

Title of Project: Hybrid Peptide/RNA Molecules for Safe and Efficient Gene Silencing in Human Embryonic Stem Cells

Amount requested: \$200,000

Principal Investigator: Yong Wang (Ph.D.)

Institution: University of Connecticut

Collaborator: Ren-He Xu (Ph.D. and M.D.), University of Connecticut Health Center

Description: This Project's purpose is to develop hybrid peptide/RNA molecules for enhancing siRNA delivery into human embryonic stem cells to achieve efficient and safe gene silencing effect.

Project Summary: Safe and efficient siRNA delivery into human embryonic stem cells (hESCs) is critical for hESC research and applications. However, current methods and reagents used for siRNA delivery into hESCs suffer from many limitations. Therefore, there is a clear need to develop novel methods and reagents to improve siRNA delivery into hESCs. The objective of the proposed research is aimed at developing novel hybrid molecules for siRNA delivery without the need of using viruses, transfection reagents, or physical tools. Our hypothesis is that the peptides will perform like keys to open the closed doors in the cells and therefore achieve the efficient delivery. The hypothesis will be tested through three specific aims:

- 1. Synthesis and characterization of hybrid peptide/RNA molecules;*
- 2. Characterization of binding, internalization and distribution of hybrid molecules;*
- 3. Evaluation of gene silencing effect.*

This will be the first time to develop bifunctional hybrid molecule-based delivery system for siRNA delivery. The hybrid peptide/RNA molecules are expected to provide not only a platform to enhance gene silencing in hESCs, but also a convenient tool of doing temporal control of gene silencing in hESCs. Therefore, the success will benefit various hESC studies and applications. The success will also enhance the applicant's collaboration with hESC scientists and thereby to further strengthen hESC research and applications in the state of Connecticut. The proposed research is expected to be finished in two years.

Title of Project: Derivation and Functional Characterization of Heart Cells from Human Embryonic Stem Cells

Amount requested: \$200,000

Principal Investigator: Yibing Qyang

Institution: Yale University

Collaborators: Drs. Christopher Breuer, Teruo Okano, Frederick Sigworth, Barbara Ehrlich and George Richerson.

Description: The goal of this project is to derive and characterize cardiovascular progenitor cells and differentiated cardiomyocytes; and use these cells to study human heart development and in future clinical applications.

Project Summary: Heart disease is the leading cause of mortality in the developed world. Human embryonic stem (ES) cells may provide an ideal source for production of heart cells for cell-based therapy. A mixture of cells enriched for heart cells can be isolated from human ES cell culture, but due to their heterogeneity they fail to engraft and may cause abnormal electrical activity after implantation. Thus, it is critically important to isolate pure cell populations enriched for cardiac cells that will engraft and promote cardiac repair. I propose studies to compare and contrast two highly purified human ES cell-derived cell populations: ventricular cardiomyocytes (VCM) and cardiovascular progenitor cells (CPC). Another challenge for cell-based therapy is to improve upon routes of delivery because cell suspension injection often results in poor engraftment. Tissue engineering has the capacity to greatly alleviate this problem. Using a recently developed cell sheet technology, I propose to establish and characterize engineered heart tissues in cell culture using these human ES cell-derived CPC and VCM. This research will lead to the production of pure, well-characterized CPC and VCM, and novel engineered heart tissues from human ES cell cultures, which ultimately could be used for cell-based therapy for heart failure. In addition, pure CPC and VCM derived from human ES cell cultures will provide an unprecedented cellular model to dissect human heart development at the level of CPC function and VCM maturation. Furthermore, CPC and VCM will provide ideal cells for cardiotoxicity screening which is important for many therapeutic applications in cardiac medicine. There are many stem cell scientists throughout Connecticut who would like to derive heart cells from human ES cells for cell-based therapy for heart failure as well as for basic research into human heart development; and I will be happy to share with them my discoveries and expertise in order to facilitate their research.

Title of Project: The Influence of Aberrant Notch Signaling on Rb Mediated Cell Cycle Regulation in Megakaryopoiesis & Acute Megakaryoblastic Leukemia

Principal Investigator: Stephanie Massaro, MD MPH
Institution: Yale University School of Medicine

Description: This Project's purpose is to identify the mechanisms underlying the development of Acute Megakaryoblastic Leukemia, a disease that disproportionately affects infants and children.

Project Summary: Acute Megakaryoblastic Leukemia (AMKL) is a rare form of leukemia that affects megakaryocytes, which are platelet-making blood cells. The disease most commonly strikes very young children. Approximately 30% of pediatric patients diagnosed with AMKL are infants who have a specific genetic abnormality that involves two genes, RBM15 and MKL1. These genes may play important roles in normal blood cell development. However, when they are incorrectly linked together, they may contribute to leukemia development by altering Notch signaling, a normal cell signaling pathway responsible for cellular growth and maturation. This aberrant Notch signal may cause a failure of the tumor suppressor protein Rb and result in uncontrolled growth of immature megakaryocytes. This abnormality is associated with an extremely poor outcome, with an average survival time of only eight months from diagnosis despite aggressive medical therapy.

Given that AMKL primarily afflicts infants, we believe this leukemia arises during fetal blood development. Thus, it is essential to study the interactions of RBM15 and MKL1 with regulators of cell growth, such as Notch and Rb, using human embryonic stem cells (hESCs) in order to understand their impact on fetal blood maturation and leukemia development.

The aims of this proposal are to 1) establish the pattern of embryonic megakaryocyte development and test the hypothesis that RBM15 and MKL1 are differentially expressed during hESC-derived megakaryopoiesis, 2) define the role of Notch in hESC-derived megakaryopoiesis and to determine whether RBM15 and/or MKL1 impact Notch signaling, and 3) demonstrate the downstream effects of Notch signaling on Rb mediated cell cycle regulation during hESC-derived megakaryopoiesis.

Methods have been established to derive megakaryocytes *in vitro* from hESCs. Briefly, we will evaluate the cellular and genetic characteristics of these megakaryocytes as they develop in culture. We will manipulate this system in order to stimulate Notch signaling and to examine specific downstream targets, including regulators of the cell cycle in the resultant megakaryocyte population.

Title of Project: Investigating the role of nuclear RNA quality surveillance in embryonic stem cells

Amount requested: \$200,000

Principal Investigator: Sandra Wolin, M.D., Ph.D., Professor of Cell Biology and of Molecular Biophysics and Biochemistry
Institution: Yale University School of Medicine

Description: This Project's purpose is to investigate the role of noncoding RNA quality control pathways in ES cell function

Project Summary: The objective of this exploratory project is to determine the contributions that nuclear RNA surveillance pathways make to ES cell pluripotency and self-renewal. The genomes of mouse and human embryonic stem (ES) cells are hyperactive, with many sequences expressed at levels up to 10-fold higher than differentiated cells. The expressed RNAs include many RNAs that may be deleterious, such as transcripts of intergenic regions, variant noncoding RNAs and pseudogenes. This low-level promiscuous transcription, by keeping many tissue-specific genes in a transcriptionally competent state, is thought to be important for maintaining ES cell pluripotency.

As ES cells must balance the need to keep many genes transcriptionally competent with the need to protect themselves from deleterious transcripts, nuclear RNA surveillance may play a key role by targeting intergenic transcripts and variant defective noncoding RNAs for decay. Although the role of these pathways in ES cells is unknown, a nuclear poly(A) polymerase, TRAMP, functions with an exoribonuclease complex to degrade intergenic transcripts and unstable mutant noncoding RNAs in yeast. In vertebrate nuclei, the ring-shaped Ro protein binds the ends of misfolded noncoding RNAs in its central cavity and targets them for decay. Consistent with a role for Ro in sequestering defective RNAs in ES cells, Ro binds variant misfolded U2 snRNAs in mouse ES cells, but is not detected bound to these RNAs in other cells. Our first aim is to determine the spectrum of RNAs targeted by the TRAMP pathway in mouse and human ES cells and by the Ro pathway in human ES cells. Using RNA interference to deplete components, followed by examination of specific classes of noncoding RNAs, we will determine which RNAs are targets of these pathways. Our second aim is to determine if RNAi-mediated knockdown of Ro and/or TRAMP impairs ES cell function. Following depletion of either Ro or TRAMP components, we will determine if these pathways are required for ES cell self-renewal or pluripotency. The proposed studies should elucidate an important aspect of ES cell biology and advance our understanding of the role of nuclear RNA surveillance pathways in mammalian cells.

Title of Project: Can Natural Neuromodulators Improve the Generation of Nerve Cells From Human Embryonic Stem Cells?

Amount requested: \$200,000

Principal Investigator: Srdjan D. Antic, M.D., Assistant Professor of Neuroscience

Institution: University of Connecticut Health Center

Description: This Project's purpose is to improve the yield (number), purity, maturity and survival rate of stem cell-derived neurons for replacement therapy in Parkinson's disease.

Project Summary: The cause of Parkinson's disease is degeneration and death of small group of neurons (dopaminergic neurons) that release a substance called dopamine. Human embryonic stem cells (hESCs) may potentially serve as a renewable source of neurons. Specifically, neurons derived from hESCs may replace the degenerated dopaminergic neurons in patients with Parkinson's disease. Several research groups were able grow dopamine-releasing neurons in the dish and then transplant these neurons into an animal model of Parkinson's disease. However, several major obstacles still preclude clinical application. First, the efficacy of nerve cell production is low hence the number of transplantable DA neurons is inadequate. Second, DA neurons are heavily contaminated with pluripotent cells, which often cause undifferentiated tumors (teratomas) in the host animals. Finally, transplanted DA neurons die in massive numbers. Poor viability of differentiated DA neurons after transplantation is a major problem.

Here, we propose a novel method for improving the procedures for nerve cell generation from human embryonic stem cell lines. Our ideas are based on well-known positive effects that naturally occurring neurotransmitters (neuromodulators) exert on neuron proliferation, migration, differentiation and maturation in the embryonic brain, at very early stages of brain development. We anticipate that natural neuromodulators (dopamine, norepinephrine and serotonin) when applied in culture dish, in laboratory conditions, would increase the survival and maturation of hESC-derived neurons. One of our primary goals is to determine which federally in-eligible hESC line available at the UConn Health Center Stem Core has the greatest potential to generate DA-releasing neurons. This result would have important practical implications for further proliferation of human stem cell research among Connecticut neuroscientists.

Two factors increase the significance of our proposal. First, most of the previous studies relied on anatomical and histochemical measurements. However, the project can be considered a success only if hESC-derived neurons are fully functional. That is, if they are making synaptic contacts, generating proper nerve impulses and releasing substantial amounts of dopamine. For this reason, in the present study in addition to anatomy and histochemistry, we propose a detailed physiological characterization of neuronal functional properties such as electrical activity, fluctuations of intracellular calcium, synaptic transmission and release of dopamine. In the proposed project we have designed laboratory tests that have never been used for evaluation of hESC-derived neurons. Physiological measurements, including fast cyclic voltammetry, patch-clamp recordings and optical imaging of intracellular calcium signaling are the strength of our laboratory. Second, the prospect for clinical application of neuromodulators is not unrealistic. If our experiments determine that an increased dopaminergic, noradrenergic or serotonergic tone is indeed beneficial for hESC-derived neurons, such elevations can be achieved in human hosts (recipients of hESC-derived DA neurons) with drugs which are already approved for clinical use (dopamine, serotonin and norepinephrine reuptake inhibitors). In fact, these are the most commonly prescribed drugs in the USA.

Title of Project: A human cell culture model of Angelman syndrome for drug screening

Amount requested: \$200,000

Principal Investigator: Stormy Chamberlain, Ph.D.
Institution: University of Connecticut Health Center

Description: This Project's purpose is to develop a human neuronal cell culture model to study and identify therapies for Angelman syndrome.

Project Summary: Angelman syndrome (AS) is a human neurodevelopmental disorder characterized by mental retardation, lack of speech, ataxia, and seizures. We propose to develop a human cell culture model of this disorder by making AS pluripotent stem cells and differentiating them into neurons. First, two different pluripotent stem cell lines will be generated to model AS: induced pluripotent stem (iPS) cells from AS patient fibroblasts and hESCs in which the AS gene, *UBE3A*, has been knocked down using a small hairpin RNA (shRNA). Secondly, these pluripotent cells will be differentiated into neurons to determine how *UBE3A* expression is regulated during human neuronal development. From studies in mouse embryos it is known that *UBE3A* is expressed only from the maternally inherited chromosome in neurons and from both chromosomes in all other cell types. This has never before been examined in human cells. Thirdly, we have observed that many cell types derived from the mouse model of AS are resistant to cell death caused by oxidative stress. This phenotype may also be responsible for the symptoms of AS. The human AS neurons described above will be subjected to oxidative stress to determine whether they are resistant. If AS neurons are resistant to oxidative stress, we will use this phenotype to screen for drugs that may reverse resistance to oxidative stress.

Title of Project: Novel response to RNA editing in human embryonic stem cells

Amount requested: \$200,000

Principal Investigator: Ling-Ling Chen

Institution: University of Connecticut Health Center

Description: This Project's purpose is to understand the consequences of RNA editing in human embryonic stem cells and its role in the maintenance of self-renewal and pluripotency.

Project Summary: The focus of this project is to understand how RNA editing influences human embryonic stem cell (hESC) growth and differentiation. In the nuclei of differentiated cells, double stranded RNAs (dsRNAs) are recognized and edited by members of ADAR (adenosine deaminases that act on RNA) family of enzymes, which deaminate adenosines to inosines, thus changing mRNA structure and coding potential. Some dsRNA structures within 3'-untranslated regions (3'-UTRs) of mRNAs are edited at many positions. We recently published that such promiscuous editing inhibits gene expression by causing the edited mRNAs to be retained in the nucleus. We have discovered that hESCs have robust ADAR activity, but do not retain edited RNAs in the nucleus. One edited mRNA that is not retained is for the protein LIN28, which is specifically expressed in hESCs and is important for pluripotency and self-renewal. The altered nuclear retention pathway in hESCs appears to be due to a defect in the assembly of nuclear structures called paraspeckles. Upon differentiation, paraspeckles return and the editing/retention pathway is re-established. Importantly, we have found that a large nuclear noncoding RNA called hNEAT1 plays a critical role in paraspeckle assembly, and is not expressed in hESCs, but is induced upon differentiation. In this proposal, we will further characterize the editing-associated nuclear retention pathway in hESCs. We will examine this pathway in a number of hESC lines, including induced pluripotent stem cells. Further, we will study the role of hNEAT1 RNA in stem cell growth and differentiation. Finally, we will identify genes whose expression is affected by the editing-associated nuclear retention pathway in hESCs and during differentiation. Taken together, these studies will not only address the questions of how and why hESCs respond differently to editing, but will also provide us an opportunity to demonstrate for the very first time the biological function of a large noncoding nuclear RNA.

Title of Project: Transcriptional control of keratinocyte differentiation in human ES cells

Amount requested: \$200,000

Principal Investigator: Valerie Horsley
Institution: Yale University

Description: This Project's purpose is to determine the mechanisms that control keratinocyte differentiation from human embryonic stem cells

Project Summary: As the body's largest organ and its first defense against external pathogens, the skin is crucial for life. The skin creates an external barrier to our external environment but can be compromised during injury, burns, cancers and genetic skin diseases. As a treatment for these skin disorders, human embryonic stem (hES) cell replacement therapies have great potential. Skin grafts can be generated ex vivo from patient skin keratinocytes but generating enough cells for tissue engraftment is time consuming and the grafts have limited potential since they do not form hair follicles or sweat glands. Thus, cell-based therapies are not currently used extensively in the clinic to treat burns or other genetic diseases that compromise skin. hES based therapies might be able to overcome these current limitations because large numbers of cells are able to be generated and they may have a broader differentiation spectrum. However, current methods to generate keratinocytes from hES cells are inefficient. Uncovering the specific mechanisms that drive keratinocyte differentiation in hES cells will allow us to exploit these mechanisms to generate large numbers of keratinocytes from hES cells for therapeutic purposes to generate tissue replacements for skin disorders like burns, extensive wounds and genetic abnormalities.

The transcriptional control of lineage specification is well established for many cell types. We hypothesize that specific transcription factors are required for the direction of keratinocyte fate in human ES cells. Interestingly, studies of early stage epidermis in the mouse identified that transcription factors are highly enriched during epidermis formation. The objectives of this proposal are to 1) define the transcriptional network that defines keratinocyte specification of hES cells and 2) analyze the function of specific transcription factors during keratinocyte formation in hES cells. We will generate reporter lines for the two stages of keratinocyte differentiation from ES cells and use these lines for analysis of candidate and whole genome expression of transcription factors during keratinocyte differentiation from hES cells. For our second aim, we will functionally analyze the role of four candidate transcription factors that are enriched in developing mouse epidermis in keratinocyte formation in hES cells using gain- and loss-of function studies. These experiments will dissect the mechanisms of human skin epithelial cell differentiation and potentially allow us to more efficiently generate differentiated keratinocyte cell populations for use in human therapies.

Title of Project: Evaluation of homologous recombination in hESC and stimulation using viral proteins

Amount requested: \$ 200,000

Principal Investigator: April Schumacher
Institution: University of Connecticut Health Center

Description: This project's purpose is to improve gene targeting in human embryonic stem cells.

Project Summary: In order to capitalize on the potential of embryonic stem cells (ESCs) to treat human injury and disease, researchers will have to target specific genes to generate the modified cells necessary for therapy. Unfortunately, the frequency of gene targeting that has been achieved in human ESCs is far below the level necessary for efficient gene replacement. Most of the information about the cellular pathway that is used to exchange specific segments of DNA for gene targeting comes from studies using mouse ESCs or other human cell lines; however, our knowledge of these pathways in human embryonic stem cells is much less advanced. For instance, we do not know how efficiently genetic exchange will occur. In addition, it may be necessary to develop methods to increase the frequencies of genetic exchange in order to enhance the therapeutic potential of this technology. The goals of this proposal are twofold: First, we plan to evaluate the frequency of the genetic exchange events in human ESCs compared to other human cell lines. Second, we will test the hypothesis that a newly identified viral protein can significantly improve the efficiency of gene replacement and gene targeting in human ESCs.

Title of Project: Induction and differentiation of beta cells from human embryonic stem cells

Amount requested: \$200,000.00

Principal Investigator: Kevan C. Herold, M.D.

Institution: Yale University

Collaborators: Caihong Qiu, Ph.D., Octavian Henegariu, Ph.D.

Description: This Project's purpose is to develop an approach to differentiate human embryonic stem cells into functional, insulin producing beta cells.

Project Summary: Type I diabetes (T1D) is an autoimmune disease with strong genetic susceptibility, characterized by a gradual infiltration of the pancreatic islets with auto-reactive T-cells, progressive decrease in β -cell mass, impaired insulin secretion and finally hyperglycemia and insulin-dependence. A true "cure" of the disease will require prevention of the autoimmune process as well as regeneration or replacement of the lost β -cell mass. New immunologics, such as anti-CD3 monoclonal antibody (mAb), and others, may arrest β -cell destruction, but there has been little evidence for spontaneous recovery of normal β -cell function even when the immune process ceases. Human embryonic stem cells (hESC) could provide a limitless source of cells needed for replacement of lost β cells. Promising results, using hESC cells to produce β -cells have been described using a series of cell culture manipulations. There were two problems with the differentiated cells, however. First these cells did not respond in a normal manner to glucose, the primary secretagogue in vivo. Second, transplantation of the cells into mice led to acquisition of glucose responsiveness, but tumors were also induced. The factors responsible for differentiation of the β -cells in vivo were not identified. Moreover, the need for transplantation of the cells into mice and the development of tumors would preclude use of the cells in patients. We have recently found that co-culture of mature islets with β cell precursors can deliver signals to induce glucose responsiveness. The goals of the proposed studies are therefore to establish methods for differentiation of hESC into insulin+ cells at the Yale Stem Center facility. We will use hESC lines that will be available in the Yale Stem Cell Core. We will test whether transcription factors tagged with fluorescent labels can be used to visualize cell differentiation in vitro, and whether this visualization system will allow us to identify cells that acquire glucose responsiveness and separate them from cells that develop into tumors. Finally, we will test whether mature islets can induce glucose responsiveness of differentiated hESC.

Title of Project: Neural Stem Cell Responses to Hypoxia

Amount requested: \$200,000

Principal Investigator: Qi Li, PhD

Description: This Project's purpose is to examine human neural stem cell-endothelial cell interactions in response to hypoxia with the goal of developing potential therapeutic approaches.

Project Summary: Chronic sublethal hypoxia (CSLH) caused by premature delivery is a significant cause of neurodevelopmental handicaps among low birth weight premature infants. 30 to 50% of the 60,000 low birth weight infants born each year are reported to develop cerebral palsy, mental retardation or significant neurological problems by school age. The deleterious effects of low Oz in the perinatal period are thought to be the consequences of several aberrations: (1) altered endothelial apoptosis, survival and angiogenesis, (2) loss of neurons, glia, and their progenitor cells due to excessive apoptosis and (3) altered neuronal differentiation and synaptogenesis. Patient IQs improve variably over time and recovery from neurodevelopmental handicaps is thought to depend upon the responses of neurovascular niches (the Sub-Ventricular Zone (SVZ) and the Sub-Granular Zone), distinct neurogenic regions, differentially responsive to insult and stimuli. Having determined that two mouse strains (C57BL/6 and CD1) span the range of responses to and recovery from CSLH, we propose to utilize brain-derived microvascular endothelial cells (BEC) from these two mouse strains in co-culture with human NPCs to assess the roles of endothelial cells in modulating the behavior of huNPCs at the neurovascular niche. Our hypothesis is that variable endothelial cell responsiveness in the SVZ of these two mouse strains will determine, in part, the responsiveness of the huNPCs to hypoxia. Additionally, utilizing in utero transplantation of huNPCs into E14 C57 and CD1 pup SVZ regions, we will assess the roles of the SVZ environment in modulating NPC survival, proliferation, differentiation and migration. This study utilizes murine BEC co-cultured with human embryonic stem cell derived neural progenitor cells in an in vitro model of the SVZ and human NPC transplantation into the SVZ regions of C57 and CD1 E14 pups in an in vivo model to elucidate the roles of endothelial cells in SVZ NPC responses to hypoxia. This study will lead to insights into the dynamic BEC-NPC interactions that modulate neural stem cell behavior and eventually lead to the development of novel therapies.

Title of Project: Molecular profiling and cell fate potential of hESC-derived early neural crest precursors

Amount requested: \$200,000.00

Principal Investigator: Martín I García-Castro

Institution: Yale University

Description: This Project's purpose is to characterize early neural crest precursors (Pax7-expressing cells) derived from hESCs, and to generate new tools for the study of human neural crest development.

Project Summary: Neural crest cells (NCCs) are multipotent migratory stem cells critical for the development of vertebrate organisms. NCCs generate, amongst many other different cells, bone, cartilage, muscle, dermis, and teeth in the cranial or head region. In addition NCCs generate melanocytes, the pigmented cells that protect us from damaging ultra violet light, as well as most of the cells of the peripheral nervous system, special heart cells, secretory and supportive cells of various glands amongst other derivatives [1,2]. The astonishing capacity of NCCs to generate such a broad range of derivatives is unfortunately matched by their participation in human birth defects [3]. About one third of all congenital birth defects are directly linked to neural crest flaws [4], and some NCC-related conditions, like cleft lip/palate, occur as often as 1 in 1000 births [3]. NCCs are also associated with various aggressive and frequent tumors, including melanoma and neuroblastoma [3]. The enormous differentiation potential of neural crest stem cells promises to be of great value for therapeutic purposes for degenerative diseases and for severe traumas alike. In order to use these cells for clinical purposes, it is critical to improve our understanding of their basic biology. Very limited work has directly addressed early neural crest events during human development. Recent studies based in the differentiation of human embryonic stem cells (hESCs) have identified neural crest precursors of relatively late stages, (comparable to migratory neural crest cells) that display a limited capacity to differentiate and to maintain this capacity after proliferation [5-9]. We propose to investigate earlier events related to the human NCCs, using human embryonic stem cells (hESC) with the aim to obtain and manipulate NCC precursors from early stages capable of broader differentiation and proliferation capacities. We will characterize the expression of a battery of molecular markers expressed by NCCs at early stages of development and we will interrogate their differentiation potential. Furthermore, we propose to generate new tools to prospectively isolate early NCC precursors, and to test their differentiation potentials *in vitro* and *in vivo*. The tools here proposed will benefit human research beyond the neural crest realm, as the candidate molecules to be used are also relevant for muscle development and homeostasis, as well as for cancer research.

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7. Motohashi, T., Aoki, H., Chiba, K., Yoshimura, N. & Kunisada, T. Multipotent cell fate of neural crestlike cells derived from embryonic stem cells. *Stem cells (Dayton, Ohio)* 25, 402-410 (2007).
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9. Zhou, Y. & Snead, M.L. Derivation of cranial neural crest-like cells from human embryonic stem cells. *Biochemical and biophysical research communications* 376, 542-547 (2008).

Title of Project: Williams Syndrome Associated TFII-I Factor and Epigenetic Marking-Out in hES and Induced Pluripotent Stem Cells

Amount Requested: \$500,000

Principal Investigator: Dashzeveg Bayarsaihan, Ph.D.

Institution: University of Connecticut Health Center

Description: This project's purpose is to investigate epigenetic marking-out in the Williams syndrome-derived iPS cells.

Project Summary: The generation of patient-specific induced pluripotent stem (iPS) cells is a critical step toward understanding the molecular basis of any particular human disorder. This proposal is designed to investigate the epigenetic status of Williams syndrome-derived iPS cells. Williams syndrome (WS) is a complex disorder with distinctive features including craniofacial defects, mental retardation, microcephaly, and short stature. Recent findings of WS patients have pointed to *GTF2I* as the prime candidate gene responsible for these clinical features. *Gtf2i* inactivation in mice causes craniofacial, neural tube and skeletal defects. The TFII-I transcription factor, a product of *GTF2I*, regulates a specific subset of chromatin-modifying enzymes including H3K27 methyltransferase EZH2, a member of the Polycomb Repressive Complex 2, the Class I histone deacetylase complex NuRD and NSD1, H3K36 methyltransferase, respectively. Therefore, TFII-I deficiency might disturb embryonic developmental program by changing the histone code of target genes at very early stages of development. We hypothesize that TFII-I is required for maintaining the correct spatial and temporal expression of a specific subgroup of epigenetic marker genes. As a result, TFII-I modulates epigenetic marking-out that is necessary in normal development. Two Specific Aims are proposed to test this hypothesis. (1) Characterize the epigenetic profile in iPS cells derived from the Williams syndrome patients (WS-iPS) compared to control; and (2) Define the TFII-I molecular mechanisms of gene regulation in embryonic stem cells. Ultimately, combined computational, genomic and biochemical studies will significantly increase our understanding of the epigenetic marking-out and explain some of the WS clinical features.

Title of Project: Stem Cell Therapy for spinal cord injury in the nonhuman primate.

Amount Requested: \$500,000

Principal Investigator: Jeffrey D. Kocsis, PhD

Institution: Yale University

Collaborator: Masanori Sasaki, MD, PhD

Description: This Project's purpose is to study the potential of neurosphere cells derived from human embryonic stem cells transplanted to the demyelinated spinal cord of the nonhuman primate for myelin and improve impulse conduction.

Project Summary: Cellular transplantation of human neurospheres (hNS) derived from adult and embryonic stem cells have been shown to remyelinate axons in rodent models of demyelination. A key question is whether hNS cells derived from human embryonic stem cells (hNS-hESCs) can remyelinate the adult nonhuman primate (NHP) spinal cord. One experimental impediment to address this issue is that endogenous myelin repair in rodents is typically robust and rapid, and because hNS-hESCs may require several weeks or months to mobilize *in vivo* to myelinate axons, endogenous repair may compete with the transplanted cells. We have developed a persistent demyelinating model system in the NHP spinal cord where virtually no endogenous repair is observed over the first six months after demyelinating lesion induction. We propose to transplant hNS-hESCs into the persistent demyelinated NHP spinal cord and study survival and the myelinating potential of these cells over the course of a year. The results will allow us to determine if hNS-hESCs can remyelinate the NHP spinal cord and if the remyelination contributes to improved conduction velocity.

Two Specific Aims will be addressed:

Specific Aim 1. Remyelination potential and time course of nonhuman primate spinal cord axons by transplantation of hESC-derived neurospheres.

Specific Aim 2. Ionic channel and electrophysiological analysis of hNS-hESCs remyelinated axons.

The significance of the proposed studies is that will allow us to determine the efficacy of transplantation of neural progenitors derived from human embryonic stem cells to remyelinate the adult nonhuman primate spinal cord. This result has implications for cell based therapies in demyelinating and other neurological disorders.

Title of Project: Mechanisms of Stem Cell Homing to the Injured Heart

Amount requested: \$500,000

Principal Investigator: Linda H. Shapiro, PhD
Institution: University of Connecticut Health Center
Co-PI: Bruce T. Liang, MD

Description: This Project's purpose is to investigate the signals that guide stem cells to the damaged heart following a myocardial infarction to facilitate repair and new blood vessel growth.

Project Summary: Stem cells have the amazing capacity to contribute to the growth and healing of many different types of tissues. This ability is critically dependent on the cells successfully finding the damaged tissue and effectively incorporating into the site. Currently stem cells are generally injected into the site of injury to increase the chances of correct cell delivery, but intracardiac injection is quite invasive and carries a certain degree of risk. Stem cell therapy would be greatly simplified if the cells could be injected into the bloodstream and allowed to 'home' or find their way to the damaged tissue. Therefore, investigating mechanisms of stem cell homing and approaches to enhance this process would lead to more efficient, safer and less invasive therapy. It is known that both the blood vessels of injured tissues and the traveling stem cells display a number of unique molecules on their surfaces that allow them to recognize and attach to each other to begin the process of integrating into the damaged tissue. Interestingly, stem cells will bypass healthy blood vessels that lack these molecules in search of vessels with the correct "address", thus preventing incorrect positioning. While a few of these molecules have been identified, clearly stem cell homing is so complex that more of them must exist in order to regulate this intricate process. We have identified a molecule called CD13 that is found on both stem cells of many lineages and in the damaged heart vessels following myocardial infarction, suggesting that it could serve as such a recognition molecule. Importantly, we have also seen that CD13 participates in the attachment of other types of circulating cells to blood vessels and so could logically participate in stem cell homing and attachment at sites of myocardial injury. Finally, we have devised a method to improve the ability of CD13 on the circulating cells to recognize and attach to injured blood vessel walls and so we may be able to enhance stem cell homing to tissue damaged by myocardial infarction. Therefore, using mouse models of myocardial infarction and human stem cells, we propose to investigate the role of CD13 in stem cell homing to the injured heart and our ability to enhance homing by increasing CD13's adhesive capacity on stem cells, thus advancing toward the goal of less invasive, systemic stem cell therapy.

Title of Project: Genome-wide screen to identify hESC-specific DNA transcription elements

Amount requested: \$500,000

Principal Investigator: Richard Sutton

Institution: Yale University School of Medicine

Collaborators: Mike Snyder (Yale); Sherman Weissman (Yale)

Description: This Project's purpose is to identify, in a genome-wide manner, DNA enhancer elements active in and specific to hESC.

Project Summary: Human embryonic stem cells (hESC) have enormous potential in treating illness in man. Much is known about the genes are turned on in these cells, but little is known about what controls the genes being turned on and off. We have developed a new method in which small pieces of human DNA are placed in a 'vector' based upon human immunodeficiency virus or HIV. To cover all human DNA this requires 10 million vectors. These vectors can be made as defective viruses in cells, and then transferred to hESC such that each hESC only gets one. If the piece of DNA is read as active, then a special fluorescent protein is made, which allows us to identify and collect those cells. The pieces of 'active' DNA may then be amplified and deciphered by using new sequencing methods, and when compared to other cells it may be determined which pieces are active only in hESC. A subset of the DNA pieces may be recovered and tested again in hESC and other cell types to confirm the results. A few of the DNA pieces may be subdivided to determine what parts make them active in hESC. At the end of four years, we hope to have a better understanding of which DNA pieces are best able to turn on genes in hESC. It should be pointed out that the method is general enough to apply to any human cell type that can be cultured.

Title of Project: Molecular function of Lin28 in human embryonic stem cells

Amount Requested: \$500,000

Principal Investigator: Yingquan Huang

Institution: Yale University School of Medicine

Description: The Project's purpose is to dissect the molecular function of Lin28, a protein that is highly expressed in human embryonic stem cells and that has been used, in combination with other three factors, to reprogram human fibroblasts to induced pluripotent stem (iPS) cells.

Project Summary: Highly expressed in human and mouse embryonic stem cells, Lin28 is among four factors (including Oct4, Sox2 and Nanog) that can together reprogram human skin cells to induced pluripotent stem cells, or iPS cells. Despite its critical role in human embryonic stem (hES) cells and in the projection of iPS cells, the molecular function and mode of action of Lin28 are largely unknown. We have recently discovered that the mouse Lin28 plays an important role in the regulation of expression of genes important for the maintenance of the unique proliferation properties of ES cells. Specifically, we found that Lin28 selectively binds to a subset of messenger RNAs (mRNAs) and enhances their ability to make proteins. These mRNAs encode proteins important for ES cell proliferation. We propose to use the knowledge we have gained with mouse ES cells to extend the analysis of Lin28 to hES cells. Thus, we have developed four specific aims: 1) to characterize the expression of Lin28 in hES cells throughout the cell cycle; 2) to characterize the interactions between Lin28 and its associated mRNAs, in order to gain insight into how this protein selects its targets and regulates mRNA function in the cellular milieu; 3) to identify Lin28-interacting proteins that may work in concert to mediate Lin28 function; and 4) to perform a genome-wide search for new Lin28 targets to establish a more comprehensive and global understanding of Lin28 function. We anticipate that results derived from these studies will shed new light on the molecular function of Lin28, thus not only contributing greatly to the general hES cell field of research, but also to the technology of generating patient-specific iPS cells.

Title of Project: Therapeutic differentiation of regulatory T cells from iPS and hES for immune tolerance

Amount requested: \$499,998

Principal Investigator: Zihai Li, M.D., Ph.D. (PI), Ren-He Xu, M.D., Ph.D. (co-investigator)
Institution: University of Connecticut Health Center

Description: This Project's purpose is to generate regulatory T cells from stem cells for treatment of autoimmune diseases.

Project Summary: Human embryonic stem (hES) cells can be cultured and propagated long-term outside of the body, and under proper conditions, differentiate into all cells and tissues in the body. Recently, skin cells and other adult cells have been reprogrammed into inducible pluripotent stem cells (iPS) with a defined set of genes including Oct4, Sox2, Klf4 and c-Myc. One of the main challenges of the immune system is to maintain the balance of fighting against germs but leave self tissues unharmed. Scientists have found that such a balance is in part made by a key regulatory T cell (Treg) in the body. Bearing a unique gene called Foxp3, Tregs can suppress self-reactive immune responses and have emerged as a promising therapeutic modality for autoimmune diseases such as diabetes, lupus, arthritis and inflammatory bowel diseases.

In order to generate unlimited number of Tregs for therapeutic purpose and for understanding their basic mechanism of action, we propose to derive Tregs from both pluripotent hES and iPS cells. We also wish to understand if Foxp3 can "push" stem cells to differentiate into Tregs more efficiently. A series of sophisticated molecular and functional studies of these Tregs will be conducted. We believe that our study may not only uncover a novel method for generating unlimited number of Tregs for therapeutic purpose of a variety of diseases, but also reveal the fundamental mechanisms of Treg development. Although many scientists have shown that blood cells can be generated from stem cells outside of the body, no study has specifically addressed the issue of Treg development from stem cells. This is also the first in-depth study to compare Tregs generated from different source of stem cells. Our proposal is thus novel and significant.

Title of Project: Prevention of spontaneous differentiation and epigenetic compromise of human ES and iPS cells.

Amount requested: \$499,956

Principal Investigator: Theodore Rasmussen, Ph.D., Associate Professor
Institution: University of Connecticut, Center for Regenerative Biology
Collaborators: Winfried Krueger, Asst. Prof., U. Conn., Center for Regenerative Biology
Dennis Wright, Assoc. Prof., U. Conn., Dept. of Pharmaceutical Science

Description: This project will identify methods and compounds that will inhibit the spontaneous loss of epigenetic quality of human ES and iPS cells in culture.

Project Summary: Human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPS cells) are of great promise for medicine because they can be coaxed to differentiate into all cell types in the human body. Therefore, they offer the possibility to provide immunologically-matched transplantable cells to alleviate human degenerative diseases and organ damage. Unfortunately, both hESCs and iPS cells frequently undergo spontaneous and irreversible alterations during their culture *in vitro* that compromise their quality and utility for clinical applications. These alterations are often epigenetic in nature, meaning that though the DNA sequence may remain unaltered, gene expression becomes mis-regulated.

The object of this grant is to identify methods and chemical compounds that will prevent the spontaneous loss of quality of human ES and iPS cells. If such improved methods and compounds are identified, then the quality of *in vitro* culture of these promising cells will be greatly increased.

The starting point for this grant is provided by previously awarded seed grant entitled "Pragmatic Assessment of Epigenetic Drift in Human ES Cells". This grant allowed my lab to gain insights into the specific changes that occur when hESCs become altered in culture. In this application for expanded support, I propose to engineer hESCs and iPS cells to contain fluorescent reporter genes that will indicate both the maintenance of pluripotency, and its loss for epigenetic reasons. This reporter gene platform will be used as a platform to find culture conditions and chemical compounds that stabilize the pristine pluripotent state of human ES and iPS cells. In addition, the research may lead to methods to increase the efficiency of the iPS procedure, and to reverse degraded states of spontaneously compromised human ES and iPS cells.

Title of Project: Development of iPS cells to study craniometaphyseal dysplasia in humans

Amount requested: \$500,000

Principal Investigator: Alex Lichtler, PhD, Ernst Reichenberger, PhD (Co-PI)

Institution: University of Connecticut Health Center

Collaborator: Stormy Chamberlain PhD

Description: The purpose of this project is to develop and investigate inducible pluripotent stem cells (iPS) from patients with the untreatable bone disorder craniometaphyseal dysplasia (CMD).

Project Summary: This application is designed to develop human inducible pluripotent stem (iPS) cells from skin biopsies of patients with a rare bone disorder, craniometaphyseal dysplasia (CMD), and to stimulate them into becoming typical mature bone-forming cells (osteoblasts). CMD is a debilitating genetic bone disorder with sometimes fatal overgrowth of skull bones for which no treatment exists. It affects children and progresses throughout life. We have promising data from a mouse model for CMD but it is currently impossible to translate our findings to the human system because bone cells from patients become only rarely available. Our motivation is to explain the mechanisms that lead to this debilitating disorder with the hope that treatment can be found based on this knowledge. We will obtain skin cells from patients and normal volunteers to create iPS cells, a method that has already been tested by co-workers of this grant application. The challenge is to identify iPS lines, which can then be stimulated in cell cultures to become bone-forming osteoblasts. The other big challenge is to analyze those cells with sophisticated methods and to compare their bone-forming properties with other embryonic stem cell cultures, normal osteoblast cultures and with normal bone. We will compare our differentiating iPS cells to certain human ES cell lines, which have shown some capacity to differentiate to osteoblast-like cells. Bone-forming properties of these cells have, however, not been studied with all the methods that we will use. The most promising ES cell line to be used is "unregistered" and we will be able to study this line only with "non-federal" funds. It is to our advantage that Dr. Lichter is already involved in a project funded by the State of Connecticut Stem Cell grant program to improve differentiation of human ES cells into osteoblast-like cells.

In summary, we expect the proposed studies to lead to a thorough understanding of 1) how to stimulate stem cells to become osteoblasts, 2) how to do quality assessment of those differentiating hES and iPS cells, and 3) what are the differences between patient and control osteoblasts. These studies will not only help CMD research, but will provide new tools for renewable sources of osteoblasts from patients with other human skeletal disorders, such as osteogenesis imperfecta or osteoporosis.

Title of Project: *piggyBac* Transposon for Genetic Manipulation and Insertional Mutagenesis in Human Embryonic Stem Cells

Amount requested: \$500,000

Principal Investigator: Tian Xu

Institution: Yale University School of Medicine

Collaborator: Caihong Qiu

Description: This Project's purpose is to use the *piggyBac* transposon to genetically manipulate human embryonic stem cells and screen for human embryonic stem cell transformation and malignancy by insertional mutagenesis.

Project Summary: The ability to perform genetic manipulations in human embryonic stem cells (hESCs) is of central importance to study hESC biology. However, researchers lack critical methodologies to manipulate the hESC genome. Development of efficient technologies to genetically manipulate hESCs will enable researchers to address many interesting and fundamental aspects of hESC biology that are currently difficult to approach. To efficiently manipulate the hESC genome, we propose to use the newly developed, bipartite *piggyBac* (*PB*) transposon system for mammals as a tool for insertional mutagenesis. There are multiple reasons why *PB* should be developed as a tool for functional annotation of the hESC genome. It transposes efficiently in a genome-wide manner in mouse embryonic stem cells and all human cell types tested. *PB* also can carry antibiotic-resistant or visible markers and can be easily mapped upon insertion into the genome. In addition, we have shown that *PB* can induce loss-of-function as well as gain-of-function mutations, and that it can interrogate not only protein-coding genes but also small RNA genes and regulatory regions. Finally, *PB*-based genetic manipulations permit the utilization of forward genetic screens to address complex biological processes controlled or driven by several genetic components or pathways in an unbiased fashion. These advantages suggest that *PB*, as a tool, will have wide applicability to the study of many aspects of hESC biology. As an example of a complex process that can be addressed using *PB*, we will use *PB* insertional mutagenesis to screen for hESC transformation and malignancy. We will use the soft agar assay for transformation as well as injection into immune-compromised mice for malignancy. While cancer research has identified a host of oncogenes and tumor suppressors important for transformation and malignancy in terminally-differentiated cells, hESCs may require fewer genetic alterations or a different set of genetic mutations altogether. The identification of genes that drive transformation and malignancy in hESCs will elucidate interesting mechanistic differences between the biology of pluripotent cells and terminally differentiated cells. In addition, it will provide crucial information to researchers about the kinds of genetic alterations that should be avoided in hESC cells for therapeutics.

Title of Project: Brain Grafts of GABAergic Neuron Precursors Derived From Human and Mouse ES Cells for Treating Temporal Lobe Epilepsy

Amount Requested: \$499,988

Principal Investigator: Janice R. Naegele
Institution: Wesleyan University, Department of Biology
Collaborators: Laura B. Grabel, Gloster Aaron

Description: This Project's purpose is to generate GABA progenitors from human and mouse ES cells and test their efficacy for suppressing seizures in a mouse model of temporal lobe epilepsy.

Project Summary: We proposed a thorough set of in vitro and in vivo experiments to test our hypothesis that ES cell grafts of GABA progenitors will integrate and differentiate into the brains of mice with epilepsy, where they will provide lasting suppression of seizures. Our published work and preliminary studies not only demonstrate the feasibility of the proposed work, but also show our commitment to understanding the functional roles of GABA neurons in normal brain circuits and in abnormal conditions such as epilepsy. We have two main objectives.

Objective 1: We will use a three-prong approach to induce mouse and human Embryonic Stem (ES) cell lines to generate GABAergic progenitors. 1A: to generate GABAergic progenitors by stably transfecting ESNPs with plasmids encoding transcription factors *Dlx1/2*, *Nkx2.1*, and *Lhx6/7*, previously found to specify embryonic GABAergic neurons. 1B: to generate GABAergic progenitors by treating ESNPs with the conditioned media from the embryonic ganglionic eminence. 1C: to generate GABAergic progenitors using a protocol established for embryonic neurons by sequential exposure of ESNPs to small molecular antagonists of sonic hedgehog, fibroblast growth factor (FGF), brain derived neurotrophic factors (BDNF), and differentiation molecules (valproic acid and dbcAMP). Objective 2: We will determine whether engrafted ES-derived GABA progenitors suppress seizures in mice. 2A: Compare the frequency and severity of seizures using video-EEGs in mice over a period of weeks following and initial bout of status epilepticus in the systemic pilocarpine model of TLE. 2B: Analyze video-EEGs after stereotaxic transplantation of GABA progenitors into the hippocampus, entorhinal cortex, or substantia nigra in mice with TLE. 2B. Characterize differentiation of GABAergic transplants in different host brain locations by immunohistochemistry and electron microscopy in mice with TLE. 2C: Test the function of the GABAergic transplants by calcium imaging in living hippocampal slices from mice with TLE. 2D: Optimize graft survival in mice with seizures using anti-apoptosis agents. Mice will receive continuous intraventricular infusions of the caspase-3 inhibitor z-DEVD-fmk or saline (control) via Alzet minipumps.

Title of Project: MicroRNA regulation of hESC fates

Amount requested: \$500,000

Principal Investigator: Jun Lu

Institution: Yale University

Collaborator: Dr. Caihong Qiu, Technical Director, Yale hESC Core Facility

Description: This Project's purpose is to investigate the roles of microRNAs in hESC fate determination.

Project Summary: The power of human embryonic stem cells (hESCs) to become any given cell type in a human body is the underlying principle to develop hESC-based therapies to replace damaged cells in human diseases. In order to make any desired cells, hESCs need to undergo sophisticated changes to switch from a stem cell state to a committed path toward another cell type, a fate switch process that is not fully understood. Prior research efforts have been focusing on genes that make proteins. However, such protein-making genes only make up less than 2% of the human genome, leaving the majority of the human genome poorly explored. Recently, we and others have produced data to support the idea that a class of new genes, termed microRNAs, deserves strong attention in hESC research. Unlike protein-making genes, microRNAs do not make proteins themselves, but control the levels of protein production in cells.

In this proposal, we hypothesize that microRNAs contribute to the hESC fate switch process. We aim to systematically produce a roadmap of microRNA-based regulation in hESCs and to identify microRNAs that modulate the hESC fate switch. We propose to accomplish these objectives through four achievable steps. We will first chart all the early cell types produced by hESCs when they undergo cell identity transformation, by documenting all the microRNA changes during these processes. We will then look for microRNAs that may reinforce or compromise the stem cell state, by testing their ability, one by one, to regulate important known protein factors. We have already identified candidate microRNAs that can do this. So in the third step, we will test whether these candidate microRNAs can mold hESCs into other cell types. Finally, additional candidate microRNAs that have potentially different molding ability will be explored and studied in detail. We anticipate that the proposed work will lead to critical knowledge in a previously underappreciated dimension in hESC research, providing important clues to novel hESC-based therapies.

Title of Project: Continuing and Enhancing the UConn-Wesleyan Stem Cell Core

Amount requested: \$2,500,000

Principal Investigator: Ren-He Xu, M.D., Ph.D.

Institution: University of Connecticut Health Center

Collaborators: Dr. Robert Kosher, UCHC; Dr. Shijiang Lu, Advanced Cell Technologies; Dr. Timothy Kamp, University of Wisconsin-Madison; Dr. Zack Z. Wang, Maine Medical Research Center; and Dr. Yi Zhang, University of North Carolina

Description: This Project's purpose is to provide existing and new services critical for Connecticut scientists to study human embryonic stem (ES) cells as well as induced pluripotent stem (iPS) cells, promising sources to make therapeutically useful cells or tissues to treat degenerative diseases.

Project Summary: The University of Connecticut-Wesleyan Stem Cell Core is supported by a Core Facility grant from the Connecticut State Stem Cell Research Program. The overall objective of the grant is to enable the Core to serve Connecticut scientists by deriving, banking, and providing human embryonic stem cell (hESC) lines, technologies, and training on the culture and differentiation of hESCs, a promising source to make therapeutically useful cells or tissues to treat degenerative diseases.

The Core has stocked 9 hESC lines, and provided these hESCs to over 20 laboratories at University of Connecticut, Wesleyan University, and Yale University. The Core has successfully derived two new hESC lines CT1 and CT2 from donated embryos and trained 100 researchers statewide and beyond. Researchers are using our services to study not only hESCs but also induced pluripotent stem (iPS) cells. iPS cells are an ES-like cell type derived from cells in our body, like the skin cells, by using a cocktail of rejuvenating factors. iPS cells so far hold the highest promise for patient-specific cell therapy. We have also derived iPS cells from human fibroblast.

To continue our services and further evolve our missions we are applying for continued funding for our Core Facility. In this application, we propose:

1. Continuing our current services to support research on hESCs.
2. Supporting iPS cell research by providing training or direct services for iPS derivation and characterization, and continuing to derive, bank, and distribute normal and diseased iPS cells.
3. Enhancing our services by providing state-of-the-art deep sequencing technologies for detailed analysis of both human ES and iPS cell lines derived in the Core.

Through these services, we will assure that scientists funded by the state stem cell program continue to obtain full support from the UConn-Wesleyan Stem Cell Core to achieve their research goals, and helping translate stem cell research from laboratories to clinical trials.