MEMORANDUM

Date: January 21, 2011

From: Alan Trounson, PhD
CIRM President

To: Independent Citizen's Oversight Committee

Subject: Extraordinary Petition for Application RT2-01913

Enclosed is a petition letter from Dr. Guoping Fan of UCLA, an applicant for funding under RFA 10-02, CIRM Tools and Technology II Awards. This letter was received at CIRM on January 20, 2011 and we are forwarding it pursuant to the ICOC Policy Governing Extraordinary Petitions for ICOC Consideration of Applications for Funding.

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January 20th, 2011

Robert Klein, J.D., Chair
Independent Citizens' Oversight Committee
Alan Trounson, Ph.D.
President and Chief Scientific Officer
California Institute for Regenerative Medicine

Re: Extraordinary Petition for
RT2-01913: A novel protein delivery platform to promote stem cell reprogramming and differentiation
Principal Investigator: Guoping Fan, Ph.D.
Institution: University of California, Los Angeles

Dear Mr. Klein and Dr. Trounson and Distinguished Members of the ICOC,

Thank you for the opportunity to submit this letter of Extraordinary Petition with regards to the review of our project "RT2-01913: A novel protein delivery platform to promote stem cell reprogramming and differentiation". While we appreciate the Reviewer's comments on the proposal, both PI and co-PI would really like to take this opportunity to clarify some issues raised in review that may merit the outcome of our grant.

The proposed project described studies to develop engineered nanocapsules to effectively deliver transcription factors to achieve cellular reprogramming and differentiation. The reviewers felt that the proposal addressed the need for a non-viral cellular reprogramming system and provided a logical research plan, but were divided regarding the following issues: innovation, potential toxicity/efficacy issues and lack of details for potential difficulties encountered. The proposal therefore was almost rated in the tier one category. We would like to address these comments and take this chance to clarify these aspects of our proposal.

The first division between reviewers was in regards to the innovation of this project: **"Reviewers were divided as to whether the proposed research is innovative; although some reviewers felt the technology incorporated novel ideas, others questioned the novelty of the nanocapsule system for protein delivery and thought that other aspects of the proposed technology provided little advantage beyond those approaches already in development in other labs."** We would like to address the novelty issue by first stating that a detailed manuscript from our labs on the delivery of nuclear proteins (most of the preliminary data are included there) has been accepted for publication in *ACS Nano* (Biswas, *ACS Nano* 2011 In press). The reviewers for this manuscript offered many comments owing to the novelty and advantages of this technology. One reviewer stated that the nanocapsule is unique from other methods because "*it has a thin crosslinked outer layer that is biodegradable*

(digestion by furin, a ubiquitous protease in many cells)" and "*the system developed could have significant contribution to the drug/gene delivery literature*." Another praised the many advantages the endoprotease-mediated system has over current technologies including "*release from the early endosome into the cytosol without going through potential degradation and exocytosis*" as well as "*release into the cytosol in their native form upon disassembly of the nanocapsules*" in contrast to covalently modified proteins which may result in alterations of structure and function.

We believe that our engineered nanocapsules have many advantages in comparison to current approaches. To date, protein-induced pluripotent stem cells have been described by the use of protein transduction domains including 9R or 11R tags which are covalently linked to OSKM proteins (Zhou et al., Cell Stem Cell 2009; Kim et al., Cell Stem Cell 2009). The limitations of protein transduction domains include proteolysis and degradation of the protein cargo, lack of targeting capability and inefficient escape from the endosomal vesicles (Murriel et al., *Exp. Op. Drug Deliv*. 2006). In fact Kim et al. themselves acknowledge the ineffectiveness of the 9R-tagged method in stating that the generation of iPS cells "is very slow and inefficient and requires further optimization." In our approach and paper, we demonstrate nuclear uptake of proteins within 4 hours of delivery which shows the rapid nature of NC uptake and degradation intracellularly. The nanocapsule design includes many free amine groups on the surface of the capsule allowing easy modification to include targeting ligands in contrast to PTDs which lack targeting capabilities. **In summary, we strongly believe that our system is highly innovative since we have successfully incorporated many elements lacking from other approaches including a non-covalent robust carrier to help protect the protein cargo, biodegradability in response to a specific enzyme, efficient endosomal escape into the cytosol, rapid cellular uptake and an ideal surface chemistry for covalently linking targeting ligands.**

The next area of concern for the reviewers was that we did not address potential toxicity or efficacy problems related to the synthesis of protein nanocapsules: "**The applicant did not address potential toxicity or efficacy problems related to protein modification during encapsulation."** We have shown in our manuscripts that our engineered protein nanocapsules are able to be delivered to a variety of cell lines including the immortalized HeLa, the highly regenerative hAFDC and the essential structural MEF (Gu et al., Nano Letters 2009; Biswas et al., ACS Nano 2011) We did not observe any cytotoxicity of nanocapsules up to concentrations of ~2 μM indicating that in general, these nanocapsules do not have a strong tendency to decrease cell viability. We also showed that protein cargos of different sizes and tertiary structures can be encapsulated and released reversibly without loss of bioactivity, including the 27 kDa beta barrel eGFP; the 51 kDa Klf4 that has three zinc finger regions; and the 64 kDa caspase-3 which is a heterotetramer. In each of these cases, we demonstrated that there is no loss of protein activity by showing identical secondary structure of eGFP before and after NC formation and degradation using circular dichroism, nuclear localization ability of Klf4 after degradation intracellularly using immunostaining and confocal imaging and apoptotic activities of caspase-3 using imaging to visualize hallmark apoptotic properties such as cell membrane blebbing and shrinkage and by performing the TUNEL assay and observing the formation of nick end DNA. **We believe we have demonstrated that our engineered nanocapsules are relatively non-toxic in a variety of cell lines and the diverse encapsulated proteins do not lose bioactivity during the entire nanocapsule formation, cellular uptake and intracellular degradation process. This point was also echoed by the reviewers of the paper.**

The third area of concern raised in the review was the lack of details for potential difficulties encountered: "**The proposal lacked sufficient consideration of potential pitfalls and alternative approaches."** For example, the reviewers noted that "the applicant did not adequately address potential difficulties related to the intracellular half-life of proteins following nanocapsule-mediated delivery". Dr. Yi Tang's laboratory has worked extensively with these protein nanocapsules and employs various optimization techniques regularly to enhance delivered protein effect. As described in the proposal (page 8), we can vary the synthesis parameters to achieve optimal protein delivery to allow maximum bioactivity. We can alter the preparation method of nanocapsules from an *in situ* polymerization or emulsion-based method. We will also synthesize nanocapsules with varying crosslinking densities and use dynamic light scattering and scanning electron microscopy to dynamically characterize the size and surface charge change of nanocapsules upon the degradation either by furin or glutathione. The Tang laboratory can also visualize protein delivery using confocal microscopy. Furthermore, the fact that protein-induced pluripotent stem cells have been generated using multi-arginine tags indicates that successfully delivered nuclear transcription factors are able to drive reprogramming before losing activity (Zhou et al., Cell Stem Cell 2009; Kim et al., Cell Stem Cell 2009). Another concern raised by reviewers was "the lack of a systematic approach to control levels of cellular protein delivery to achieve optimal reprogramming". On page 8-10 of our proposal, we describe the dual-luciferase assay as a means to test the efficacy of the protein nanocapsules in the cell lines we wish to reprogram and we will use optimal synthesis parameters as a starting point for reprogramming. We plan to improve efficiency of reprogramming with the use of 6 recombinant proteins OSKMNL and additional small molecules such as HDAC inhibitors and G9a inhibitors (page 9-10 of proposal). We have worked extensively with generation of iPS cells in our lab and we therefore possess all the expertise to optimize reprogramming as well as directed differentiation as seen in our recent publications in stem cell research (Shen et al., Human Molecular Genetics 2006; Fouse et al., Cell Stem Cell 2008; Shen et al., PNAS; Liao et al., et al., Human Molecular Genetics, 2010). **In summary, we feel that both the PI and co-PI have sufficient expertise in their fields to overcome potential difficulties in the project and have routinely employed optimization techniques for protein nanocapsule synthesis and reprogramming.**

We thank the committee for reviewing our proposal and are inspired by the positive response we received: **"*Reviewers found the proposal to be focused and carefully designed with methodologies, strategies and analyses that are logical and appropriate to complete the specific aims. They further agreed that the applicant provided quantitative measures of success and included convincing preliminary data supporting the ability of the team to carry out the proposed research."*** We hope we have satisfactory addressed the three major issues that the reviewers.

We thank you for your time in reviewing our appeal and we hope that you would find out that our grant application merits a favorable consideration.

Sincerely yours,

Guoping Fan, PhD
Associate Professor