fragile X mental retardation syndrome.

Project Summary

Fragile X syndrome (FXS), which affects approximately 1/4000 males and 1/8000 females, is the leading heritable form of mental retardation and is associated with a variety of learning disorders and behavioral problems. The syndrome is caused by the loss of the fragile X mental retardation protein, called FMRP. Despite enormous efforts on the study of FMRP and the pathogenesis of the disease for the past decade, the function of FMRP and the molecular basis of FXS remain largely unknown. Conclusions derived from studies on nonhuman species including mice, may provide valuable insights into how FMRP might work in cells, but cannot address many of the specific aspects of the human disease. Given that FXS most likely arises from the absence of FMRP during early neural development, it is thus pivotal to perform the studies during the appropriate developmental time windows. Since performing experiments in early developing human brains is impossible, the studies proposed here will allow us to fill this gap by addressing the molecular mechanisms of the disease in early state neurons derived from human embryonic stem cells (hESC). The ability of hESC to differentiate into neural stem cells and neurons in tissue culture dishes thus offers an excellent model system for studying how FMRP may influence its target gene function at the very earliest stages of brain development. Experiments of this sort have not yet been reported. In this proposal, we will genetically modify unregistered hESC lines so that they cannot produce FMRP, a situation that mimics a developing FXS human fetus. We will then examine its effects on the expression of genes that are specifically regulated by FMRP using a variety of molecular, cellular and biochemical methods. We believe that results derived from the proposed studies will set the platform for future more in-depth studies and hold the promise of helping to develop new strategies to combat FXS.

Year 1 Update

The ultimate goal of the proposed research is to understand the molecular mechanism of FXS in order to aid in the development of effective interventions. FXS is caused by a mutation in the FMRI gene that leads to the loss of expression of its encoded protein FMRP. FMRP is an RNA-binding protein that functions through interaction with mRNAs. We proposed to identify mRNA targets regulated by FMRP in neuronal progenitor cells derived from hESCs. This would enable us to gain novel molecular insights into how this disease may develop in the very early stage of human brains. To facilitate target identification, we proposed to create and characterize FMRI gene – knockout (FMRI-KO) hESC lines in year one to be used to derive negative control materials for our next step experiments in year two which involve the functional validation of targets. In this report, we describe how we have successfully made a targeting construct that is to be used to permanently silence the FMRI gene in the hESCs.

06SCA05**CT Stem Cell Research Proposal**

Title of Project: **Quantitative analysis of molecular transport and population kinetics of stem cell cultivation in a microfluidic system**

Amount requested: \$200,000; Amount funded: \$200,000

Principal Investigator: **Tai-Hsi Fan**

Institution: **University of Connecticut**

Collaborators: Joanne C. Conover, Dept. of Psychology and Neurobiology & Center for Generative Biology, University of Connecticut and Xudong Yao, Dept. of Chemistry, University of Connecticut

This Project's purpose is to design and characterize a miniaturized cell culture environment to facilitate stem cell proliferation.

Project Summary

Understanding how biological cells grow, proliferate, and respond to a perturbed microenvironment is the key to the success of maintaining a specific cell line. For stem cells, as well as other cells, this understanding is essential in designing an ideal cell culture system. Although the molecular origins that direct stem cell differentiation are not yet fully understood, it is known that stem cells are sensitive to their growing environment, and in certain conditions, they can differentiate into cells of all germ layers with the potential to replace damaged cells. In this proposed research, our goal is to design and characterize a miniaturized culture system with ideal conditions for the self-renewal of undifferentiated hESCs. Using a miniaturized system can reduce the sample size, the system response time, and the expenditure of precious biochemical reagents. Miniaturized systems can also simplify the design and process for real-time monitoring. In this project, fluorescent microscopy and Fourier transform infrared spectroscopy will be applied to correlate physical and chemical information; including cell morphology, growth kinetics, concentrations of the secreted proteins, and the consumption of nutrients and growth factors in space and time. The microfluidic testbed is designed for continuous medium perfusion and mixing of nutrients and growth factors in the culture medium. The microscale reaction chamber will house a porous gel matrix that can encapsulate hESCs to avoid oversized cell colony growth and a polymer-coated attenuated total reflection crystal will be integrated into the *in situ* Fourier transform infrared spectroscope for quantitative determination of organic compounds in the culture medium, which will be further analyzed by an off-line mass spectrometer. We will establish theoretical models to explore the ideal conditions for maintaining the stability of a sustained hESC line. This research will (1) fill a strong need of understanding small scale transport phenomena involved in stem cell regenerative processes, (2) provide insight into stem cell biology, and (3) suggest optimized settings of control parameters for a large scale cultivation system for use in clinical applications. To be able to achieve the project goal and foster interdisciplinary collaboration at UConn, this project involves faculty members with expertise in stem cell biology, microfluidics, analytical chemistry, biotransport phenomena, and applied mathematical modeling.

Year 1 Update

This research focuses on the design and analysis of a small-scale perfusion culture system that has controllable shear stress and mass transfer rates for the culture of undifferentiated hESCs. The study is of fundamental importance for a better understanding of physiological conditions required for the microenvironment,

especially the mechanisms sustaining stem cell proliferation and differentiation. Our model is important for predicting stem cell behavior and properties that can be translated to large-scale biomedical applications relevant to tissue reconstruction.

Using a microfluidic cell culture system will reduce the cell colony size and the expenditure of precious biochemical reagents (several milliliter culture medium is sufficient for a week-long experiment) and will provide opportunities to investigate short-term cell responses to the environmental change by real time monitoring of cellular level activities. The in-house fabricated fluidic device provides better control of the microenvironment to which the cell is exposed and provides a promising advantage for *in-situ* chemical analysis when integrated with a variety of chemical sensors. Through the integrated analysis and experimental design, real-time detection of cell proliferation kinetics can be achieved. Cell behavior, in small-scale perfusion culture systems, can be very different from the results obtained by using conventional culture dishes, and therefore, providing many opportunities for advancing the field of stem cell biology.

Through our interdisciplinary collaborations, progress has been made toward generating a prototype microfluidic chamber for culturing stems cells. Experimental setting and protocols have been established based on using murine embryonic fibroblast cells as the prototype for cell seeding and culturing. Our quantitative analyses included cell morphology, proliferation kinetics, secretion and transfer of specific growth factors. These are important for the design and settings of control parameters for a scalable stem cell culture system for large-scale applications.

06SCA09**CT Stem Cell Research Proposal**

Title of Project: **Cell Cycle and Nuclear Reprogramming by Somatic Cell Fusion**

Amount requested: \$200,000; Amount funded: \$200,000

Principal Investigator: **Winfried Krueger**

Institution: **University of Connecticut Health Center**

Collaborator: Dr. Theodore Rasmussen

This Project's purpose is to examine the cell cycle dependency of nuclear reprogramming of somatic cells by ES cells.

Project Summary

Somatic cells can be reprogrammed to acquire stem cell properties through fusion with embryonic stem cells. Based on the observations from somatic cell nuclear transfer (SCNT), the required biochemical activities are not permanently associated with the Chromosomes. Only 1 out of 100,000 somatic cells is covered to a stem cell by this ES cell mediated somatic reprogramming (EMSR). This efficiency is low compared to the success rate of about 1 in 100 for SCNT and the hybrid cells carry two sets of chromosomes. One difference between SCNT and EMSR is the absence of a defined oocyte cell nucleus at the time of the oocytes chromosome removal prior to transfer of the somatic nucleus. In ESCs, reprogramming activities are localized in the nucleus and may be retained there by a membrane called the nuclear envelope. Breakdown of the nuclear envelope during cell division may facilitate release of the reprogramming activities and therefore, arresting ES cells at a specific stage during cell division may increase the yield of hybrid cells. In SCNT, terminally differentiated donor cells are less suitable, suggesting that somatic cell division may also impact reprogramming. The two sets of chromosomes in hybrid cells limit their therapeutic utility and make it difficult to identify the set of chromosomes that gives rise to gene expression. Since the reprogramming activities seem to be soluble, diploid hybrid cells might be generated simply by removal of all ESC chromosomes prior to the fusion event. My proposal aims to measure: (Objective 1) the dependency of hES cell fusion mediated reprogramming on the human foreskin fibroblasts (hFFs) cell cycle and, (Objective 2) the dependency of reprogramming in cell cycle optimized hFFs on the hESCs cell cycle and on hESCs devoid of metaphase chromatin. Activation of reporter genes from the somatic donor cells, consisting of green fluorescent protein topaz and Geneticin resistance genes under control of the stem cell specific promoters Rex1 and Oct4, respectively, will be used to assess visually as the ratio of GFP/Geneticin hybrid cells to input cells. Comparative profiling of gene expression in ESCs, hFFs and hybrid cells will be used for monitoring of reprogramming on a gene by gene basis. The experiments will be performed initially with the approved human ES cell line WA09/H9 in collaboration with Dr. Ted Rasmussen who will also act as the supporting PI, and make use of unapproved ES cell lines once the ES Cell Institute becomes operational.

Year 1 Update

We have created transgenic reporter candidate cells from human foreskin fibroblasts that are required for the quantitative assessment of cell cycle effects on reprogramming efficiency. We are now assessing the potential for activation of the stem cell specific reporters by their fusion mediated reprogramming in a cell cycle independent fashion. We have mastered the culture of the hESC line H9/WA09 on mouse embryonic fibroblasts and matrigel and have adapted these cells to the growth on human foreskin fibroblast feeder layers as a step towards their culture

with media lacking animal components. Conditions for stage specific cell cycle arrest are being established with both H9 cells and a human foreskin fibroblast derived reporter cell line as soon as activation through fusion mediated reprogramming of the transgenes in the somatic reporter cell candidates has been validated. We have modified our approach to also include induced pluripotent stem cell-like cells (IPSCs) alongside H9 cells and a non-approved line that we are currently attempting to obtain from the Hovatta or Cellartis laboratories. The comparison of the capacity for somatic reprogramming of IPSCs with established hESC lines in a cell cycle stage specific manner will further aid in the assessment of the clinical utility of IPSCs.

06SCA12**CT Stem Cell Research Proposal**

Title of Project: **Magnetic Resonance Imaging of Directed Endogenous Neural Stem Cell Migration**

Amount Requested: \$199,975; Amount Funded: \$199,725

Principal Investigator: **Erik Shapiro, Ph.D.**

Institution: **Yale University School of Medicine**

This Project's purpose is to use magnetic resonance imaging to assess the effect of combining the delivery of various chemicals into the brain to steer the migration of neural precursor cells to areas of potential need.

Project Summary

Neural stem or precursor cells hold the promise for treatment of a myriad of diseases. At the cutting edge of this research is the idea that steering the migration of one's own neural precursor cells may be useful as a treatment option. Indeed, it has been demonstrated that delivery of various chemicals into the brain can alter and drive migration patterns of one's own neural precursor cells away from their normal routes, to areas of potential need. Additionally, these directed cells have demonstrated significant benefits in several disease models such as ALS and Huntington's disease. To date, few studies have combined the delivery of these chemicals to enhance directed migration of precursor cells. Furthermore, no study has had the capability to monitor these migratory events non-invasively, in real time. Clearly, the successful implementation of this and other stem/precursor cell therapies in humans would be aided by the use of non-invasive methods for tracing migration and engraftment of cells.

The overall objective of this proposal is to use both magnetic resonance imaging (MRI) and immunohistochemistry (IHC) to evaluate the effect of combining the delivery of various chemicals into the brains of adult rats to increase cell migration to desired areas of the brain. We hypothesize that combining the administration of chemicals that increase the birth of new stem cells, with factors that attract stem cells into areas where they normally do not migrate, will lead to increased deviant cell migration, compared to the administration of chemicals that attract stem cells alone. The rationale for this combination therapy is that the beneficial effect of steering one's own stem cells to areas of disease will likely be related to the number of cells that get there. Additionally, by using MRI, in combination with gold standard IHC to quantify the numbers and timing of cell migration, these processes can be followed in real time, serially over several weeks, and non-invasively.

We expect that combined delivery of chemicals that attract migrating cells and stimulate stem cell birth will increase the number of cells that migrate to desired locations in the brain, compared to administration of one chemical alone. Additionally, we will be the first group to use non-invasive imaging to serially monitor, in living animals, the rate of migration as well as the number of newly arriving cells. These studies will set the stage for a comprehensive evaluation of these treatment methodologies in various disease and injury models.

Year 1 Update

This grant proposes to use MRI methodologies to monitor the migration of adult neural precursor cells in the brain. Furthermore, we intend to steer these immature neurons to ectopic sites in the brain, where they could potentially be beneficial for a

variety of diseases including stroke, Parkinson's disease or ALS. The core technological principal of the research is the incorporation of iron oxide particles into neural stem cells. When the stem cells divide to make young neurons, particles can be transferred to these cells. As they move through the brain, MRI can detect these cells at single cell resolution.

The initial characterization of this baseline migration is currently being carried out in the Molecular and Cellular MRI Laboratory, in close collaboration with the Yale Stem Cell Center and Magnetic Resonance Research Center. This involves a linear combination of MRI, image processing, tissue pathology and statistical analysis. As early experiments resulted in variable cell labeling, a rigorous study evaluating label titration in the brain is concurrently being carried out. This titration, the first of its kind, will greatly enhance our ability to longitudinally monitor cell migration in the brain by reducing background and increasing cellular specificity.

06SCA18**CT Stem Cell Research Proposal**

Title of Project: **Lineage Mapping of Early Human Embryonic Stem Cell Differentiation**

Amount Requested: \$200,000; Amount Funded: \$200,000

Principal Investigator: **Craig E. Nelson**

Institution: University of Connecticut

Collaborators: Luke Achenie, Ph.D., David Goldhamer, Ph.D., Brent Graveley, Ph.D., Ren-He Xu, M.D., Ph.D.

This Project's purpose is to map the events leading to the production of different therapeutically relevant cell types from human embryonic stem cells.

Project Summary

Realizing the full promise of stem cell therapy depends upon our ability to generate medically useful cell types from human embryonic stem cells. The primary objective our project is to create a "roadmap" of human embryonic stem cell differentiation that will serve as a key guide in the generation of the cells needed for regenerative medicine and cell replacement therapy.

It is not always widely appreciated that we know very little about early human embryonic development. Scientists cannot study human embryogenesis directly. Instead we infer what we know of early human development from what we learn by studying the development of other embryos, most notably the mouse embryo. However, increasing evidence demonstrated that critical developmental decisions can be dramatically different between human and mice. For instance, one of the earlier and most important decisions made by cells in the embryo, whether to become part of the embryo itself or become part of the placenta, is made in precisely the opposite fashion in mouse embryonic stem cells and human embryonic stem cells.

Thus, in order to make informed decisions about how best to derive medically useful cell types from *human* stem cells, we need to study the development of *human* embryonic stem cells directly. Surprisingly, perhaps, this has not yet been done in any systematic fashion because stem cell cultures are complex mixtures of many different cell types and this heterogeneity makes them difficult to study. Our lab solves this problem by using single cell analysis methods to track the identity of individual human embryonic stem cells as they develop and differentiate in culture.

Year 1 Update

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Surprisingly, perhaps, this has not yet been done in any systematic fashion because stem cell cultures are complex mixtures of many different cell types and this heterogeneity makes them difficult to study. Our lab solves this problem by using single cell analysis methods to track the identity of individual human embryonic stem cells as they develop and differentiate in culture. To do this we amplify the genetic material from single cells as they differentiate and then analyze this material by PCR and on DNA microarrays. In this way we can ascertain the precise identity of

each cell in a complex culture. By gathering this information from stem cells as they develop, we can reconstruct a map of the paths stem cells follow on their way to become mature cells suitable for cell replacement therapy. Drawing this map is a critical step toward our full realization of the great potential of stem cell medicine.

To date, we have set up a stem cell culture facility in our lab, trained several lab members in stem cell culture techniques and are in the process of obtaining all the necessary approvals for human stem cell work. We have adapted and refined single-cell PCR and microarray expression assays for stem cell work, defined the sensitivity of these assays, developed statistical methods for data analysis, and run a pilot experiment on differentiating mouse embryonic stem cells that has revealed a number of interesting genetic interactions in these early stem cell lineages. We have also constructed a map of early mouse stem cells differentiation from this data. We are now proceeding to use these methods to analyze differentiating human embryonic stem cells and illuminate the earliest decisions these cells make as they progress from stem cells to clinically relevant cell types.

06SCA26**CT Stem Cell Research Proposal**

Title of Project: **Generation of Insulin Producing Cells from Human Embryonic Stem Cells**

Amount Requested: \$200,000; Amount Funded: \$200,000

Principal Investigator: **Gang Xu, M.D., Ph.D.**

Institution: University of Connecticut

This Project's purpose is to generate insulin-producing cells from human embryonic stem cells for the treatment of diabetes.

Project Summary

Diabetes is a devastating disease that affects millions of people worldwide. According to the data from the U.S. Centers for Disease Control and Prevention (CDC), currently there are 21 million Americans suffering from diabetes and the cost for diabetes-related healthcare accounts for more than \$100 billion annually. On the other hand, the treatment for diabetes, especially type1 diabetes, is dependent upon the exogenous injection of insulin that is often associated with hypoglycemia unawareness on the part of the patient, in addition to the inconvenience of having to give themselves multiple daily injections.

Stem cell therapy provides an opportunity for curing this disease through cell transplantation of glucose-responsive, insulin-producing cells. Studies in mice have shown that mouse embryonic stem cells can be differentiated into insulin-producing cells and normoglycemia can be restored in diabetic mouse models following transplantation.

I intend to compare mouse and human embryonic stem cells (hESCs), including new nonfederally approved hESCs, for differentiation into insulin-producing beta cells. To this end, strategies, including stepwise differentiation protocols in combination with use of transcriptional factors, growth factors, and coculture with pancreatic cells, will be applied. In addition, diabetic animal models will be established at our center to validate the differentiated cells with respect to regulation of insulin secretion, teratoma formation, long-term cell survival, etc., These efforts will lead to our long-term goal of generating patient-specific nuclear transfer embryonic stem cells for the treatment of diabetes without immunosuppression.

Year 1 Update

Due 10/1/08

06SCA27**CT Stem Cell Research Proposal**

Title of Project: **Directed Isolation of Neuronal Stem Cells From hESC Lines**

Amount requested: \$184,407; Amount Funded: \$184,407

Principal Investigator: **Eleni A. Markakis**

Institution: **Yale University School of Medicine**

Collaborators: Fred H. Gage, Professor and Vi and John Adler, Chair for Research on Age-Related Neurodegenerative Diseases, The Salk Institute, La Jolla, CA

This Project's purpose is to simplify methodology for hES cell culture and standardize components of the cell lines to isolate embryonic neural stem cells.

Project Summary

Available hES cell lines and the protocols used for their maintenance are as numerous and varied as the labs that create them. Current methods for the generation and propagation hES cell lines are so detailed and often so tailored to each specific cell line that it is difficult to know whether the cell lines are in any way comparable. Current protocols for stem cell maintenance are laborious and notably divergent in their methodologies. I will use the expertise I have developed with adult-derived neural stem cells to try to isolate embryonic neural stem cells within hESC lines, and simplify their culture. In this way, I hope to make neural stem cells that are easier to maintain, standardized in their growth conditions (and their makeup), and more widely used. I will employ the density gradient centrifugation method that we use in isolating adult-derived neural progenitor cells, to purify the components of hES cell lines and pull out the fraction of cells that give rise to new neurons. This "purification" will also pull out differentiated cells from the cultures that may promote further differentiation of the line, thereby keeping the cultures purely proliferative for longer periods of time.

Year 1 Update

Our work seeks to simplify the culture of hES cells so that more laboratories may work with them to develop treatments for diseases of the central nervous system.

06SCA30**CT Stem Cell Research Proposal**

Title of Project: **Development of efficient methods for reproducible and inducible transgene expression in human embryonic stem cells**

Amount Requested: \$200,000; Amount Funded: \$200,000

Principal Investigator: **James Li, Ph.D.**

Institution: University of Connecticut Health Center, Department of Genetics and Developmental Biology

This Project's purpose is to establish methods for efficient genetic manipulation of human embryonic stem cells.

Project Summary

Human embryonic stem cells (hESCs) are an unlimited source of precursor cells that can be directed to differentiate into any type of cells for regenerative medicine and studies of toxicology and pharmacology. The promises of hESC applications depend on our knowledge and ability to drive hESC differentiation into particular cell type as desired. Genetic manipulations of hESCs are essential for the study of hESC biology and differentiation. The central purpose of this proposal is to develop an efficient and reproducible method for genetic modifications of hESCs. Currently, the prevalent methods for gene transfer (transgenesis) into hESCs, results in integration of the transgene into random chromosomal positions. These approaches lead to unpredictable expression of the transgene due to varying copy number of the transgene and position-dependent expression. Furthermore, random insertion of the transgene could disrupt the host genome. To circumvent most of these problems, we are going to develop recombinase-mediated transgenesis in hESCs. This strategy takes advantage of the ability of DNA recombinase to recognize a particular short DNA sequence (anchoring cassette) in a pre-characterized position in the genome and to efficiently introduce transgenes into this locus. Our first objective is to introduce an anchoring cassette by viral delivery into hESCs, and establish methods for targeted integration of transgenes in hESCs. The second objective our proposal is to target an anchoring cassette into a specific site of the genome by homologous recombination in hESCs. Establishment of hESC lines allowing efficient site-specific transgenesis will be instrumental for gain-of-function and cell-marking analyses. Furthermore, in combination with RNAi technology, this new form of transgenesis will facilitate lost-of-function and genetic correction studies. Therefore, these hESC lines could have broad experimental applications and should be an important recourse for the stem cell research community.

Year 1 Update

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06SCA31**CT Stem Cell Research Proposal**

Title of Project: **Embryonic Stem Cell As A Universal Cancer Vaccine**

Amount Requested: \$200,000; Amount Funded: \$200,000

Principal Investigator: **Bei Liu, M.D., (Lead PI), Zihai Li, M.D., Ph.D., (co-PI and sponsor)**

Institution: University of Connecticut Health Center

This Project's purpose is to test the idea if embryonic stem cells can be used as a universal cancer vaccine.

Project Summary

Long before embryonic stem (ES) cells were used for genetic and developmental studies, it was appreciated that cancer cells have uniformly undergone a process called "de-differentiation." This process is in fact intimately coupled with tumor formation. The resulting tumor cells bear many similarities to ES cells in that, (a) both express oncofetal antigens (molecules that can trigger immune response); (b) both divide indefinitely at the right conditions; (c) both can differentiate (become other cell types) when proper stimulation was provided. More importantly, tumor cells are clearly the problem of stem cells, which is best exemplified by a variety of leukemias.

Immune system can recognize oncofetal antigens on the tumor cells. In fact, most of the ongoing cancer vaccine trials are targeted against these antigens such as "carcinoembryonic antigen" for the treatment of colon cancer. Unfortunately, targeting one antigen alone is unlikely to generate effective anti-tumor immune responses to mediate tumor rejection due to rapid appearance of escape tumor variants. In this 2-year proposal, we propose to generate tumor vaccines from plain ES cells. The immunogenicity (the ability to induce immune responses) of these ES cells will be further enhanced by expressing heat shock protein (HSP) gp96 on the cell surface. Our laboratory has significant expertise in using HSP technology, which is currently in phase III trial against kidney cancer, melanoma and colon cancer. The ES cell-based cancer vaccines are expected to stimulate the immune system to recognize a variety of oncofetal antigens that are shared across the broad spectrum of cancers. With the proper inflammatory signal such as HSPs, we will test the concept, for the first time, that **ES cells provide a universal cell-based vaccine against cancer**. Although our approach is unlikely to induce self-reactive autoimmune diseases, we will rigorously address this possibility along with other safety concerns. The specific aims therefore, are: (1) *Determine if embryonic stem cell-based vaccines (ES cells with and without surface expression of gp96) are cross-protective against leukemia, sarcoma and breast cancers;* (2) *Study the safety of human ES cell based vaccine in mouse models with the focus on autoimmune diseases;* (3) *Full characterization of ES cell-vaccine in preparation for Investigative New Drug (IND) filing through FDA and a phase I study against advanced human cancer.* **Our study, if successfully carried out, could potentially lead to the beginning of direct clinical testing of stem-based cancer vaccine to benefit cancer patients in the State of CT within 3 years.**

Year 1 Update

Our seed grant, entitled, "Embryonic Stem Cell As A Universal Cancer Vaccine", studies the potential of ES cells to deliver de-differentiation antigens to the immune system to generate anti-tumor immunity. In the first year, we have gained approval

of all animal experiments. We have also become proficient in culturing human ES cells. Since we will use a heat shock protein, gp96 to increase the immunogenicity of ES cells, we have spent a considerable amount of time to construct expression vectors for gp96 and testing these vectors in a number of cell lines. We have also entered a strategic collaboration with Dr. Ren-he Xu, a stem cell expert in our institution. We are now ready to move on to the second phase of our experiment, i.e. testing the ability of engineered hESCs to elicit protective anti-tumor immunity in vivo.

We are focusing on two specific aims, including: *(1) Determine if embryonic stem cell-based vaccines (ES cells with and without surface expression of gp96) are cross-protective against leukemia, sarcoma and breast cancers; (2) Study the safety of human ES cell based vaccine in mouse models with the focus on autoimmune diseases.* Specific progresses in these areas are outlined as follows:

1. Establish culturing conditions of human ES cells.
2. Optimization of retroviral and lenti-viral expression vector to drive the expression of heat shock protein gp96 on cell surface.

In our proposal, we presented some data on the construction of a lenti-viral expression vector for heat shock protein gp96. We have carried out large-scale production of gp96-expressing retroviral vectors, concentrated the virus and quantified the viral titer.

06SCA34**CT Stem Cell Research Proposal**

Title of Project: **Pragmatic Assessment of Epigenetic Drift in Human ES Cell Lines**

Amount requested: \$200,000; Amount funded: \$200,000

Principal Investigator: **Theodore Rasmussen, Ph.D.**

Institution: **University of Connecticut, Center for Regenerative Biology**

Collaborator: Ren-He Xu, Ph.D., UConn Human ES Cell Core Director (Consultant)

This Project's purpose is develop practical quantitative methods to assess the quality of human ES cell lines so they can be maintained and grown without compromise.

Project Summary

It is widely recognized among stem cell researchers that human embryonic stem cell (hESC) lines can be challenging to culture, and can undergo unexpected and irreversible changes that can render them useless for further research or therapies. Reliable hESC lines can unexpectedly lose their pluripotency (the ability to form a variety of cell-types) or can undergo undesirable differentiation events in the course of cell culture. These troublesome degradations in hESC quality are difficult to detect and can cause costly losses of experimental data and hamper progress in the laboratory.

Epigenetics is a field of study that examines influences on gene expression that do not involve changes in the underlying sequence of DNA. A current hypothesis is that ESCs undergo "epigenetic drift," a poorly understood phenomenon that alters gene expression in ESCs without the accumulation of DNA-based mutations. At present, no simple methods exist to monitor the epigenetic quality of ESC lines and drift is detected only after hESCs fail to perform as expected. My laboratory has developed a novel and quantitative technology to assess the epigenetic status of cells based on their chromatin content. We will use these mechanisms to try to understand why hESCs under epigenetic drift and to try to develop simple means to detect such drift when it occurs. In addition, these studies will provide an unambiguous method to assess the relative qualities of federally-approved and non-federally-approved hESC lines. If successful, the research proposed here will make the culture and maintenance of hESCs a much more reliable process and decrease the time required to bring hESC-based therapies to the clinic.

Year 1 Update

During the previous year, the Rasmussen Laboratory initiated research under a Connecticut State stem cell grant entitled, "Pragmatic Assessment of Epigenetic Drift in Human ES Cell Lines". The overall purpose of this grant is to develop unambiguous assays that allow us to determine and monitor the developmental quality of hESC lines grown *in vitro*. All human cells contain identical content of DNA, yet cells come in many different varieties. The variety, or "type", of a cell is therefore determined largely by the set of genes that are expressed in that cell (out of the total set of genes present within the human genome). Each cells' characteristic set of genes are expressed and maintained semi-stably by an epigenetic system that operates within each cell. This system functions by mechanisms that operate through the action of proteins that physically bind to DNA in sequence-specific ways that are characteristic for each cell type. The involved proteins are called histones, and these can be

modified by small molecular additions such as methylation and acetylation, which in turn, can affect the status of expression of associated genes.

During the past year, we completed optimization and validation of a new molecular biology method that can quantitatively monitor epigenetic states in ES cells. Our experiments indicate that epigenetic status of human ES cells is quite stable over protracted growth of these cells in the laboratory. This bodes well for their future use in therapies. However, sometimes epigenetic alterations do occur in human ES cells. The methods being developed in my lab will allow us to monitor and confirm the quality of specific isolates of ES cells to ensure their utility and safety prior to their use in future cell-based therapies. We continue to expand these epigenetic analyses to cultured ES cells, and to improve the sensitivity and accuracy of our epigenetic validation methods.

06SCB03**CT Stem Cell Research Proposal**

Title of Project: **Optimizing Axonal Regeneration Using Polymer Implant Containing Human Embryonic Stem Cell-derived Gila**

Amount requested: \$602,127; Amount Funded: \$529,872

Principal Investigator: **Akiko Nishiyama, M.D., Ph.D.**

Institution: **University of Connecticut**

Collaborator: Richard Parnas, Ph.D., Institute of Material Science, UConn, Storrs

The Project's purpose is to use glial cells from human embryonic stem cells to promote regeneration in the brain.

Project Summary

Axons (nerve fibers) that are severed in the central nervous system (CNS) do not regenerate efficiently. This leads to permanent loss of function after an insult to the brain such as trauma and ischemia. CNS axons appear to have an intrinsic potential to regenerate, but their regenerative ability is limited in vivo by environmental constraints. CNS glial cells, which fill the space through which regenerating axons must navigate, play a major role in establishing the environment that is either favorable or restrictive to axons. One way to promote axonal regeneration is to fill the cavity created by damaged tissue with glial cells that provide a favorable environment for axonal growth. The goal of the proposed studies is to develop a three-dimensional cellular implant that contains human embryonic stem cell (hESC)-derived glial cells to promote axonal regeneration in the CNS. The proposal is built upon our recent observations that two types of glial cells isolated from the developing mammalian brain called astrocytes and NG2 cells promote axonal growth in culture, and that growing axons in the developing brain intimately contact NG2 cells. Our hypothesis is that immature glial cells derived from hESCs support regeneration of injured CNS axons.

We will work with Dr. Richard Parnas in the Institute of Material Science in Storrs to develop a polymer scaffold made of poly-lactic acid (PLA) into which hESC-derived glial cells will be seeded. The seeded scaffolds will be implanted into injured adult mouse brain together with microspheres that contain growth-stimulatory growth factors, and the extent of axonal regeneration will be assessed by quantifying labeled regenerating axons. We will carry out the investigation in the following three steps: (Aim 1) Study how axons behave on hESC-derived glial cells in two-dimensional culture. (Aim 2) Study how axons behave on hESC-derived glial cells in three-dimensional culture. (Aim 3) Study the effects hESC-derived glial cells on axonal regeneration following brain injury.

Year 1 Update

During the first year of the funding period, we have successfully begun to grow NIH-approved hESCs. Two members of the group were trained by the UConn Core Facility and we are currently exploring methods to grow the cells to a high density for storage and to differentiate them into neural cells.

Year 2 Update (3/1/09)

We have made significant progress during the past year. The laboratory is now adequately equipped to work with hESCs. The PI and her graduate student, Hao Zuo, attended the Workshop on neuronal differentiation of hESCs and have succeeded in generating neural stem cells from hESCs. We have optimized the lesion model are

ready to transplant hESC-derived glia when we succeed in generating glial cells from hESCs. We have also begun to collaborate with Drs. Diane Burgess in the School of Pharmacy and Robert Weiss in the School of Engineering who will provide us with microbeads and poly-lactic acid polymer, respectively, which will be used with hESC-derived glial to study the ability of hESC-derived glia to promote axonal regeneration.

06SCB05**CT Stem Cell Research Proposal****Title of Project: Directing production and functional integration of embryonic stem cell-derived neural stem cells**

Amount requested: \$998,123; Amount Funded: \$878,310

Principal Investigator: **Laura Grabel, Ph.D.**

Institution: **Wesleyan University**

Collaborators: Janice R. Naegele, Gloster Aaron, Alex Lichtler, Leonardo Aguila

This Project's purpose is to define conditions that promote embryonic stem cell differentiation into neurons, in culture and in the brain.

Project Summary

An estimated 50 million Americans each year are diagnosed with neurological disorders, including epilepsy, Parkinson's disease and Alzheimer's disease. There are currently no effective treatments for many of these conditions. Human embryonic stem (ES) cells hold great promise for the treatment of these neurological disorders. Before this promise can be realized, conditions that support the production of neural cells for transplant and facilitate functional integration of transplanted cells must be identified. Our proposal is aimed at these two goals. Objective 1 is based on our prior studies that established a role for the signaling molecule Hedgehog in production of neurons from mouse ES cells. Proposed studies will establish the mechanism whereby Hedgehog performs this function, determine how Hedgehog influences the differentiation of human ES cells to neural stem cells, and investigate how the Hedgehog pathway interacts with other pathways involved in the production of neurons. Objective 2 will examine the conditions required for effective transplant integration and is based upon our studies examining the fate of mouse ES cell-derived neural stem cells transplanted to a damaged brain. We use a mouse model of temporal lobe epilepsy in which induced excitotoxic seizures cause cell death in the adult hippocampus and stimulate production of new neurons in the dentate gyrus region. Transplanted ES-derived neural stem cells migrate to the dentate gyrus where they are able to respond to endogenous cues and become granule neurons, a cell type normally found in this region. In animals not subjected to prior seizures, however, transplanted cells typically form tumors. We, therefore, hypothesized that seizures upregulate signals that promote the migration, differentiation, and functional integration of ES-derived neural stem cells. Proposed studies will determine if ES-derived granule neurons in the hippocampus have the electrophysiological properties of endogenous granule neurons, examine the fate of human ES-derived neural stem cells, determine if the chemokine SDF-1a promotes migration of transplants to the dentate gyrus, and examine the role of Hedgehog in promoting transplant success. The results of these studies will aid in the design of human embryonic stem cell-based therapies to treat neurological disorders.

Year 1 Update

As we begin to think about designing embryonic stem cell therapies to treat neurodegenerative disorders and injuries to the central nervous system, we must understand how to efficiently generate the appropriate cell type for transplant. This requires identifying the signals that promote the transition of embryonic stem cells into neural stem cells and neurons. Towards this end, under Objective 1, we have been investigating the role of the extracellular signaling molecule Hedgehog in the production of neural stem cells from embryonic stem cells. Our previous studies indicated that mouse embryonic stem cell lines deficient for an essential component

of the Hedgehog signaling pathway were unable to generate neural stem cells or neurons in culture. To more clearly define the role of the Hedgehog signal, we turned to a mouse embryonic stem cell line engineered to express a green fluorescent tag when the cells differentiate into neural stem cells. This allows for easy quantification of this transition. Using this cell line, we now demonstrate upregulation of Hedgehog signaling during the production and maturation of neural stem cell colonies, called rosettes. Using small molecule agonists and antagonists of Hedgehog signaling, we show that Hedgehog acts not by stimulating the differentiation of embryonic stem cells into neural stem cells, but by supporting the proliferation of an intermediate precursor cell and by promoting the survival of neural stem cells. We are currently testing the role of Hedgehog in the production of neural stem cells and neurons from human embryonic stem cells. These data suggest that the addition of Hedgehog will increase the yield of neural stem cells and neurons generated by human embryonic stem cells for transplantation therapies. We understand little about the how host conditions effect the fate of transplanted ESC derived neural stem cells and neurons. Towards this end, under Objective 2, we have been studying the fate of transplanted ESC-derived neural stem cells into a mouse model of epilepsy in which animals are subjected to excitotoxin-induced seizures that damage the hippocampus, the site of learning and memory. Transplanted ESC-derived neural stem cells migrate to the dentate gyrus region of the hippocampus, where they undergo the pattern of differentiation associated with host neural stem cells in that region, and become granule neurons. Preliminary analysis suggests these cells hook up with the host circuitry and are functional. In contrast, if cells end up in the fimbria region of the hippocampus, they become oligodendrocyte support cells, a cell type prevalent in the fimbria, not neurons. These studies suggest that local signals direct differentiation of ESC-derivatives. Additional experiments suggest a role for the secreted molecule SDF-1 in the migration of transplanted cells. The results from these studies will help in the future design of human ESC-based therapies to treat neurological disorders.

Year 2 Update (3/1/09)

As we begin to think about designing embryonic stem cell therapies to treat neurodegenerative disorders and injuries to the central nervous system, we must understand how to efficiently generate the appropriate cell type for transplant. This requires identifying the signals that promote the transition of embryonic stem cells into neural stem cells and neurons. Towards this end, under Objective 1, we have been investigating the role of the extracellular signaling molecule Hedgehog in the production of neural stem cells from embryonic stem cells. Our previous studies indicated that mouse embryonic stem cell lines deficient for an essential component of the Hedgehog signaling pathway were unable to generate neural stem cells or neurons in culture. To more clearly define the role of the Hedgehog signal, we turned to a mouse embryonic stem cell line engineered to express a green fluorescent tag when the cells differentiate into neural stem cells. This allows for easy quantification of this transition. Using small molecule agonists and antagonists of Hedgehog signaling, we showed that Hedgehog acts not by stimulating the differentiation of embryonic stem cells into neural stem cells, but by supporting the proliferation of an intermediate precursor cell and by promoting the survival of neural stem cells. We have recently turned to the human embryonic stem cells and developed a rapid, direct protocol for their differentiation into neural stem cells and neurons. We are now studying what signals influence this fate.

We understand little about how host conditions affect the fate of transplanted ESC-derived neural stem cells and neurons. Towards this end, under Objective

2, we have been studying the fate of transplanted ESC-derived neural stem cells into a mouse model of epilepsy in which animals are subjected to excitotoxin induced seizures that damage the hippocampus, the site of learning and memory. Transplanted ESC-derived neural stem cells migrate to the dentate gyrus region of the hippocampus, where they undergo the pattern of differentiation associated with host neural stem cells in that region, and become granule neurons. Preliminary analysis suggests these cells hook up with the host circuitry and are functional. In contrast, if cells end up in the fimbria region of the hippocampus, they become oligodendrocyte support cells, a cell type prevalent in the fimbria, not neurons. These studies suggest that local signals direct differentiation of ESC-derivatives. Additional experiments suggest a role for the secreted molecule SDF-1 in the migration of transplanted cells. Recent analysis reveals a decrease in tumor formation if the ESC-derived neural cells are isolated free of the undifferentiated cells prior to transplant. We are now transplanting human ESC-derived neural progenitors. The results from these studies will help in the future design of human ESC-based therapies to treat neurological disorders.

06SCB08**CT Stem Cell Research Proposal**

Title of Project: **dsRNA and epigenetic regulation in embryonic stem cells**

Amount requested: \$1,000,000; Amount funded: \$800,000

Principal Investigator: **Gordon G. Carmichael, PI; Asis K. Das, Co-Investigator**

Institution: **University of Connecticut Health Center**

This Project's purpose is to understand how gene expression in human embryonic stem cells is regulated by double-stranded RNA and develop a versatile new technology for stem cell transgenesis.

Project Summary

The ultimate goal of this project is to elucidate some of the fundamental mechanisms that govern stem cell self-renewal, pluripotency and differentiation in humans. Embryonic stem cells derived from human blastocysts (hESCs) are endowed with two remarkable features. They have the capacity for self-renewal, i.e. the potential to differentiate into virtually all the cells of the human body. hESCs represent the only experimental system available to unfold the underlying mechanisms of human development. hESCs also have the awesome potential for regenerative medicine – our ability to heal tissues and cure individuals from heritable genetic disorders. To fulfill these dreams, however, we need not only discover the key molecular players that govern “stemness”, but also develop facile technologies with which to manipulate the stem cells and their genes. We are proposing experiments to advance each of these seminal areas of stem cell research.

Epigenetic reprogramming is a hallmark of embryonic development. We have recently discovered an intriguing connection between dsRNA, RNA editing and heterochromatin that may be altered in hESCs. It is therefore important to learn how dsRNA is involved in the regulation of chromatin in stem cells, and how this pathway changes during cell differentiation. What keeps the ES cell chromatin in its wonderfully flexible state and can we find some underlying key to the switch? If we can do this, we might be able to modulate it to our liking.

Specifically, this project has three aims: 1) to characterize the underlying silencing machinery related to ADAR and vigilin. 2) to test models for RNA-mediated establishment of heterochromatin in hESCs. 3) to develop a versatile vector system for manipulation of stem cells that will not only enable us to carry out the experiments in aims 1 and 2, but also to generate stem cell lines for future basic research on development and differentiation, as well as disease therapy. Once developed, the technology and cell lines will be made available to the Connecticut stem cell community.

Year 1 Update

The goal of this project is to elucidate some of the fundamental mechanisms that govern stem cell self-renewal, pluripotency and differentiation in humans. Of particular interest to us are the pathways by which embryonic stem cells respond to double-stranded RNA molecules (dsRNA). In particular, we are interested in the dsRNA response pathways of interferon induction, RNA editing and establishment of heterochromatin via the protein vigilin. Although culturing of human embryonic stem cells in our labs began later than we anticipated, a number of goals we originally set forth for year one of this grant was nevertheless accomplished. Through this progress, we now have a great deal of new and interesting information about several

aspects of dsRNA regulation in hESCs. Studies supported by this award led to two manuscripts that have been submitted for publication. We examined in detail the expression in hESCs of proteins involved in dsRNA response pathways. Interestingly, one of these pathways, the interferon response pathway, appears to be missing in these cells. This is not only scientifically interesting, but also has practical implications relating to how dsRNAs can be utilized for gene silencing in stem cells. A second dsRNA response pathway, RNA editing, is robust in hESCs. We found that special vectors we have designed for future experiments, when introduced into hESCs, generate dsRNAs that are extensively altered by RNA editing. This is likely to influence the fate of these RNAs, and we are in the process of learning more molecular details about this phenomenon. Finally, we have prepared and validated constructs that will allow us to test our proposed model for the role of the protein vigilin in the establishment of heterochromatin in both stem cells and their differentiated progeny.

Year 2 Update (3/1/09)

The goal of this project is to elucidate some of the fundamental mechanisms that govern stem cell self-renewal, pluripotency and differentiation in humans. Of particular interest to us are the pathways by which embryonic stem cells respond to double-stranded RNA molecules (dsRNA). In particular, we are interested in the dsRNA response pathways of interferon induction, RNA editing and establishment of heterochromatin via the protein vigilin. In the past year we accomplished a number of stated goals, but also made several new discoveries that will guide future studies. Through this progress, we now have a great deal of new and interesting information about several aspects of dsRNA regulation in hESCs. Studies supported by this award have led so far to three published papers and a fourth that has been submitted.

We examined in detail the expression in hESCs of proteins involved in dsRNA response pathways. Interestingly, one of these pathways, the interferon response pathway, is missing in these cells. This is not only scientifically interesting, but also has practical implications relating to how dsRNAs can be utilized for gene silencing in stem cells. A second dsRNA response pathway, RNA editing, is robust in hESCs. Normally, editing is associated with preventing some messenger RNA molecules from being exported from the nucleus to the cytoplasm. This is true for differentiated cells, but not for hESCs. While studying the reason for this altered response pathway we made some discoveries that should shed new light on factors involved in pluripotency and differentiation.

06SCB09**CT Stem Cell Research Proposal**

Title of Project: Alternative Splicing in Human Embryonic Stem Cells

Amount requested: \$1,000,000; Amount funded: \$880,000

Principal Investigator: **Brenton R. Graveley**

Institution: University of Connecticut Health Center

This Project's purpose is to identify alternative splicing events that correlate with and/or maintain human embryonic stem cells in their undifferentiated state and those that drive differentiation.

Project Summary

Cells and organisms function based on the expression patterns, actions, and interactions of thousands of genes and their products. In order to fully understand how stem cells work and to develop the power to differentiate human stem cells into specific cell types for therapeutic use, it is essential to determine their complete gene expression program. Many studies have been conducted to elucidate the transcription profile of different stem cell populations including human embryonic stem (hES) cells. However, all of these studies have overlooked an important aspect of the gene expression program of these cells – alternative splicing. Alternative splicing is one of the most important mechanisms by which gene expression is regulated and as many as 75% of human genes encode alternatively spliced transcripts. The main goal of this proposal is to determine the alternative splicing events that occur in undifferentiated hESC cells and in hES cells undergoing differentiation into different cell types as well as the roles of specific RNA binding proteins such as Musashi1, in controlling these alternative splicing events. This research project will allow us to obtain a more thorough understanding of the gene expression program of hES cells which is essential knowledge for the long term goal of directing the differentiation of hES cells into specific cell types.

Year 1 Update

Cells and organisms function based on the expression patterns, actions, and interactions of thousands of genes and their products. In order to fully understand how stem cells work and to develop the power to differentiate human stem cells into specific cell types for therapeutic use, it is essential to determine their complete gene expression program. Many studies have been conducted to identify the genes that are specifically turned on in human embryonic stem (hES) cells to endow them with their amazing potential to generate all possible cell types. However, all of these studies have overlooked an important aspect of the gene expression program of these cells - alternative splicing. Alternative splicing is a process by which the quality and function of a gene that is turned on can be altered thus increasing the diversity of how our genes work. This process can, for instance, make a given gene function one way in one cell type, but in a completely opposite way in another cell type. Alternative splicing is one of the most important mechanisms by which gene expression is regulated and as many as 75% of human genes use this process of gene regulation. The main goal of this project is to identify the alternative splicing events that occur in undifferentiated hES cells and in hES cells undergoing differentiation into different cell types as well as the roles of specific RNA binding proteins such as Musashi1, in controlling these alternative splicing events. This research project will allow us to obtain a more thorough understanding of the gene expression program of hES cells which is essential knowledge for the long term goal of directing the differentiation of hES cells into specific cell types.

During the first year of this project we have developed a technology that allows us to examine the alternative splicing of all 25,000 human genes at once. Using this technology, we have analyzed alternative splicing in undifferentiated hES cells and in hES cells that have been induced to differentiate by the addition of a signaling molecule called BMP4. While we still need to fully analyze and validate the data we have obtained, our results suggest that the process of alternative splicing does indeed have an important role in hES cell differentiation. Thus, a more thorough analysis of this process will significantly increase our understanding of the gene expression program in hES cells which will be useful in directing hES cell differentiation for therapeutic purposes.

Year 2 Update (3/09)

Human cells function based in part upon which of the 25,000 genes are turned on and which are turned off. In order to fully understand how stem cells work and to develop the power to turn human stem cells into different types of cells such as brain cells or muscle cells, it is essential to determine which genes are turned on or off in both stem cells and brain or muscle cells. Many studies have been conducted to determine this information for human embryonic stem (hES) cells as this will inform us about how hES cells have the amazing potential to generate all possible cell types. However, all of these studies have overlooked an important aspect of gene functions - a process called alternative splicing (AS). Alternative splicing is a process by which the quality and function of a gene that is turned on can be altered - essentially, each gene can come in a variety of flavors or colors. So, it is not only important which of the 25,000 genes are turned on or off, but also which flavor or color of those genes that are turned on. These different flavors or colors, for instance, make a given gene function in different ways. It is extremely important as ~95% of human genes come in a variety of flavors/colors. The main goal of this project is to determine the flavors and colors of the genes that are turned on in undifferentiated hES cells and in hES cells that are changing into different cell types, such as brain or muscle. This project will allow us to obtain a more thorough understanding of how hES cells function, which is essential knowledge for the long term goal of directing the differentiation of hES cells into specific cell types.

During the first two years of this project, we have developed technologies that allow us to distinguish which flavors or colors are being used for all 25,000 human genes at once. We have used this technology to analyze undifferentiated H9 hES cells grown in three different conditions. While we still need to fully analyze the data we have obtained, our results suggest that there are many differences in the flavors or colors of each gene when the cells are treated differently. This will have a large impact on the field as it is critical to understand how these cells are behaving and it is clear that how the cells are grown can make a considerable difference on their properties. Thus, a more thorough analysis of this process will significantly increase our understanding of how genes function in hES cells.

06SCB11**CT Stem Cell Research Proposal**

Title of Project: **Migration and Integration of Embryonic Stem Cell Derived Neurons into Cerebral Cortex**

Amount requested: \$638,218; Amount funded: \$561,632

Principal Investigator: **Joseph LoTurco**

Institution: **University of Connecticut**

Collaborator: Rosalyn Fitch

This Project's purpose is to discover and develop ways to improve the integration of transplanted hESC derived neurons into brain.

Project Summary

The cerebral cortex is the major target of several currently untreatable degenerative and traumatic brain disorders Alzheimer's disease and stroke. It is also among the largest and most complex tissues in the body. Composed of more than 5 billion cells and at least 20 different neuronal and glial cell types, the structure and function of cerebral cortex is built upon a framework of 7 neural layers. Reconstruction of cerebral cortex by hESC transplantation will require development of methods to direct the migration of transplanted neurons into the appropriate layers and locations. This is a significant challenge and will likely require methods to direct the migration of transplanted neurons by directly manipulating the mechanisms that control the migration of transplanted cells. Many of the mechanisms that operate during normal development to ensure the appropriate migration and position of neurons in the developing brain have been discovered over the past twenty years, and we hypothesize that by manipulating these same mechanisms, that it will become possible to direct the patterns of migration of hESC-derived neurons transplanted into the brain. This proposal then focuses on a set of experiments in which known and novel migration control proteins will be manipulated in hESC-derived neurons. The migration and integration of modified hESCs will be assessed in normal and neonatal cerebral cortex damaged by ischemic insult. Hypotheses will be tested concerning the mechanism of migration of transplanted hESCs and methods will be developed to both enhance and direct migration. Together these experiments should lead directly to insights into how to improve the migration and integration of neurons derived from hESCs and may lead directly to therapeutic approaches for the damaged neonatal cerebral cortex.

Year 1 Update

The cerebral cortex is the major target of several currently untreatable degenerative and traumatic brain disorders Alzheimer's disease and stroke. If neural cell transplantation therapies are to be successful, it will necessary to direct the migration and positioning of transplanted neurons. Many of the mechanisms that operate during normal development to ensure the appropriate migration and position of neurons in the developing brain have been discovered over the past twenty years, and we hypothesize that by manipulating these same mechanisms it will become possible to direct the patterns of migration of hESC-derived neurons transplanted into the damaged or diseased brain. Toward this effort in the first year of the funding period, we have established culture of H9 human embryonic stem cells and grown them into neuronal progenitor cells. We have also constructed DNA vectors that can now be used to alter the stem cell derived neurons in ways that may improve their migration once transplanted.

Year 2 Update

The cerebral cortex of the brain is a target for major brain disorders that result in the loss of neurons. For example, Alzheimer's disease and stroke both result in widespread loss of neurons in the cerebral cortex. Such disorders may one day benefit from stem cell replacement therapies in which neurons are transplanted to repopulated neural circuits with new healthy cells. One of the greatest challenges confronting the development of stem cell therapies is to ensure that transplanted cells are appropriately positioned within a tissue or organ. This is particularly important in the case of the brain where the precise location of cells is central to normal function. In this project we will test strategies that may improve the positioning and integration of transplanted neurons derived from human embryonic stem cells. We are using information derived from studies of the normal development of the cerebral cortex to design strategies for producing the appropriate types of neurons for transplantation, and to encourage those transplanted neurons to distribute into the brain. Progress in the first two years of the project has largely been in establishing cell cultures of embryonic stem cells that give rise to cerebral cortical neurons, and in establishing transplantation protocols and methods to test these cells in animal models of neuronal degeneration.

06SCB14**CT Stem Cell Research Proposal**

Title of Project: **SMAD4-based ChIP analysis to screen target genes of BMP and TGF β Signaling in Human ES Cells**

Amount requested: \$1,000,000; Amount Funded: \$880,000

Principal Investigator: **Ren-He Xu**

Institution: **University of Connecticut**

Collaborator: Brenton Graveley

This Project's purpose is to screen target genes of BMP and TGF β signaling and analyze their role in genetic control of human ES cell fates.

Project Summary

This proposal is based on my previous and ongoing research that has identified two essential signaling pathways governing the early fates of human embryonic stem cells. The BMP pathway promotes the cells to differentiate, whereas the TGF β pathway sustains their self-renewal. We propose here to search for target genes for both pathways in human embryonic stem cells by using a global searching strategy that involves chromatin-immunoprecipitation and promoter arrays.

Year 1 Update

This project is based on previous and ongoing research that has identified two essential signaling pathways governing the early fates of human embryonic stem cells. The BMP pathway promotes the cells to differentiate, whereas the TGF β pathway sustains their self-renewal. In this project, we search for target genes for both pathways in human embryonic stem cells by using a global searching strategy that involves chromatin-immunoprecipitation (ChIP) and promoter arrays.

In year one, we have met the milestones for Aim one including 1) identification of suitable antibodies and some potential target genes for ChIP, 2) construction of plasmids to express genes to stimulate or inhibit the TGF β and BMP pathways, and 3) formulation of an all-defined medium.

Year 2 Update (3/09)

This project is aimed to decipher the molecular mechanisms that govern the early fates of human embryonic stem cells (hESCs), which include the signaling pathways stimulated by the transforming growth factor (TGF β) to sustain hESC self-renewal and bone morphogenetic protein (BMP) to induce hESC differentiation.

In year one, we completed Aim 1 and started to work on Aim 3. We examined in detail the expression of pluripotency genes in hESCs treated with molecules that stimulate or inhibit the TGF β and BMP pathways. By inhibiting the TGF β pathway in this way, we observed that the expression of the key pluripotency gene *NANOG* appears to rapidly decrease. These important findings suggest that *NANOG* is a direct target for the molecule called SMAD2/3 that is activated in the TGF β pathway. By using multiple approaches, we confirmed that SMAD2/3 controls the expression of *NANOG*. This is the first evidence that links a signaling pathway such as TGF β to a pluripotency gene in hESCs. This finding is of great significance in advancing our understanding of the mechanisms for hESC self-renewal and differentiation. A manuscript containing this information has been accepted by the prestigious journal *Cell Stem Cell*. In year two, we started genome-wide search for SMAD target genes in hESCs as proposed for Aim 2. Via collaboration with Dr. James Thomson of

University of Wisconsin-Madison, we have obtained global profiles of target genes for SMAD1/5/8, SMAD2/3, and SMAD4. Right now, we are validating the binding of these SMADs with the targets through specific protein-DNA binding assays. In addition, we started to work on Aim 3 by analyzing the biological relevance of some select target genes such as *ABCG2* in hESCs and their derivative cell types. *ABCG2* is a cell membrane transporter protein expressed in many normal and cancer stem cells, pumping out toxic chemicals from the cells. Cancer cells become resistant to chemotherapy when they express *ABCG2* or other transporters in their membranes. Unexpectedly, we found *ABCG2* is absent in hESCs but present in trophoblast and neural progenitor cells differentiated from hESCs. The *ABCG2*-expressing cells have fewer cell deaths than non-expressing hESCs in response to chemotherapeutic treatment. This work is being prepared for publication.

06SCB18**CT Stem Cell Research Proposal**

Title of Project: **Role of the leukemia gene MKL in developmental hematopoiesis using hES cells**

Amount requested: \$973,469; Amount Funded: \$856,654

Principal Investigator: **Diane Krause**

Institution: **Yale University**

This Project's purpose is to determine the role of MKL in developmental hematopoiesis using primary murine cells and human embryonic stem cells..

Project Summary

Acute megakaryoblastic leukemia (AMKL) is a type of leukemia that occurs exclusively in newborns and infants less than 2 months of age. The disease has a very poor prognosis such that the median survival is only 8 months. My laboratory has generated significant data regarding the function of a gene called MKL that is mutated in a proportion of patients with AMKL. We have demonstrated that levels of this protein change during the maturation of blood cells and we have obtained strong evidence that specific signaling pathways are involved in how MKL affects the cells to make them leukemic. Since this leukemia occurs in newborn infants, it is likely that the cancer stem cell for this leukemia arises during fetal development. We propose to build upon our data to identify more clearly the mechanism by which MKL affects blood maturation during fetal development in mice, and, most relevant to the CT stem cell initiative, we will also use human embryonic stem cells as a model of human prenatal development to study the role of MKL in blood maturation in people. This grant is highly relevant to the CT stem cell initiative because we are proposing to expand our current research on leukemia to compare and contrast data obtained during prenatal development in mice with that obtained during blood development of human ES cells.

Year 1 Update

Acute megakaryoblastic leukemia (AMKL) is a type of leukemia that occurs exclusively in newborns and infants less than 2 months of age. The disease has a very poor prognosis. Most patients survive for only 8 months. My laboratory is studying the function of the gene called MKL that is mutated in the cancer cells of some patients with AMKL. We have demonstrated that levels of this protein change during the maturation of normal blood cells and we are testing hypotheses regarding how MKL affects cells to make them leukemic. Since this leukemia occurs in newborn infants, it is likely that the cancer stem cell for this leukemia arises prior to birth during fetal development. Our studies over the past year have begun to clarify the mechanism by which MKL affects blood growth during fetal development in mice, and, most relevant to the CT stem cell initiative, we have use human embryonic stem cells as a model of human prenatal development to study the role of MKL in blood maturation in people. This grant is highly relevant to the CT stem cell initiative because we are proposing to expand our current research on leukemia to compare and contrast data obtained during prenatal development in mice with that obtained during blood development of human ES cells.

Year 2 Update

Studies funded by a grant from the State of Connecticut in the Krause laboratory are focused on how bone marrow cells become cancerous. Specifically, we study the gene called MKL1, which are mutated in a specific type of leukemia that usually

occurs in newborns and infants under 1 year of age. In the past year, we have made great progress on our funded studies. We published a manuscript on the normal function of MKL1 in adult blood formation. This was published in the *Blood*, the leading journal in our field. In other ongoing studies, we are assessing the role of MKL1 in blood formation during embryonic development using mouse and human embryonic stem cells. The next step after determining whether the role played by MKL1 in embryonic blood formation is different from that in the adult will be to assess mutations of MKL1 to determine how they promote leukemia formation.

06SCC04**CT Stem Cell Research Proposal**

Title of Project: **Directing hES derived progenitor cells into musculoskeletal lineages**

Amount requested: \$4,000,000; Amount Funded: \$3,520,000

Principal Investigator: **David W. Rowe, M.D.**

Pi of each Project of this Group Grant: **Alexander Lichtler, Ph.D., Leonard Aguila, Ph.D., Dong-Guk Shin, Ph.D., Liisa Kuhn, Ph.D., David Rowe, M.D., Mina Mina, D.D.S., Ph.D., Robert Kosher, Ph.D., David Goldhamer, Ph.D., Stephen Clark, Ph.D.**

Institution: **University of Connecticut Health Center and University of Connecticut**

Collaborator: **Lenny Shultz, Ph.D., The Jackson Laboratories, Bar Harbor, ME**

This Project's purpose is to optimize hESCs for treatment of musculoskeletal diseases.

Project Summary

Although musculoskeletal tissues such as bone, cartilage, skin and muscle have amazing reparative properties, there are limits and when they are exceeded, diseases with significant morbidity result. The most recent example is the extreme damage to the skeleton suffered by our combat troops in Iraq and Afghanistan. For both cases, the hope is to introduce cells with reparative function into the area of damage that can form new skeletal tissues that in turn will integrate and function with viable pre-existing skeletal structures. Although adult stem cells have received most of the attention to provide this cell type, there is increasing reason to believe that reparative cells derived from human ES cells will have superior regenerative properties and will be the most clinically practical source of these cells. The investigators in this application that have chosen to submit a group grant, focused on how to produce reparative cells from human ES cells, have a long track record of NIH funded interactive research programs in the area of bone, cartilage and muscle research. They bring their expertise in the early embryonic stages of skeletal development to the problem of reproducing these developmental steps in a tissue culture dish in order to direct ES cells to become skeletal tissue. Because the early stages of development are shared by all the skeletal tissues, the grant is designed to optimize the reagents, personnel and ideas between all investigators (project 1). Essential to the early stages, are two projects that isolate pure populations of cells (project 2) based on colors that can be assigned to cells as they reach a certain level of development. From the isolated cells, the genes that are active at each level of development can be assessed by gene chip studies (project 3) and for testing their ability to acquire full development in models of tissue repair (projects 5-long bone; 6-facial bone; 7-cartilage; 8-muscle; 9-dermis). Another important component is the availability of scaffolds to mold the repair cells to form a particular structural feature (project 4). The members of this team will meet regularly to inter-relate interpretations of experiments performed within each project utilizing video conferencing that will link laboratories at the Farmington and Storrs campuses. We view this grant as an opportunity to begin human based ES cell research and acquire sufficient experience in preclinical small animal models to advantage us for acquiring funds to apply this knowledge to large animal studies and, eventually, human trials.

Year 1 Update

Bioinformatics has become an essential component of modern life science research because the amount of data being generated with the advanced life science research instrumentations far exceeds the limits of manual handling of produced data. Particularly, the use of microarray (a.k.a. DNA chip) technology generated multiple data points for each of the 40,000 human genes from one chip experiment. Scientists repeat experiments with varying experimental conditions. Furthermore, they repeat each experiment multiple times in order to gain statistical confidence in their findings. Analysis of microarray experiment data requires application of a series of computer programs. During the first year we have thoroughly examined the software packages that are currently available for microarray data analysis. We have identified strengths and weaknesses of those software tools. We have found that one class of software tools called clustering programs are not adequate for our stem cell data analysis, particularly because these existing programs perform poorly for the time-series data. We have developed a new method of clustering, called Pattern-Based Clustering, and this program works far better than the commonly used ones, such as the k-means clustering program.

We have also developed two innovative methods of carrying out meta-analysis of microarray data. One is called Pathway analysis which is an attempt to review how the analyzed gene expressions are compared to the known, reported gene regulation information. The other is called Gene Ontology analysis which is a method of finding genes belonging to certain functional categories. Our GO analysis suggests that this publicly available software module does indeed provide some interesting bioinformatics insight to the scientists. It helps scientists uncover genes whose functions are unknown for the tissue type they were investigating.

Our bioinformatics effort is also being used to optimize the working protocol for the multiple scientists who need to work as a group. After witnessing the poor performance of microarray for low expressing genes, the stem cell group came up with the idea of using Low Density Array (LDA) chips. LDA is a new emerging technique which allows the scientists to examine genes whose expression levels are too low to be detected using the microarray technology. LDA is far more sensitive than microarray, but its limitation is that it is far more expensive than microarray. LDA can be effective if its cost can be contained by sharing its use. We have developed a website that enables the scientists to enter their preferences of genes to be included in LDA. Once all the wish list genes are entered, the website collates their preferences and prioritizes which subset of genes should be included in the custom LDA in a manner that minimizes the cost and maximizes the gene list coverage.

Year 2 Update (3/09)

Administration

During the second year, this project worked closely with members of the group grant to promote an environment that fosters a better understanding of the existing procedures for making bone progenitor cells from human ES cells. We built tools (web based databases) to deposit and retrieve results from experiments that can be shared by members of the group grant. We meet regularly by video conference to discuss success and failures, and to make plan for subsequent experiments. The resources of this core has developed databases to share reagents and methods for directing hES and iPS cells into an early lineage cells that will be useful to all members. We share a common interest in finding the best way to produce this early

lineage cells from which each member will take them to their final cell type of interest.

Project 1: Skeletal Mesenchymal Progenitor Cells

The basic goal of our project is still to develop improved methods to get human embryonic stem cells (hESC) to change into cell types that can be used to repair defects in bone, cartilage, skin or muscle. The reason that we want to do this is because the currently available methods for producing these cell type are generally not very efficient. Also, we want to be able to monitor intermediate steps that the cells go through along the way, as they go from being totally undifferentiated cells that can become any cell type in the body, to becoming a very specific cell type. One of the first steps in becoming a bone, cartilage, skin or muscle cell is to become a mesoderm cell, and one of our main goals is to develop ways to easily detect when a hES cell has differentiated into a mesoderm cell. We had previously created a viral vector that contains the promoter for a gene called brachyury, which is expressed in mesoderm cells, linked to the gene for green fluorescent protein. We had also developed hES cells that contain the vector. In this year of funding we have been trying to show that, as we expect, the GFP is turned on in these cells if they become mesoderm. One of the main ways of demonstrating this is to show that the cells that are green also make brachyury protein. This has been more difficult than we expected, because the methods for detecting brachyury protein that we find in scientific papers have not worked well when we try them. This may be because, in some cases, the antibodies used by other laboratories may have been used up, so we have to use new antibodies, which may not work as well. We may have to try several new antibodies before we find one that works. However, we have shown that several protocols that were previously shown by other laboratories to cause hES cells to become mesoderm also induce expression of the brachyury-eGFP reporter construct. Thus, we expect that when we solve these technical problems we will be able to show that the brachyury-GFP does come on in mesodermal cells. The experience we have obtained should allow us to make good progress in developing new differentiation protocols once we have solved the technical problems. In other studies we were able to convert skin cells from patients with osteogenesis imperfecta, a genetic disease that causes weak bones that break very easily. We plan to use these cells in experiments designed to learn more about how the mutations cause the disease, and also to do the beginning testing of a possible strategy for treating one form of the disease.

Project 2: Phenotyping and Isolation of hES Derived Cells of the Musculoskeletal Lineage

One of the requirements to use defined stem cells in regenerative therapies is the development of methods to identify them and isolate them from other cells that would not contribute to the repair process or that, in some instances, could turn into potential cancer cells. Molecules expressed in the surface of cells serve as a signature to identify, characterize and isolate different cell types. Antibodies elicited against these molecules can bind to them with high specificity serving as powerful reagents for the study of their expression and distribution. These antibodies are produced by immunizing experimental animals with purified molecules or with whole cells expressing them in their surface. These antibodies can be tagged with fluorescent molecules making them ideal reagents to visualize, dissect and isolate live cell populations.

The main purpose of this project is to generate new antibodies that would allow the identification of stem cells capable to contribute to the regeneration of the cartilage,

bone, and muscle. Human embryonic stem cells are directed to differentiate into these tissues and as they progress into differentiation, cells are harvested and injected into mice that will generate specific antibodies against cell surface molecules specific for different stages of human development including the desired type of stem cells. At this moment we have already generated a battery of over 200 antibodies that bind hESCs and other cells representing very early stages of human development. Using differential screening techniques that include the cross examination with commercial antibodies of known specificities, we are now analyzing the binding patterns of these new antibodies. We will use these antibodies to study the diversity of early stem cells, and as a reference to antibodies recognizing cells more committed to form bone, cartilage and muscle. We are actively working on the generation of additional antibodies against later stages of differentiation. We expect these antibodies will be critical tools to isolate stem cells for clinical use and if so they will have the potential to be commercialized.

Project 3: Microarray and Genetics Networks

The microarray technology (a.k.a. DNA chip) produces expression patterns of thousands of genes at once. Use of the microarray technology is essential for the stem cell researchers as it provides systematic ways of understanding how stem cells differentiate and progress over different time points. During the first year, we have recognized the problems of using the conventional clustering computer programs in analyzing time-series stem cell gene expression patterns. We have thus engaged in developing a new method of clustering called Pattern-Based Clustering, and conjectured that our new method would work better than the commonly used existing clustering programs.

During the second year, we have made a significant progress toward making our newly invented Pattern-Based Clustering program robustly function in analyzing stem cell gene expression patterns. We have observed that this computer software program is very effective in helping biologists derive interpretations from the gene expression studies. We have discovered that this computer software is particularly strong in aiding the researchers to understand why a particular group of gene expression patterns are clustered together. We are currently investigating ways of associating the discovered pattern with the biological knowledge that is readily available from other public resources in order to further help the researchers derive biological meanings from the identified clusters of gene expression patterns.

Project 4: Biomimetic Surfaces for Efficient and Stable Stem Cell Differentiation

There is an increasing prevalence of degenerative diseases of the bones and joints among our aging population, along with battlefield trauma and other extensive injuries. These conditions cannot heal on their own and current treatments are often unsatisfactory. Stem cells could potentially be used to regenerate these diseased or damaged tissues, but directing the stem cells to convert (differentiate) to the needed tissue is still a technical challenge.

Since all cells respond to their immediate physical environment, the goal of Project 4 is to create biomaterial surfaces that will help direct stem cells to regenerate the needed bone, cartilage, tendon or muscle tissue. This work is done in close collaboration with the other projects that study the biology of stem cells.

During the first year, flat (2-dimensional) collagen and hydroxyapatite surfaces were formed. These mimic the environment in our target tissue, so we expect cells contacting these biomaterial surfaces to more rapidly transform into bone, muscle, etc. The experimental surfaces were thoroughly characterized to insure the proper chemistry, purity and structure. Experiments with special mouse cells that fluoresce when they start becoming bone showed successful attachment and growth and initial experiments on differentiation of the cells were promising.

During the second (most recent) year, the methods for quantifying the amount of differentiation were refined. These special methods allowed us to observe that differentiation to bone forming cells is accelerated by the hydroxyapatite surface and how the cells spread more uniformly on the surface and do not need to cluster, as usually observed, to form mineral (bone). This could lead to biomaterials that accelerate bone formation for an injury or disease. Additionally, a common problem with trying to convert stem cells to a desired final tissue type is that the stem cells do not adhere to most biomaterial surfaces, a necessary first step. We demonstrated that by attaching molecules that bind to molecules on the exterior of the human embryonic stem cell on the surface of our biomaterials, we could promote adhesion of stem cells to surfaces.

Project 5: Optimizing mesoderm derived bone cell differentiation from hES

During the second year, this project collaborated heavily with other members of the group grant to better understand existing procedures for making bone progenitor cells from human ES cells and to build reagents that will allow us to optimize existing or develop new protocols for this goal. In addition, we made significant progress in developing the mice that will be used to test the ability of the bone progenitor cells to make a bone in the intact animal. A new direction that builds on these two capabilities is the production of iPS cells from fibroblasts previously obtained from

subjects with Brittle Bone disease (osteogenesis imperfecta). The goal is to assess the quality of bone that is formed from these cells when directed into the bone lineage from the iPS cells and implanted into the mouse model. Success in that effort will bring us closer to the possibility of correcting a gene defect from an affected subject and then treating the individual with their own corrected cells. The tools and experience that we have been afforded by the State Stem Cell Grant has given us the ability to pursue this possibility that otherwise would not have been possible from traditional funding sources.

Project 6: Craniofacial Sciences Optimizing neural crest derived bone cell differentiation from hES cells

Our long-term goal is to develop effective cell-based therapies for missing/defective skeletal structures in the craniofacial complex. The cells that contribute to the facial skeleton including the bones and the cells that form the teeth, are derived from a special group of embryonic cells called cranial neural crest cells that are different from cells that give rise to the skeletal elements in the appendicular or axial skeleton. A significant body of evidence suggests that differences in the embryonic origin in bones of the craniofacial and appendicular skeleton have significant influences on various properties of skeletal tissues. Thus, effective cell-based therapies for skeletal tissues in the craniofacial complex are dependent on isolation and identification of stem/progenitor cells capable of regeneration of skeletal tissues with structural, morphological and mechanical properties similar to craniofacial skeleton *in situ*. To address these issues we are optimizing conditions for generation and identification of neural crest progenitors from human embryonic stem cells. We plan to examine the formation of bone and teeth from these cells.

Project 7: Cartilage Differentiation from hES Derived Progenitor Cells

Degenerative diseases of cartilage particularly osteoarthritis are among the most prevalent and debilitating chronic health problems in the United States and are one of the main causes of decreased quality of life in adults. Osteoarthritis affects most people over the age of 65, and indeed it is estimated that about 90% of the population over the age of 40 exhibits some form of cartilage degeneration in their joints resulting in pain and immobility. Treatment of degenerative cartilage diseases is difficult because of the limited capacity of the tissue for self-repair. Because of their unlimited capacity for self-renewal while maintaining the ability to differentiate into multiple cell types, human embryonic stem cells (hESCs) are a potentially powerful tool for the repair of cartilage defects. Induced pluripotent stem cells (iPSCs) also offer great potential for cartilage repair. iPSCs are human cells such as skin cells that have been reprogrammed to a stem cell state. Like hESCs, iPSCs can self-renew indefinitely and differentiate into multiple cell types. Significantly, iPSCs have the long term potential to be used for patient-specific cell therapy. Fulfilling the potential of hESCs and iPSCs for treatment of degenerative cartilage diseases such as osteoarthritis will require the development of methods for directing their differentiation into cartilage cells. During the current year of our project we have optimized culture conditions that promote the progressive and uniform differentiation of hESCs and iPSCs into cartilage cells. The progressive uniform differentiation we have achieved has allowed us to identify by gene expression profiling hESC-derived cells in different stages of their differentiation into cartilage cells. We are thus poised to test the abilities of hESC and iPSC-derived cartilage cells and their precursors to repair damaged or diseased cartilage.

Project 8: "A Mouse Model to Study the Myogenic Potential of HESCs"

Skeletal muscle exhibits an enormous regenerative capacity. Muscle injury stimulates mitotically quiescent satellite cells to re-enter the cell cycle and execute the myogenic program, leading to regeneration of the damaged muscle. Regeneration is also elicited in Duchenne muscular dystrophy, where a mutation in the *dystrophin* gene results in several muscle necrosis. Although a robust regenerative response initially maintains adequate muscle function in young patients, satellite cell replicative capacity is drastically diminished with age, leading to a rapid decrease in regenerative ability and irreversible progression of the disease.

Cell-based therapies for muscle degenerative diseases such as Duchenne muscular dystrophy have shown some promise in animal models. Thus, committed myogenic progenitor cells (myoblasts) injected into dystrophic leg muscles of the mouse will fuse with damaged host muscle and with each other, resulting in the production of dystrophin+ muscle fibers and improve muscle function. Injected myoblasts, however, are unable to efficiently produce satellite cells – an essential requirement for long-term therapeutic benefit – possibly because satellite cells are developmentally more primitive. Poor survival of injected myoblasts also will limit their treatment effectiveness for the large muscle masses encountered in a clinical setting. A major objective of the present proposal is to evaluate and optimize the ability of hESC-derived mesodermal stem/progenitor cells to repair skeletal muscle and contribute to the satellite cell niche. Optimization of directed differentiation protocols and isolation of cells with myogenic capacity will be aided by the development of reporter constructs to monitor lineage progression. hESC-derived myogenic cells will be tested to repair muscle in new and existing models for muscular dystrophy.

Project 9: Use of hES cell derived fibroblasts for therapy of cutaneous wounds

The skin is the largest organ of the body. It serves as the first line of defense for unwanted pathogens and participates in thermoregulation. It is frequently subjected to environmental insults to which in most settings it can readily repair. However, in a variety of disease states, the skin cannot complete the repair process leading to the presentation of chronic wounds. This is particularly problematic in the elderly and diabetic patients. Wound healing is a complex physiologic response to the skin injury. Delayed wound healing has a huge economical impact on our population. It is estimated that delayed wound healing has a cost of more than \$20 billion to the United States health care system. Although recent breakthroughs with growth factor therapy have made significant progress in wound healing therapy, more than 50% of chronic wounds are resistant to any mode of therapy. Among the many factors contributing to the delayed wound healing, impairment in the production of cytokines and growth factors by local inflammatory cells and dermal fibroblasts with reduced angiogenesis have been implicated. The objective of this research program is to identify cellular reagents that can be used to improve the resolution of a cutaneous wound. The use of cell based therapy is a major objective in the emerging field of regenerative medicine. In this research program murine models will be utilized to determine if the application of specific cells to a cutaneous wound will improve the wound healing process. The long range objective is to use human embryonic stem (hES) cells or reprogrammed patient specific somatic cells in the treatment of chronic skin lesions.

Optimizing mesoderm derived bone cell differentiation from hES cells

Over 10 million Americans are currently carrying at least one major implanted medical device in their body. Among these implants, those for repair of bone fracture

and other damage constitute a large proportion and play an essential role in more than 1.3 million bone-repair procedures per year in the USA. Bone tissue engineering is a new emerging field, which has major potential to improve human health by repairing and maintaining existing bone or generating new bone. Three-dimensional biodegradable tissue-engineering scaffolds have become a promising alternative approach for bone repair. The scaffold provides a framework for cell attachment, proliferation and differentiation; formation of extracellular matrix; and templating new bones into various shapes. During the process of new bone tissue formation, the scaffolds gradually degrade and are replaced by regenerated host tissue. Thus, scaffolds have the advantages of autografts – the “gold standard” for grafting materials, but are not restricted by supply. With this approach, however, the success of bone repair is heavily dependent on the design of the scaffold. Despite many early successes, there are few bone tissue-engineering scaffolds available on the market for clinical use, and significant challenges still remain in the success of long-term bone repair. In this study, a series of tissue engineering scaffolds have been prepared using different fabrication techniques. By adjusting the processing parameters, we have been successful in controlling the pore size, porosity, degradation rate and mechanical properties of these scaffolds. The scaffolds were loaded with progenitor cells, and implanted into mouse calvarial sites together with a positive control. Based on the in vivo outcomes, we have carefully modified scaffold design and fabrication process. The progenitor cell loaded scaffolds have demonstrated good bone forming ability at the calvarial site within four weeks of implantation. Therefore, our new one-step coprecipitation scaffold can be used a potential skeletal tissue repair material.

06SCD09**CT Stem Cell Research Proposal**

Title of Project: **Human Embryonic Stem Cell Core Facility at Yale Stem Cell Center**

Amount requested: \$5,000,000; Amount funded: \$2,500,000

Principal Investigator: **Haifan Lin**

Institution: **Yale University**

Collaborator: Diane Krause

This Project's purpose is to establish core laboratory facilities for research with human embryonic stem cells.

Project Summary

We propose to establish a human embryonic stem cell (hESC) core facility for stem cell research at Yale School of Medicine to serve stem cells researchers through the state of Connecticut. The Stem Cell Core Facility will be housed in the Yale Stem Cell Center (YSCC) located in the newly built Amistad building. The proposed core facility will have three components: a hESC culture core laboratory, a cell sorting core, and a confocal microscope core. The basic service function of the Core Facility will include:

- Assembling a repository of hESC lines.
- Expanding and distributing hESC to scientists in Connecticut.
- Training researchers how to grow hESC lines and how to use them to produce specific types of cells represented in various tissues in our bodies.
- Monitoring the genetic stability of hESC lines and their abilities to proliferate and produce various cell types.
- Providing a central administrative mechanism for assuring compliance with federal, State of Connecticut, and University guidelines for research involving hESCs.

The more advanced service and research functions will include:

- Providing shared resources needed for cell and molecular studies of hESCs, including equipment for cell culture, microinjection, cell imaging, and cell sorting.
- Facilitating relevant studies of other types of stem cells supported by the State of Connecticut funding.
- Developing new hESC technology, including investigation of hESC culture conditions without helper cells (i.e., feeder cells) and without sera.
- Providing tools and advice for modifying genes in hESC, and creating new hESC lines and hES cell lines with inducible gene expression for disease studies.
- Coordinating educational activities, including bimonthly bioethics seminar series and a monthly journal/data/technology club.

Year 1 Update

The YSCC has made rapid progress in achieving its proposed goals. Within the first six months, we successfully recruited excellent staff for all of the funded positions. Furthermore, we have developed hESC research regulatory compliance policies and procedures, purchased equipment and supplies, and moved into the new building on Amistad Street in New Haven. Moreover, beyond the scope of the \$2.5 million funding, we established a FACS Core and a Genomics Core by securing support from the Yale School of Medicine.

Year 2 Update

The human embryonic stem cell core facility at the YSCC is playing a key role in building an infrastructure for investigators to conduct stem cell research on hESC and iPS cells. The Core is also finding new and more efficient ways to culture hESC and differentiate them into other cell types.

06SCD02**CT Stem Cell Research Proposal**

Title of Project: **Human ES Cell Core at University of Connecticut and Wesleyan University**

Amount requested: \$5,000,000; Amount Funded: \$2,500,000

Principal Investigator: **Ren-He Xu**

Institution: **University of Connecticut Health Center**

Collaborators: Drs. James Thomson, Xiangzhong Yang, Nirupama Shevede, Igor Slukvin, Timothy Kamp, Jon Odorico, Brenton Graveley

This Project's purpose is to establish a human embryonic stem cell (hESC) core facility.

Project Summary

Our overall objective is to meet the research needs and demand for hESCs and training in hESCs in Connecticut and contribute to furthering stem cell therapies for human disease. This proposal is to expand a human embryonic stem cell core facility that is being established by investigators at the University of Connecticut and Wesleyan University. We will stock, expand, and provide both federally approved and non-approved hESC lines to all eligible scientists in Connecticut. We will also provide training on the stem cell culture and differentiation, quality control, and technical support to all users. Subject to IRB and ESCRO approval, we will derive new hESC lines from extra embryos donated to IVF clinics. To promote stem cell research, we will organize on-campus workshops and reach out to scientists, students and members of the community.

Year 1 Update

Our overall objective is to meet the ever-increasing demand by Connecticut scientists for hESCs and stem cell-related training and services, and help advance stem cell-based therapies to treating human diseases.

Based on this objective, we have stocked nine hESC lines, and provided the stem cells to more than 30 laboratories at the University of Connecticut, Wesleyan University and Yale University. We have held 11 training sessions and trained more than 70 researchers statewide on the stem cell culture. We have also provided quality control and technical support to stem cell researchers. We have obtained

Approvals by the Internal Review Board and Embryonic Stem Cell Research Oversight and received donated embryos to derive new hESC lines from extra embryos donated to in vitro fertilization clinics. Our subcores for cell sorting and gene delivery have also started to provide services to stem cell researchers. To promote stem cell education, we have continued cross-campus stem cell seminars teleconferenced to UConn Storrs, Wesleyan and Yale, and organized three outreach seminars other colleges in the state.

Year 2 Update

The overall objective of the University of Connecticut and Wesleyan University Stem Cell Core supported by the Core Facility grant is to meet the ever-increasing demand by Connecticut scientists for hESCs, now also induced pluripotent stem (iPS) cells, and stem cell-related training and services, and help advance stem cell-based therapies to treat human diseases. Based on our success in Year 1, we continue to achieve new milestones in Year 2, which are summarized as follows:

We derived two new human embryonic stem cell lines named CT1 and CT2, which were distributed to eligible researchers along with the 9 existing stem cell lines stocked in the Core. Another two lines, CT3 and CT4 were derived recently and are under validation. We also derived more than 10 iPS cell lines from human fibroblasts and established the iPS cell derivation and characterization services to help colleagues with this cutting-edge technology.

We continued our services to scientists who received stem cell grants in the last two rounds of grant applications, as well as scientists who were poised to extend their research to stem cells. We held 5 training sessions with 30 trainees (in addition to over 70 trained in Year 1) on the hESC culture, and held a Neural Differentiation Workshop with 14 trainees. Many of the trainees obtained new grants in the 3rd round of applications.

We hosted 10 cross-campus stem cell seminars teleconferenced to UConn Storrs and Wesleyan, one summer student workshop, and four outreach seminars at colleges and high schools. In addition, we planned our first Blood and Cardiovascular Differentiation Workshop for September 17, 2009.

Dr. Laura Grabel, as the co-PI of this grant, continued to lead the stem cell educational programs. She organized the four outreach seminars and summer student workshop. She also instructed in the Neural Differentiation Workshop. Dr. Leonardo Aguila, the subcore director for FACS services, continued to provide excellent services to identify stem cell markers in human ES and iPS cells and sort the cells and their derivatives based on markers of interest. Dr. Alexander Lichtler, the subcore director for transgenesis, provided lenti- and retroviral vectors to colleagues for somatic cell reprogramming to derive iPS cells, while continuing to help colleagues deliver exogenous genes or silence endogenous genes in human ES cells through viral transduction.

Our accomplishments in the past two years were highly appreciated by colleagues and praised by the public. The press highlighted our success in hESC derivation. Our extraordinary performance and impressive new proposal convinced both the state stem cell reviewer committee and advisor committee, who awarded us a renewal grant. This new grant will enable us to continue and enhance the Core missions from 2010-2013 and serve colleagues who received state stem cell grants in the last three rounds of applications from 2007-2009.

06SCE01**CT Stem Cell Research Proposal**

Title of Project: **An Integrated Approach to Neural Differentiation of Human Embryonic Stem Cells**

Amount requested: \$4,335,769; Amount funded: \$3,815,478

Principal Investigator: **Michael P. Snyder**

Institution: **Yale University**

This Project's purpose is to study neuronal differentiation from human embryonic stem cells with the ultimate goal of improved therapeutic strategies for treating neurodegenerative diseases.

Project Summary

With the derivation of human embryonic stem (hES) cells, came the desire to produce clinically relevant cell types *in vitro* for repairing tissue damage caused by injury, disease and aging. This proposal uses a multifaceted approach integrating genomic, proteomic, and genetic experiments to elucidate the molecular events that control neural cell differentiation using hES cells as a model system. Understanding neural differentiation by hES cells has enormous potential in treating neural degenerative diseases, as the availability of unlimited numbers of various types of neurons is a first step toward exploring the potential of hES cells in preclinical models of such diseases. Our goals are:

1. Globally determine the genes and proteins that are expressed and regulated during neural differentiation from hES cells.
2. Identify mRNAs negatively regulated by the Pumilio family translational repressors whose homologs in genetic models are master regulators of the stem cell fate.
3. Establish a high-throughput platform to identify, at a genome-wide scale, genes important for neural differentiation from hES cells by RNAi and over-expression analyses.
4. Characterize the functionality of neural progenitors derived from hES cells for their stem/progenitor cell properties, especially with regard to their ability to balance self-renewal and differentiation and their homeostatic properties during development.
5. Establish a genomic and proteomics core to be used by the entire state of Connecticut.

We expect this project to provide significant insight into the process of neural cell differentiation and identify key components involved in this process. Such information has enormous value in identifying therapeutic targets.

Year 1 Update

We have hired personnel for performing the project and made significant advances in our research. This includes: 1) Profiling of RNAs expressed in hESCs; 2) Profiling of proteins expressed in hESCs; and, 3) Development of Methods for Mapping Transcription Factor Binding Sites.

We have begun to profile protein and RNA that is expressed in hESCs. This will profile a list of components that are expressed in these cells and potentially describe new components involved in hESC self renewal and differentiation. Our studies will also help us understand how hESCs are regulated during cell differentiation into

neural cells. This information is ultimately expected to be valuable for controlling hESC differentiation for therapeutic purposes.

Year 2 Update

We have established a state of the art core facility that will allow us to comprehensively map the important parts of our DNA that are responsible for stem cells to be stem cells and to differentiate into other cell types such as neural cells. This facility will allow us to map the functional elements in the DNA (i.e. the genes) as well as the regulatory elements that control the expression of these genes. This information will be extremely valuable for controlling and manipulating stem cell differentiation into different cell types.

The core facility that we developed will not only help researchers at Yale map these important elements in our DNA but are potentially accessible to others in the State of Connecticut. As such, we expect it to be a very valuable resource for many stem cell researchers.

Project 1: Genomic and Proteomics Analysis of Neuronal Cell Differentiation

We have profiled protein and RNAs that is expressed in hESCs and during the development of our nerve cells. These studies will also help us understand how hESCs are regulated during cell differentiation into neural cells. This information is ultimately expected to be valuable for controlling hESC differentiation for therapeutic purposes.

Project 2: Translational Regulation of Neural Differentiation in hESC

The difference of human embryonic stem cells and their daughter neural cells is controlled by a set of genes that are either active in the stem cells or in the neural cells. However, often these genes are equally active in producing their corresponding messenger RNAs. What causes the ultimate difference in the gene activity is whether these messenger RNAs guide the production of their corresponding proteins. We have shown in model organisms that this level of regulation is essential in controlling the ability of stem cells to maintain their own identity and to produce specialized cells, such as neural cells. Yet to our knowledge, no one has been studying this important mechanism. Our progress has allowed us for the first time to systematically embark on this study. What we have achieved so far has allowed us to demonstrate the function of a few 'master' proteins, such as Pum1, Pum2, and mammalian PIWI proteins, in controlling this process. These proteins then will be effective therapeutic molecules or targets for us to harness the directed differentiation of hESCs to desired type of neural cells for eventual medical applications.

Project 3: Screening for Global Identification of Genes

During the last year we have been exploring two aspects of stem cell work. In one project we are using a system for depressing the expression of individual genes in stem cells, and studying which genes are necessary for the viability of these cells or for their ability to differentiate into neural cells. We are attempting to set up a system in which we can do this in one large experiment for a large fraction of all the genes in the genome, using viral infection of cells. We have established which viruses will work and are well on the way towards having a rapid and efficient method of measuring which genes are depressed in surviving or differentiating cells.

In another aspect of the work, we are starting with blood precursor cells and trying to increase their efficiency for long term repopulation of bone marrow after

chemotherapy or other ablative treatments. There are interesting results in model systems but we hope in the next year, to begin to extend this to normal cells that could be used to transfuse patients.

Project 4: Mechanisms for Balancing Self-Renewal and Differentiation

Stem cells are defined by the ability to produce more stem cells (self-renew) and the potential to generate differential offspring capable of carrying out various tissue functions. During the extended period of organogenesis and tissue maintenance, there is an essential need for a proper balance between self-renewal and differentiation, since a failure in either task can lead to malformation and malfunction of tissues and organs. Asymmetric cell division is a process by which a cell divides to produce two different daughter cells. Conceptually, stem cells can simultaneously self-renew and differentiate by dividing asymmetrically to produce one daughter cell that remains as a stem cell and another that differentiates. Stem cells, however, may also need to divide symmetrically to produce two stem cells, which can quickly expand their population when responding to tissue injuries, or to generate two differentiated cells, when large numbers of stem cells are no longer necessary towards the end of organogenesis and tissue repair. In other words, understanding the division patterns of stem cells – particularly how they are regulated under normal and pathological conditions – may facilitate a key goal of stem cell research, which is to repair or replace damaged tissues by introducing exogenous stem cells or expanding endogenous populations. Our research attempts to probe the mechanisms that regulate the behavior of stem cells by changing their patterns of division, as a means to find ways to expand stem cells when tissue repair becomes necessary or to eliminate stem cells in cancers, which are believed to be diseases of stem cells.

Project 6: Neuronal Cell Differentiation Core

The major goal of this small core facility is to differentiate hESCs into various nerve cells so that the four labs working on this project can use them to perform various proposed experiments. The purpose of this small facility is to provide technical assistance so that scientists can work more efficiently to carry out their research in understanding the properties of hESCs and their descendants.