CALIFORNIA INSTITUTE FOR REGENERATIVE MEDICINE

MEMORANDUM

Date: April 26, 2010

From: Alan Trounson, PhD
CIRM President

To: Independent Citizen's Oversight Committee

Subject: Extraordinary Petition for Application RB2-01523 **(LATE SUBMISSION)**

Enclosed is a letter from Dr. Edward De Robertis, an applicant for funding under RFA 09-02, CIRM Basic Biology II Research Awards. This letter was received at CIRM on April 25, two working days prior to the April ICOC meeting. We are forwarding it as a LATE SUBMISSION pursuant to the ICOC Policy Governing Extraordinary Petitions for ICOC Consideration of Applications for Funding.

Dr. De Robertis suggests that the final scientific score by the Grants Working Group may have been influenced by what he believes are two incorrect impressions by reviewers: 1) that little justification for the use of human embryonic stem cells (hESCs) was presented and 2) that the PI has only recently started working with hESCs.

Indeed, reviewers were tasked with judging proposals based on the proposed use of human stem cells and the mechanistic knowledge gained and impact made towards human stem cell biology. The reviewers and applicant agree that the studies proposed are innovative, well designed, and the knowledge gained would be broadly applicable if successful. However, we believe that reviewers were appropriately concerned that the proposed studies are not of particular importance to hESCs since the observed phenomena appear conserved among many cell types including immortalized cell lines. Thus, the knowledge gained would be of general interest and significance in cell biology but not unique to stem cells. The applicant did note in the application that, like other stem cells, hESCs appear to have a high level of endogenous Wnt signaling that would make them appealing as a model of study. Reviewers suggested, however, that such studies could be similarly if not more efficiently carried out in other cell types including mouse embryonic stem cells. Thus, there was little compelling justification in the application to convince reviewers that these experiments should be carried out in hESCs.

The applicant highlights what he believes is a mistake by reviewers that "a significant number of experiments involve the model organism rather than hESCs". In fact, many of the proposed experiments do involve studies in Xenopus (frog) oocytes in each of the experimental aims and appropriately noted by reviewers. Given the objectives of this RFA, reviewers appropriately considered the relative focus of proposed studies on human stem cells. The fact the applicant is

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an expert in Xenopus is not relevant and was not considered negatively by the reviewers as suggested by the applicant.

The applicant claims in the petition that his laboratory has worked with hESCs since 2006 in response to reviewers' criticism that the PI has only recently started working with hESCs. The application describes the role and experience of the lab manager who took a course in stem cell culture in 2006 and has since maintained a hESC line in the lab for general use. The preliminary data also references a published experiment performed in hESCs that was a catalyst for this proposal. However, the biographical sketch does not show evidence of additional experience with hESCs and reviewers felt that the proposal to conduct shake-off synchronization experiments in hESCs was an indication of inexperience with these cells. We believe that reviewers appropriately recognized the applicant's significant experience in Wnt signaling and use of the Xenopus model and that his experience with hESCs is still recent and developing.

Overall, our review of this petition suggests that comments by reviewers were appropriate and carefully considered the objectives of the RFA despite an otherwise meritorious proposal.

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.

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April 25, 2010

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
RB2-01523: Wnt/GSK3 as a general regulator of protein half-life in human embryonic stem cells

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

Thank you for the opportunity to present this extraordinary petition requesting that CIRM Basic Biology Awards II grant RB2-01523 be considered for funding at the next ICOC meeting. I greatly appreciate the transparency in the CIRM grant evaluation process. In particular, the detailed Review Summary forwarded by Dr. Gilberto Sambrano was very informative.

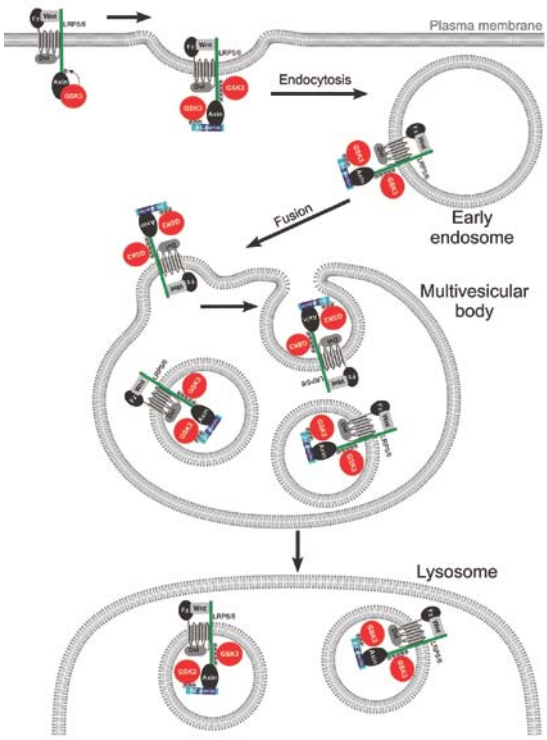
The scientific merits of our project were evaluated by the Grants Working Group on February 22-23, 2010 and received a final score of 63. In this extraordinary petition I am respectfully requesting ICOC to consider moving this application into the category recommended for funding, in case funds were still available for the Basic Biology II RFA.

This letter explains the rationale for this request for special consideration. The final score may have been influenced by the incorrect impressions that "there was little justification for the use of human embryonic stem cells (hESCs)" and that "the PI has only recently started working with hESCs". In fact, hESCs are central to this proposal and we have been committing resources from our lab into research in hESCs since 2006. We are fully committed to this new field. I will also attempt to convince the ICOC Committee, which includes a wide range of California medical interests, that funding this project would not only benefit basic stem cell biology but would also bring additional international contacts and distinction to CIRM's mission.

1) Scientific Background

The Review Summary correctly states that: "This proposal focuses on understanding the mechanism by which the Wnt signaling pathway regulates human embryonic stem cell (hESC) pluripotency and self-renewal. The central hypothesis is that Wnt regulates the stability of a number of proteins by inhibiting their phosphorylation by Glycogen Synthase Kinase 3 (GSK3). The applicant proposes a novel cellular mechanism for this inhibition by which Wnt signaling causes GSK3 to be sequestered in intracellular compartments. This sequestration would prevent target protein phosphorylation and subsequent degradation." As shown in the figure below, our working hypothesis is that when the Wnt growth factor binds to its receptors on the

plasma membrane it causes their internalization. The GSK3 enzyme, shown here in red, attaches to these receptors and eventually becomes entrapped in small membrane vesicles called multivesicular bodies (MVBs) which become degraded in organelles called lysosomes. We provide extensive preliminary evidence in favor of this model, which provides a novel cell biological paradigm for the regulation of growth factor signaling. In this view, the Wnt signal is caused by the removal of GSK3 from the cytoplasm.



GSK3 is an enzyme that adds phosphates to hundreds of target proteins, in general triggering their degradation. GSK3 was discovered because it inhibits Glycogen Synthase (GS), a key enzyme in glucose metabolism and diabetes. Insulin inhibits GSK3 by adding a phosphate via activation of a different enzyme called Protein Kinase B. Wnt signaling would also regulate GS, as well as many other cellular proteins, but through a different mechanism requiring GSK3 sequestration within intracellular membrane organelles.

2) Significance for hESC biology

The reviewers "found the proposal to be quite innovative" and "appreciated the novelty of this proposal's central hypothesis and supporting preliminary data". "However, they were not convinced of the significance of the proposal for hESC biology". As explained in the original application, Wnt signaling is key to stem cell biology. To be maintained in culture, mES cells require Wnt and Lif, neural stem cells Wnt and FGF, mammary gland stem cells Wnt and EGF, and blood stem cells Wnt and SCF. Therefore, high Wnt signaling is the common denominator of all stem cells and is a fundamental cornerstone of stem cell biology.

3) The rationale for performing the experiments in hESCs

The reviewers thought "there was little justification for the use of hESCs and that most of the experiments could just as easily be performed in somatic, transformed or non-human cells". We discovered the relationship between GSK3 and protein stability by finding that certain proteins targeted for degradation were asymmetrically distributed between daughter cells in 90% of self-renewing hESC divisions (Fuentealba et al., PNAS 2008). Other cell lines have much lower frequencies of asymmetries, and we would have not made this discovery were it not for using hESCs. The asymmetry results from the unequal distribution of MVBs and lysosome organelles. Only in hESCs these organelles can be visualized as vesicle-like particles in the cytoplasm (shown in Fig. 1 of Supplementary Materials submitted to Dr. Sambrano). Therefore, hESCs have unique properties (probably due to their high levels of endogenous Wnt signaling) that make them essential for the proposed studies. Most of the proposed experiments cannot be performed in other cells.

Another mistake was that "a significant number of experiments involve the model organism rather than hESCs". This refers to our use of embryos of the frog *Xenopus* to prove that the intracellular membrane trafficking machinery is required for the sequestration of GSK3 and Wnt signaling. We would like to respectfully note that much of what is presently know about the Wnt

signaling pathway has been deduced from microinjection experiments in frog embryos. That the PI is an expert in the field of amphibian embryology should not be considered a demerit, but rather a valuable asset for the successful completion of this exciting project.

4) Qualifications of the PI for working on hESCs

The reviewers "described the principal investigator (PI) as a leader in the field of signal transduction biology". I would like to respectfully add that the PI is a developmental biologist who understands the nexus between stem cell biology and embryonic development. As a postdoc he worked six years with Sir John Gurdon in Cambridge, UK, and discovered that oocyte-type genes were activated in somatic nuclei after injection into amphibian oocytes (De Robertis and Gurdon, PNAS, 1977). This was the first molecular demonstration of nuclear reprogramming, and a landmark study in what now is called stem cell biology. More recently, we have studied how the earliest cell differentiation decisions are taken in the vertebrate embryo. The first cells to leave their pluripotent state are those on the dorsal side, which form Spemann's organizer tissue. The PI has elucidated the molecular mechanisms of these initial cell differentiation decisions in the embryo while working here in California, at the UCLA School of Medicine, for the past 24 years.

As past-President of the International Society for Developmental Biology (2002-2006) I have excellent international connections in this field, which is inseparable from that of stem cell biology. I am also a corresponding member of the Latin American Academy of Sciences for, although born in the USA to Hispanic parents, I was raised in Latin America. Another important international platform is membership in the Pontifical Academy of Sciences, The Vatican. It consists of only 80 academicians from all the sciences, who serve as scientific advisors to the Pope (five are Californians, but only David Baltimore and I are biologists).

Finally, the "reviewers commented that the PI has only recently started working with hESCs and might benefit from collaboration with an investigator with expertise in this area". There is an oversight here. As explained in the grant, we started working on hESCs long before this application, when my lab manager attended the UCSF Stem Cell Training Course in 2006. Since then, we have continuously kept hESC cultures and prepared MEF feeder cells by gamma irradiation. There is much expertise in stem cells at UCLA. I share the floor with Owen Witte, and meet with Kathrin Plath and Bill Lowry weekly at our "Embryology Club" (all CIRM grant recipients). In response to reviewers' concerns, I have secured agreement from Dr. Michael Teitell (mteitell@mednet.ucla.edu), also a CIRM grantee, to provide us with mentorship and collaboration. We have assembled a top-notch team of trainees. I am confident we will be able to overcome technical pitfalls as they arise and accordingly plan alternative approaches. For example, if shake-off synchronization proves difficult it will suffice to analyze dividing isolated hESCs clones. The reviewers also mention that "no CVs are presented other than the PI's". I regret this, but understood from the instructions that the description in Basic Awards II form page 24 (and in pages 7-12 as well) was all that was requested for trainees.

In summary, I thank the ICOC for consideration of this extraordinary petition for funding of grant RB2-01523. A positive response would substantially advance our understanding of basic stem cell biology and further strengthen the leadership of the State of California in the field of Regenerative Medicine.

Sincerely yours,

Edward M. De Robertis, M.D., Ph.D.

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

RB2-01523: Wnt/GSK3 as a general regulator of protein half-life in human embryonic stem cells

Recommendation: Not recommended for funding
Final Score: 63
First Year Funds Requested: $456,844
Total Funds Requested: $1,370,532

Public Abstract (provided by applicant)

Human embryonic stem cells (hESCs) have the remarkable capacity of limitless self-renewal. This property is known to be controlled by signaling of a growth factor called Wnt. This proposal investigates the molecular mechanism by which Wnt causes self-renewal. Current conversational wisdom is that Wnt only prolongs the half-life of a protein called beta-Catenin.

Here we propose the hypothesis that Wnt regulates the stability of a multitude of proteins, all of them characterized by receiving phosphates from an enzyme called Glycogen Synthase Kinase 3 (GSK3). In this view, Wnt would be a general metabolic signal that instructs cells to slow down protein degradation by inhibiting GSK3 activity.

How is GSK3 inhibition achieved? This is a key unanswered question in the Wnt signaling field. We propose a new cellular mechanism by which the GSK3 enzyme is sequestered inside intracellular vesicular organelles (called multivesicular bodies) after Wnt signaling. If this GSK3 sequestration hypothesis can be proven, it would constitute an important contribution to stem cell research.

Human embryonic stem cells are essential for these investigations because they naturally have very high levels of Wnt signaling. In addition, they have asymmetric mitotic divisions. We will develop methods to investigate why some hESCs differentiate, losing their astonishing self-renewal potential. The experiments proposed will help understand how Wnt signaling maintains the pluripotent state in hESCs.

By investigating the hypothesis that Wnt signaling functions as a general regulator of protein stability, we hope to significantly advance the field of human embryonic stem cell research and regenerative medicine.

Statement of Benefit to California (provided by applicant)

The State of California benefits from the proposed basic research by strengthening its leadership role in worldwide stem cell research. This project would provide employment for five full-time researchers, who will obtain training in cutting-edge molecular and cell biology research.

It has been our experience that trainees leave our laboratory always for better high-tech jobs. Many go into the biotech industry and others into teaching at the college level. Funding this project will benefit California by producing an improved and highly trained workforce. It will also strengthen our already excellent university research system.

Review Summary

This proposal focuses on understanding the mechanism by which the Wnt signaling pathway regulates human embryonic stem cell (hESC) pluripotency and self-renewal. The central hypothesis is that Wnt regulates the stability of a number of proteins by inhibiting their phosphorylation by glycogen synthase kinase 3 (GSK3). The applicant proposes a novel cellular mechanism for this inhibition by which Wnt signaling causes GSK3 to be sequestered in intracellular compartments. This sequestration would prevent target protein phosphorylation and subsequent degradation. In Aim 1, the applicant proposes to identify proteins whose stability is regulated by Wnt signaling and investigate potential asymmetric allocation of GSK3-phosphorylated proteins during hESC division. Aim 2 focuses on a specific protein identified in the preliminary studies that has GSK3 phosphorylation sites and may be regulated by Wnt signaling. The applicant proposes to study the regulation of this protein in a model organism and in hESCs. Finally, in Aim 3, the applicant proposes to test the hypothesis that Wnt signaling induces GSK3 sequestration and investigate potential underlying mechanisms.

Reviewers found the proposal to be quite innovative, as it is based on a novel hypothesis that Wnt can cause GSK3 to be sequestered away from its substrates. However, they were not convinced of the significance of the proposal for hESC biology. Reviewers agreed that there was little justification for the use of hESCs and that most of the experiments could just as easily be performed in somatic, transformed, or non-human cells. While the proposed research is quite significant for cell biology in general, reviewers did not feel it was likely to have a major impact on the fields of stem cell biology or regenerative medicine.

The reviewers found the research plan to be well designed and supported by sound preliminary data. However, they noted that a significant number of experiments involve the model organism rather than hESCs. Reviewers also raised a few concerns about the experimental design. They cautioned that certain methods proposed in hESCs, including uniform expression of epitope-tagged proteins via lentiviral transduction and synchronization of hESC cultures may be technically difficult. Demonstration of facility with these methods in the preliminary data would have been helpful. Reviewers were also concerned that potential pitfalls and alternative strategies are not discussed in the research plan.

Reviewers described the principal investigator (PI) as a leader in the field of signal transduction biology with an excellent publication record. They noted that the research team appears adequate to advance the project but no CVs are presented other than the PI's. Reviewers commented that the PI has only recently started working with hESCs and might benefit from collaboration with an investigator with expertise in this area.

Overall, while reviewers appreciated the novelty of this proposal's central hypothesis and supporting preliminary data, they were not convinced that there is a strong rationale for performing the experiments in hESCs over other model systems.

PROGRAMMATIC REVIEW

A motion was made to move this application into Tier 1, Recommended for Funding. Reviewers summarized their reviews and discussed whether there is a strong case to conduct the proposed work in hESCs. While reviewers appreciated the scientific questions posed in the application, they agreed that the work would be better suited for simpler model systems. The motion failed.

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