CALIFORNIA INSTITUTE FOR REGENERATIVE MEDICINE

MEMORANDUM

Date: April 21, 2010

From: Alan Trounson, PhD
CIRM President

To: Independent Citizen's Oversight Committee

Subject: Extraordinary Petition for Application RB2-01567

Enclosed is a letter from Dr. Eric Kurzrock and Dr. Jan Nolta, applicants for funding under RFA 09-02, CIRM Basic Biology II Research Awards. This letter was received at CIRM on April 20, five working days prior to the April ICOC meeting, and we are forwarding it pursuant to the ICOC Policy Governing Extraordinary Petitions for ICOC Consideration of Applications for Funding.

Drs. Kurzrock and Nolta highlight several points that they believe reviewers may have overlooked in their application. I have reviewed the petition in consultation with the scientific staff (referencing reviewer comments and the submitted application as necessary) and conclude that reviewers adequately and appropriately considered the application and there is no evidence that the items highlighted by the applicants were overlooked. Instead, these appear to be differences in expert opinion between the applicants and reviewers.

The applicants and reviewers both agree that the innovative aspect of this application rests on the fact that few labs are working on this problem. Reviewers would agree that replicating and optimizing the protocol for differentiation of hESCs to definitive endoderm (DE) is an important step that any lab initiating these studies should take, but the reviewers appropriately claim that this is not a novel idea and within the application, the applicants do not offer any advantage of their approach that would make it novel or that would have a significant scientific impact on the field.

We believe that reviewers fairly judged that critical details were missing regarding experiments in Aim 3. Reviewers expressed concern about a lack of critical experiments or preliminary data in support of either over-expressing or inhibiting key urothelial developmental genes that would provide confidence that the aim could be achieved and would produce meaningful results. Amongst several criticisms, the applicants focus in their analysis of reviewers' comments on the one pertaining to the stages of differentiation. Although the application does include temporal analyses of gene expression during differentiation, the reviewers were referring to a lack of information regarding stages of differentiation for the gain and loss of function experiments.

Reviewers would have liked the applicants to analyze and identify active factors released in the co-cultures in order to generate more defined culture conditions. It seems the applicants and reviewers agree that this is a worthwhile activity and also that these experiments were not included in the application.

Reviewers noted that the application repeatedly listed FoxA2 and HNF3β as two of four independent markers to be used for expression analysis, but these are two names for the same marker, so the proposal would actually use only 3 markers. We agree that this is perhaps a minor criticism but is nevertheless accurate and could reasonably cast doubt on the applicants' knowledge of this specific pathway.

The summary states that one reviewer thought it would be critical to quantify DE cell percentages. The applicants agree, and refer to the experimental plan for Specific Aim 1, to determine yield of the differentiation protocol. Staff reviewed the reviewer's detailed comments, and note that the criticism pertained to Aim 3, where the effect of candidate genes on marker expression will be analyzed. The experimental plan for Aim 3 did not refer to quantifying DE cell percentages prior to RT-PCR.

Overall, our review of this petition suggests that reviewers carefully considered the proposal and no critical details were overlooked that would have impacted the score or recommendation for this application.

CIRM staff will be prepared to provide further analysis, should that be requested by any member of the committee.

The enclosed letter represents the views of its author(s). CIRM assumes no responsibility for its accuracy.

In addition, a copy of the CIRM Review Summary for this application is provided for reference.

Eric A. Kurzrock, M.D.
Jan Nolta, Ph.D.
U.C. Davis Stem Cell Program

April 20, 2010

Robert Klein, J.D., Chair of ICOC
Alan Trounson, Ph.D., President of CIRM

Re: extraordinary petition
RB2-01567: Differentiation of Human Embryonic Stem Cells into Urothelium

Dear Mr. Klein and Dr. Trounson,

Thank you and the CIRM staff and reviewers for considering our application. We are submitting this petition due to our belief that the review of the application was extraordinary and that the reviewers had simply overlooked important details that were indeed present in the application. Since we also serve on national review committees, we certainly understand that reviewers are under immense time pressure to review many applications and we mean no disrespect. But we would like to have a chance to point out the factual errors in the review.

We appreciate your attention and effort afforded to our application.

Sincerely yours,
Eric Kurzrock, M.D.
Jan Nolta, Ph.D.

Review Summary: The goal of the proposed studies is to develop a protocol for the differentiation of human embryonic stem cells (hESC) into urothelial cells, a unique cell type lining the bladder that might prove useful for treating patients with bladder defects or cancer. In Aim 1, the principal investigator (PI) intends to improve the efficiency of hESC differentiation into definitive endoderm (DE). Next, the PI proposes to enhance the efficiency of hESC-derived DE differentiation into urothelium via inductive signaling from bladder epithelium and mesenchyme. For the third Aim, the PI will employ overexpression and knockdown approaches in order to verify the function of candidate genes in this process. Finally, in Aim 4, the PI will test the feasibility of transplanting hESC-derived urothelium into bladder.

The reviewers acknowledged that this proposal addresses an understudied and important problem that could lead to advances in the development of regenerative therapy for bladder repair. In general, this is an innovative project in that very few labs are addressing this topic, and reviewers found that some of the experimental approaches, i.e. the co-culture system and bladder transplantation models, were interesting. (break)

Comments from investigators: The reviewers are correct. CIRM has no previous or on-going investigations regarding the urinary tract, urothelium, or bioengineering bladder tissue for spina bifida or cancer patients. NIH has less than 5 on-going grants studying urothelial differentiation, in particular uroplakin expression, and only one project regarding stem cells, which is from our laboratory and involves tissue-specific adult cells, not embryonic.

Review (continued): On the other hand, other aspects of the proposal lacked novelty, especially Aim 1, since others have already published data and protocols on DE generation from hESC. As a result, reviewers were uncertain what advantages the proposed protocol would offer over alternative methodologies and therefore questioned the extent to which this work would have impact. (break)

Comments from investigators: Specific aim 1 is to improve the efficiency of induction of hESC differentiation into definitive endoderm (DE), which is the precursor of epithelial tissue within the bladder, liver and pancreas. As cited in the preliminary aims, we have been developing a protocol for induction of undifferentiated H1 and H9 ES cells into mesoendoderm and then DE by tailoring the substrate, medium conditions and Activin A concentration.

We completely disagree with the reviewers stating that “reviewers were uncertain what advantages the proposed protocol would offer over alternative methodologies and therefore questioned the extent to which this work would have impact.” Our preliminary work has already demonstrated a less expensive protocol, decreased Activin A, (50ng/ml), with a cost savings of $1,200 per experiment (induction of DE and flow sorting enrichment of DE by CXCR4). This translates to a massive decrease in the budget over a 3-year period (at least $50,000) with improved efficiency. In addition, we tested and need to further explore use of other substrates, besides MEF, since they will impact the later differentiation of DE into urothelium. MEF may not be an ideal substrate for translational purposes. Thus we strongly disagree with the reviewers questioning the impact of improving and studying alternative strategies of inducing DE as described in aim 1.

Review (continued): While the proposal was well written, the reviewers identified a number of weaknesses in the research plan that led them to question its overall feasibility. First, several lines of investigation, such as those in Aim 3, were not adequately supported by the preliminary data, leaving the impression that this effort might be somewhat premature. A similar opinion was expressed for Aim 4, which reviewers praised for its translational aspirations but cautioned that the more basic aspects of urothelial differentiation have yet to be worked out. Reviewers found the experimental design to be overly ambitious, noting that the extensive gain and loss of

function experiments proposed for Aim 3 lacked a clear rationale for candidate gene prioritization and were missing critical details regarding the stages of differentiation to be analyzed. (break)

Comments from investigators: There is no literature on the "stages" of differentiation of urothelium, which is what we propose to better define through our carefully planned experiments. Morphologically, we and others have characterized the uroplakin expression *in vivo* and we have induced the expression *in vitro* by changing the balance of proliferation/differentiation with EGF and troglitazone. There is no literature on differentiation of an embryonic cell to an immature urothelial cell. The mechanisms and steps from an undifferentiated (or possible urothelial stem) cell to a mature urothelial cell is unknown and is a major aspect of our current related but non-overlapping NIH grant on enriching and characterizing adult urothelial stem cells.

We clearly state in the experimental plan for aim 3: "At prescribed time-points, differentiation of human DE will be arrested and cellular RNA will be analyzed for the urothelial differentiation products, UPIa, UPIb, UPII and UPIII. Although their pattern of expression in adult tissue is defined, with UPIb being considered the most immature, subtype temporal expression during human development has never been demonstrated. ……The expression of these 20 candidate genes will be examined during urothelial differentiation and temporally linked to UP expression (urothelial differentiation) as well as the loss of DE marker expression ….At prescribed time-points, differentiation of human DE will be arrested and cellular RNA will be analyzed for the urothelial differentiation products…"

We disagree with the reviewer's opinion that we were not detailed enough regarding the "stages" of differentiation to be analyzed and dedicated as much space as possible to this aspect of the application within the 4-page limit of the experimental plan.

Review (continued): Considering the ultimate goal is to derive clinically relevant urothelium from hESC, reviewers were surprised that no attempts to systematically analyze and identify the active factors released from the co-cultured bladder cells were proposed. (break)

Comments from investigators: We absolutely considered evaluating the proteins released by the co-cultured urothelial and mesenchymal cells. A proteomic assay of the conditioned-medium was weighed against a genomic assay. However this type of strategy could also be deemed a "fishing expedition" and we decided that it would be better suited to internal pilot grant funding, which could complement and leverage potential funding by CIRM.

Review (continued): Reviewers also noted that the applicant mistakenly listed 2 different DE markers to be analyzed although they represent the same gene. As much of the proposal hinges on the ability to efficiently achieve DE differentiation, the reviewers worried that this technical error might be indicative of the applicant's inexperience with this pathway. (break)

Comments from investigators: Yes, we listed both names of FoxA2 gene, formerly known as HNF3β, rather than stating FoxA2/HNF3β. It is a far stretch to imply that we are inexperienced, considering our successful and efficient induction of DE, demonstrating expression of FoxA2 and Sox17, and minimal ectodermal or mesodermal differentiation, and our modifications and improvements to the protocol in short course.

Review (continued): To ensure interpretable results, a reviewer emphasized that it would be critical to quantify the percentage of DE or urothelial cells prior to polymerase chain reaction-based marker analyses. (break)

Comments from investigators: We agree. We clearly state that cells will be quantified by IHC and flow cytometry. As described in the preliminary data section: "Markers of DE differentiation (Sox17 & FoxA2) were clearly identified in the majority of cells in most colonies after 10 days of Activin A (A-A) exposure (Fig. 3 & 4). Markers of meso-endoderm (Brachyury & Sox7) and ectoderm (Sox1 & Zic1) differentiation were scantly identified in few colonies (not pictured due to the extreme space limitations)."

Also described in the experimental plan: "We will continue the current protocol, scale up the quantity and more closely analyze and quantify the yield of DE cells with markers … using IHC and RT-PCR …. We will analyze our cells to determine if the yield is equivalent to D'Amour, with greater than 80% SOX17+ cells that are AFP- (not visceral), brachyury low (minimal meso-endoderm) and low SOX1 and ZIC1 (minimal ectoderm). ….To further isolate DE, we will sort $CXCR4^+$ cells …

Review (continued): The PI is an associate professor and holds a leading clinical position; based on his/her urological background, the reviewers were convinced s/he would be very committed to this specialized research program. The PI has been productive and has assembled a very capable group of collaborators who have been already working together on this project for several years. The co-investigator has considerable experience in stem cell differentiation and has published widely. Reviewers did note that insufficient lab personnel were dedicated to this complex project. (break)

Comments from investigators:
Thank you. We dedicated as much of the budget to personnel as possible within the limitations of this particular RFA, since the supply budget for culturing these cells is quite high, even with the significant reductions in cost that we have been able to achieve through our modified culture methods. We would like to re-iterate that some of the data can be compared to that obtained through our NIH – funded research to examine adult urothelium, however neither the PI nor the co-PI have funding for embryonic stem cell culture, so potential funding of this grant would move the field forward significantly.

Comments from investigators: In summary, we believe the reviewers missed some critical points that were present in our application and thus did not fairly assess the application. This application is ambitious. We realize this but we also know that, if we were for some unforeseen reason unable to meet our milestones, the potential funding would not be continued further, thus the ambitious nature should not be of extremely high risk to CIRM. We attempt to investigate an area of stem cell science that is void in the literature, but critically important to a large number of patients throughout California. Considering the pathology and numbers of people affected by bladder cancer and bladder disorders such as spina bifida, it is mind-boggling that the urinary tract has been given little to no attention in the stem cell community. We have developed an experimental plan that both tackles the mechanisms and genes involved with urothelial differentiation, and also seeks to enrich these cells and test them in an animal model that simulates a potential clinical application for both tissue regeneration and cancer treatment. Proposed studies would be conducted under "good laboratory practices" to make them more directly applicable to the clinic for future application to patients who so desperately need bladder augmentation or replacement therapies.

REVIEW REPORT FOR CIRM RFA 09-02: BASIC BIOLOGY AWARDS II

RB2-01567: Differentiation of Human Embryonic Stem Cells into Urothelium

Recommendation: Not recommended for funding
Final Score: 65
First Year Funds Requested: $441,360
Total Funds Requested: $1,328,400

Public Abstract (provided by applicant)
Augmentation or replacement of the bladder is often necessary for the treatment of adults with bladder cancer and children with spinal cord injury or spina bifida. Current surgical techniques utilize segments of intestine or stomach as a substitute for bladder wall. Use of intestinal segments is associated with many complications including infection, stones, salt imbalance, and most concerning, cancer. An ideal substitute for bladder wall would be bioengineered bladder tissue. Ideally, a bioengineered graft would consist of cells that are genetically normal and free of cancerous mutations, promote blood vessel growth, survive long-term and regenerate. Stem cells appear to be the ideal solution for bioengineering tissue.

Preliminary clinical trials have demonstrated the feasibility of using bioengineered tissue for bladder augmentation. The bladder is lined by a very unique cell type called "urothelium". The ability to induce human embryonic stem cells (hESC) or induced pluripotent stem cells (iPSC) into urothelium would provide a major advancement in the tissue engineering field, scientifically and clinically. In addition, deciphering the mechanisms of hESC to urothelial differentiation would facilitate investigation of deviated differentiation into urothelial cancer stem cells; the "seeds" of bladder cancer.

Bladder cancer is the fourth most common type of cancer and caused 15,000 deaths last year. Treatment often requires removal of the bladder. Like other tumors, bladder cancer is believed to originate from the transformation of stem cells into cancer stem cells (CSCs). Potential markers of urothelial CSCs have been identified. Surprisingly, the scientific community has not yet addressed the study of normal human urothelial stem cells and differentiation of hESC to urothelium. The investigation of mechanisms and markers involved in the differentiation of hESC into urothelium will yield important facts about normal and abnormal differentiation and will ultimatley help predict the malignancy of bladder cancers and improve treatments.

Our specific aims are to induce the differentiation of hESC into urothelium via cell signaling. We will also investigate the genes involved in this process. And, we will test the feasibility of transplanting hESC-derived urothelium into a bladder.

This investigation will lead to advances in stem cell biology in an important area not addressed by other scientists. The successful completion of this project will improve human health, indirectly through increased knowledge of differentiation pathways relevant to normal development and neoplasia, and directly through development of novel methodologies for bioengineering tissue for adults and children with urologic disorders and cancer. We are working in a very novel field, which has a high potential to save lives and to vastly improve the quality of life for many patients who need their bladder removed or enlarged.

Statement of Benefit to California (provided by applicant)
The scientific community has not yet addressed the study of urothelial stem cells and differentiation of human embryonic stem cells (hESC) to urothelium. Our investigation of mechanisms and markers involved in the differentiation of hESC into urothelium will yield important facts about normal and abnormal differentiation and will help predict the malignancy of bladder cancers and improve treatments. This project will also advance the field of regenerative medicine. Adults with bladder cancer and children with spina bifida often need bladder reconstruction. Current surgical techniques use segments of intestine as a substitute for bladder wall. Use of intestinal segments is associated with many complications including cancer. Preliminary clinical trials have demonstrated the feasibility of using bioengineered tissue. The ability to induce hESC or induced pluripotent stem cells (iPSC) into urothelium would provide a major advancement in the regenerative medicine field, both scientifically and clinically.

Due to its high rate of recurrence, bladder cancer carries the highest lifetime cost to treat of all cancers. The successful completion of this project will improve human health, indirectly through increased knowledge of differentiation pathways relevant to normal bladder development and bladder cancer, and directly through development of novel methodologies for bioengineering tissue for adults and children with urologic disorders and cancer. These benefits will come to the citizens of California first. In addition to healthcare, this research will benefit the California economy by developing new protocols and technologies that could be adapted for other organs and tissues. Any health benefits, patents, new biotechnology or clinical trials would start in California. This research exemplifies the intent of CIRM bringing together clinical scientists with basic and translational scientists to develop stem cell treatments for the California public while at the same time advancing stem cell biology.

Review Summary
The goal of the proposed studies is to develop a protocol for the differentiation of human embryonic stem cells (hESC) into urothelial cells, a unique cell type lining the bladder that might prove useful for treating patients with bladder defects or cancer. In Aim 1, the principal investigator (PI) intends to improve the efficiency of hESC differentiation into definitive endoderm (DE). Next, the PI proposes to enhance the efficiency of hESC-derived DE differentiation into urothelium via inductive signaling from bladder epithelium and mesenchyme. For the third Aim, the PI will employ overexpression and knockdown approaches in order to verify the function of candidate genes in this process. Finally, in Aim 4, the PI will test the feasibility of transplanting hESC-derived urothelium into bladder.

The reviewers acknowledged that this proposal addresses an understudied and important problem that could lead to advances in the development of regenerative therapy for bladder repair. In general, this is an innovative project in that very few labs are addressing this topic, and reviewers found that some of the experimental approaches, i.e. the co-culture system and bladder transplantation models, were interesting. On the other hand, other aspects of the proposal lacked novelty, especially Aim 1, since others have already published data and protocols on DE generation from hESC. As a result, reviewers were uncertain what advantages the proposed protocol would offer over alternative methodologies and therefore questioned the extent to which this work would have impact.

While the proposal was well written, the reviewers identified a number of weaknesses in the research plan that led them to question its overall feasibility. First, several lines of investigation, such as those in Aim 3, were not adequately supported by the preliminary data, leaving the impression that this effort might be somewhat premature. A similar opinion was expressed for Aim 4, which reviewers praised for its translational aspirations but cautioned that the more basic aspects of urothelial differentiation have yet to be worked out. Reviewers found the experimental design to be overly ambitious, noting that the extensive gain and loss of function experiments proposed for Aim 3 lacked a clear rationale for candidate gene prioritization and were missing critical details regarding the stages of differentiation to be analyzed. Considering the ultimate goal is to derive clinically relevant urothelium from hESC, reviewers were surprised that no attempts to systematically analyze and identify the active factors released from the co-cultured bladder cells were proposed. Reviewers also noted that the applicant mistakenly listed 2 different DE markers to be analyzed although they represent the same gene. As much of the proposal hinges on the ability to efficiently achieve DE differentiation, the reviewers worried that this technical error might be indicative of the applicant's inexperience with this pathway. To ensure interpretable results, a reviewer emphasized that it would be critical to quantify the percentage of DE or urothelial cells prior to polymerase chain reaction-based marker analyses.

The PI is an associate professor and holds a leading clinical position; based on his/her urological background, the reviewers were convinced s/he would be very committed to this specialized research program. The PI has been productive and has assembled a very capable group of collaborators who have been already working together on this project for several years. The co-investigator has considerable experience in stem cell differentiation and has published widely. Reviewers did note that insufficient lab personnel were dedicated to this complex project.

In summary, reviewers found this proposal to be innovative and significant. However, an overly ambitious

scope and a premature research plan led them to question its feasibility and potential for impact.

The following scientific working group members had a conflict of interest with this application: