Final Report to the Blazeman Foundation

2011

The Blazeman Foundation funding to my laboratory allowed us to accomplish three important aims in the progress towards the understanding of amyotrophic lateral sclerosis. The first trained a young scientist in techniques of molecular genetics and cell biology. Her contributions allowed us to make important discoveries and breakthroughs in the understanding of ALS. The second major accomplished the ascertainment of one of the largest collections of skin biopsies and fibroblast cell lines from ALS patients and control subjects in a very short period of time. The third aim established de novo in my laboratory, the methodology of engineering induced pluripotent stem cell (iPSC) lines from the fibroblast cell lines obtained from ALS patients and control subjects. Five papers resulted from the funding contributions of the Blazeman Foundation. Three papers acknowledging the Blazeman Foundation Fellow have been published and include the ground breaking paper on UBQLN2. Two additional papers acknowledging the funding of the Blazeman Foundation have been completed. One is under review and the other is being reformatted for resubmission. These papers are important research milestones, one describes mechanism of disease in ALS at the molecular level implicating the autophagosome-lysosome system and the other describes a novel genetically engineered robust mouse model of

A report on the progress in ALS research funded by the Blazeman Foundation.

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FTD/ALS caused by mutations in UBQLN2. The three published papers can be quoted and links published the other two only when authorized by the journals.

Three Aims of the Blazeman Foundation Funding

1. **The Blazeman Foundation Fellow:**

The aim of training a young scientist in ALS who could acquire techniques of molecular genetics, cell biology and immunohistochemistry was accomplished with the training of Kaouther Ajroud, PhD as the Blazeman Foundation Fellow. She contributed significantly to the goals of progress in ALS research. Her assignments were two fold. One was to participate in ongoing research and learn by practically contributing to it. The second was to work closely with other scientists in the laboratory to establish techniques for engineering induced pluripotent stem cells (iPSC) in my laboratory from fibroblasts of ALS patients and control subjects. She successfully accomplished those aims by significantly contributing to both. She contributed to three papers on which is a co-author and which acknowledge funding of the Blazeman Foundation Fellow. All three papers are attached and include the breakthrough paper published in the journal *Nature* in 2011.

a) *Discovery of two parallel pathologies in ALS:* After our initial discoveries of mutations in SOD1 as the first cause of ALS in 1991 (Siddique et. al, 1991 *NEJM.*, Rosen e. al, 1993 *Nature*, Deng et. al, 1993 *Science*.), we were hopefully confident that it would lead to a common pathophysiology of all ALS. This was obviously a desired goal as it would provide a direct approach to therapy, especially given the fact we had engineered the first animal model of ALS, in fact the first genetic model of neurodegeneration (Gurney et al, (1994) *Science*). This was not to be as additional causes of ALS were discovered and etiology or causes became many, greatly increasing the complexity of the problem of ALS. The causative genes include TDP-43, FUS/TLS, optineurin and Profilin1 etc and continue to increase. We and others first distinguished the pathology of ALS linked to SOD1 was different from rest of ALS (Mackenzie et al. (2007) *Annals Neurol.*) due to the absence of TBP-43 inclusions in spinal cords of ALS patients with SOD1 mutation and in our ALS SOD1 transgenic mice. We further showed that FUS/TLS and optineurin inclusions were present in spinal cords of ALS patients but not in ALS patients or mice with SOD1 mutations. It was to the latter two important findings that the Blazeman Fellow contributed and which were subsequently published (Deng et al (2010) *Ann. Neurol.* and Deng et al (2011) *Arch. Neurol.*).

b) *Discovery of a common pathology; linking etiology to pathology and to mechanism of disease*: With our identification of two novel causative genes for ALS and ALS/dementia, SQSTM1 or p-62 (Fecto et al (2011) *Arch. Neurol*) and UBQLN2 (Deng et al (2011) *Nature*), we were able to establish a common link between pathology, cause and mechanism for the diverse forms of ALS. This was subsequently vindicated when P-62 and ubiquilin2 positive inclusions were discovered in brains and spinal cords of a subset of patients with ALS caused by an

aberrant hexanucleotide repeat expansion in the gene C9ORF72. The Blazeman Fellow contributed to the major breakthrough published in the journal *Nature* in 2011. Copy attached.

c). *Mechanism of Disease and a transgenic UBQLN2 (P497H) mouse model for Frontaltemporal lobe dementia (FTD) associated with ALS:* Identification of mutations in the UBQLN2 gene and a common ubiquilin2/p-62/ubiquitin pathology in all of ALS allowed us to rapidly identify two molecular mechanism of disease associated with UBQLN2 mutations. Neurons and other cells have two main recycling machineries, the ubiquitin proteasome system (UPS) for rapidly recycling proteins and the auophagososme-lysosme system for bulky cargo and organelles such as the mitochondria and damaged proteins. Both systems are crucial for the integrity of the synapse, the communicating mechanism of all neurons with the rest of the nervous system. Many synaptic proteins are recycled several times in 24 hours and the half-life of many a synapse is said to be 28 days. p-62 and ubiquilin2 are important in regulation of both recycling systems. We reported in our *Nature* paper in 2011 that mutations in UBQLN2 interfered with the smooth functioning of the UPS system. We have now that discovered that mutations ion UBQLN2 also inhibited the fusion of the lysosome with the autophagosome. This is the first direct evidence that that proteins accumulate in neurodegenerative disease on account of faulty repair mechanisms, leading to damage to the synapse protein recycling machinery. Thus damaged, the synapses of the motor neurons are isolated from rest of the nervous system leading to neurodegenerative condition we call ALS. We confirmed this in a new model of FTD by expressing of human UBQLN2 gene with the 497H mutation. Bothe papers cite acknowledgements to the Blazeman Foundation funding. The first paper specifically, on account of human fibroblast cultures form UBQLN2 patients and UBQLN2 knockout mice used in experiments to demonstrate the protein recycling defects in ALS. The first paper demonstrating the recycling defect is under review and the mouse model paper is being reformatted for resubmission. The recycling paper is attached in draft format and will be released to the public once permitted by the journal. Please keep it confidential till that time. We will provide you with the draft of the mouse mo0dle paper in the near future.

2. ALS patient specific fibroblast and transgenic mouse fibroblast cell lines

- Successfully established over 90 patient and control specific dermal fibroblast cell lines that include different ALS forms, including those with mutations in *SOD1, TDP43, FUS , UBQLN2 , C9ORF72* , sporadic ALS and others who have unknown causes of ALS
- Successfully established 15ALS transgenic mouse fibroblast cell lines, including different mutation forms of ALS models

3. ALS induced Pluripotent Stem Cells (iPSc) Project

Background:

In 2006 researchers in Japan reported that human skin fibroblasts could be induced to become pluripotent stem cells thus overcoming the barrier of embryonic stem cell controversies and immune rejection by the body of foreign cells. Patient's own derived stem cells could be implanted. The lead Japanese researcher was awarded the Nobel prize for this work in 2012 Further the iPSCs could be converted to cells of the nervous or other tissue or other tissue

including motor neurons. We were one of the first to recognize the importance of application this work to ALS for conversion to iPSCs and then human motor neurons for identification of specific molecular signatures that would identify late onset vs early onset, slow vs rapid and familial vs sporadic ALS. These observations could provide the basis of a new approach to therapeutics for ALS > it would for the first time allow for screening of human derived motor neuron type cells to be used in tissue culture experiments to screen a very large number of chemical compounds. Such a screen may identify \potentially useful compounds that would revert the molecular phenotype to 'normal'.

Four genes (Oct4, Klf4, Sox2 and c-Myc (OKSM) were initially identified that would induce fibroblasts to pluripotent stem cells. These were technically challenging and time consuming experiments and very few laboratories in the world had know-how to proceed. Keeping the interest of ALS patients in mind we proceeded with shoe-string funding to take on this rather challenging task. We tried several systems and finally settled on viral deliver. It took us over six months to get permission to use lentivirus vectors and over nine months to use Sendai virus vectors. After discovery of four reprogramming transcription factors, which can induce somatic cells into pluripotent stem cell, several delivery methods for these genes including viruses, plasmids, excisable vectors and minicircles have been introduced. With limitation of inserted mutagenesis risk, viral transductions have been increasingly replaced by non-virus methods such as directly protein transduction, mRNA interfering and mirRNA reprogramming. A non-integration of iPSC was preferred in our ALS study. The establishment of these methodologies in my laboratory is described below. We are now in a position to carry out large scale conversion of ALS fibroblast cell liens to iPSCs and derive spinal motor neurons from them for screening of therapeutic compounds and identification of molecular signatures corresponding to the patient genotype and phenotype.

Objective:

- To obtain non-viral integrated iPSc lines from dermal fibroblast of ALS patients;
- To differentiate iPSc to ALS patient specific motor neuron[(1)];
- To utilize differentiated motor neurons for ALS pathology study, drug discovery and potential neuron repairing study.

Accomplishments:

a). Stem cell technology preparation and practice

- Two research staffs were trained by stem cell core facility laboratory for stem cell culturing and maintenance. One stem cell working platform including dedicated sterile hood and incubator have been tested and optimized for stem cell related experiments.
- Federal registered Human stem cell line (H-7) is utilized to practice essential skills of stem cell technology in our lab setting.

b).Strategies for generating iPSc

- **Polycistronic Lentivirus Cre-excisable reprogramming**

  STEMCCA is a commercial available kit in Millipore Company comprised of all four transcription factors (OKSM) separated by self-cleaving 2A peptide and IRES sequences. This single polycistronic cassette enabled higher efficiency of reprogramming, reduced the number of viral integrations and

enable subsequent Cre-mediated excision of the viral reprogramming transgenes. The result lead to iPS cells that virtually free of the exogenous viral transgenes.

- One commercial kit contains 4 iPSc experiments needs. Two fibroblast cell lines have been transfected; the final result can be evaluated after 2-4 weeks.

- **Direct delivery of reprogramming proteins**[2]
  - Constructed separated of 4 Human reprogramming transcription genes (OKSM) into plasmid of pcDNA3.1 vector with confirmed DNA sequences and protein expressions
  - Established 4 stable HEK 293 cell lines with above 4 genes separately by enforced culture selection of neomycin (G418)
  - Collected and stored whole cell lyses that including high expression of Oct4, Sox2, Klf4 and c-Myc proteins in -80 degree
  - Carried out 4 times of protein directly deliveries experiments, each experiment last 8 more weeks, accumulated experiences by modifying and optimizing experiment. Partial reprogrammed clones are obtained after 8 weeks of protein transduction but growth of stem cell like clones were retarded in further cell culture expansion.

- **Minicircle vector with microRNA302/367 cluster derived iPSc**[3]
  - Minicircle vector contains mircoRNA302/367 cluster is purchased from SBI company, minicircle parental clone has been selected and Arabinose-inducible minicircle are collected and purified with amount of 3 milligram (~100X for one experiment needs)
  - Eletro-nucleofector and lipofectamin transfect are optimized
  - Flow Cytometry are optimized for GFP positive cells selection
  - First experiment of minicircle induce stem cell line has been carried out and result will be evaluated after two weeks.

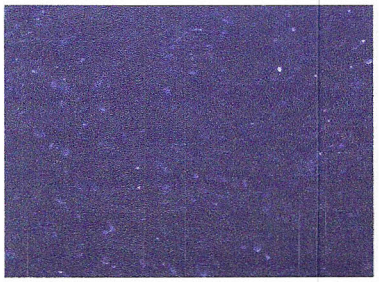
c). Generation of iPSCs from ALS patients' skin fibroblasts using STEMCCA Lentivirus programming Kit (Millipores SCR531):

We focused our strategy to generate iPSCs, on using a Cre-Excisable Constitutive Polycistronic (OKSM) Lentivirus Reprogramming Kit from Millipore SCR531. The lentivirus contains the four reprogramming genes, Oct4, Klf4, Sox2 and C-Myc cloned in unique cassette. The advantage of using this lentivirus is to decrease the risk of generating partially induced stem cells due to the incorporation one or more of the 4 genes when they are cloned individually in different lentivirus.

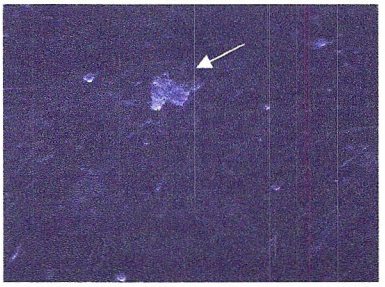
2 familial ALS patients' skin fibroblasts were used in our experiment, X-linked ALS P506T and SOD linked ALS A4V.

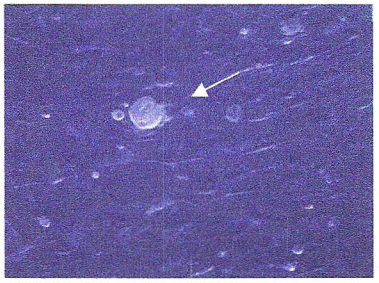
We first obtained the fibroblasts from our fibroblasts bank, cultured them in DMEM containing 10%FBS and antibiotics (Penicillin and Streptomycin). At passage tow of the culture we infected the fibroblasts according to the manufacturer procedure. We then transferred the infected

fibroblasts to a plate containing mouse embryonic fibroblasts which will be used as feeder cells. The infected fibroblasts will then incubate with the feeder cells and media containing 10ng/ml of recombinant Human FGF-2 which will enable the infected fibroblasts to become stem cells. The colonies number is estimated to 5 to 20 colonies per 6 wells plate. According to the protocol, the process to obtain stem cells colonies requires around ten to fifteen days.
We are in the process of converting the infected fibroblasts in to stem cells. So far the procedure is taking more time than what is referred in the protocol. We are in the process of optimizing our conditions to be able to obtain stem cells colonies in shorter time.

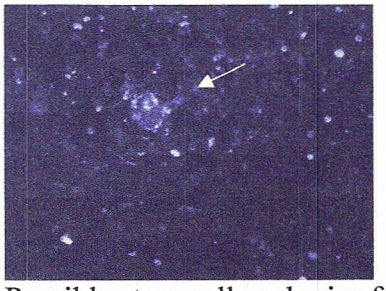


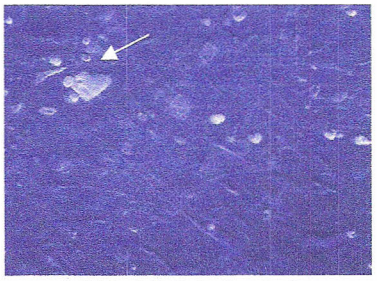
Feeder cells 4X





Possible stem cells colonies from X-linked ALS infected fibroblasts10X





Possible stem cells colonies from SOD ALS infected Fibroblasts 4X and 10X
The arrows show stem cells colonies.

Plan:

0->~6 months

- Evaluate all of three transduction strategies including Cre-excisable reprogramming, direct protein delivery and minicircle-mirRNA and choose reliable and the least risk method to induce patient specific pluripotent stem cell lines
- Plan and carry out the experiment for generating spinal motor neurons by differentiate normal iPSc or embryonic stem cell line

7->24 months

- Establish methodologies to establish iPSCs and derived motor neurons.
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Future Studies

- Patient specific spinal motor neuron pathologic study
- Patient specific spinal motor neuron mRNA analysis
- Patient specific spinal motor neuron proteomic profiling study

Alternative strategy:

- MircoRNA directly convert fibroblast to neuron(cortical neuron but not motor neurons- need watch the progress) [4]
- mRNA interface to generated iPSC[5]

References for iPSCs:

1) Differentiation of spinal motor neurons from pluripotent human stem cells
Bao-Yang Hu & Su-Chun Zhang Nature Protocols 4, 1295-1304 20 August 2009 doi:10.1038/nprot.2009.127

2) Generation of Human Induced Pluripotent Stem Cells by Direct Delivery of Reprogramming Proteins
Dohoon Kim, Chun-Hyung Kim, Jung-Il Moon, Young-Gie Chung, Mi-Yoon Chang, Baek-Soo Han, Sanghyeok Ko, Eungi Yang, Kwang Yul Cha, Robert Lanza et al. 5 June 2009 Cell Stem Cell 4(6) pp. 472 - 476

3) A nonviral minicircle vector for deriving human iPS cells
Fangjun Jia, Kitchener D Wilson, Ning Sun, Deepak M Gupta,Mei Huang, et al Nature Methods 7, 197-199 7 February 2010 doi:10.1038/nmeth.1426

4) MicroRNA-mediated conversion of human fibroblasts to neurons
Andrew S. Yoo, Alfred X. Sun, Li Li, Aleksandr Shcheglovitov,Thomas Portmann, Yulong Li, Chris Lee-Messer, Ricardo E. Dolmetsch, Richard W. Tsien & Gerald R. Crabtree Nature (2011) 13 July 2011 doi:10.1038/nature10323

5) Highly Efficient Reprogramming to Pluripotency and Directed Differentiation of Human Cells with Synthetic Modified mRNA
Luigi Warren, Philip D. Manos, Tim Ahfeldt, Yuin-Han Loh, Hu Li, Frank Lau, Wataru Ebina, Pankaj K. Mandal, Zachary D. Smith, Alexander Meissner et al. 5 November 2010 Cell Stem Cell 7(5) pp. 618